LARISSA MATTOS TREVIZANO

MELHORAMENTO POR EVOLUÇÃO DIRIGIDA DA TERMOESTABILIDADE DA XILANASE xyn*A* DE *Orpinomyces* sp. PC-2

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Agrícola, para a obtenção do título de *Magister Scientiae*.

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APROVADA: 29 de abril de 2009.

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A Deus,

Aos meus pais, Maria das Graças e Robson,

Aos meus irmãos Rômulo e Robson,

Ao meu namorado e amigo Gillian.

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BIOGRAFIA

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RESUMO

TREVIZANO, Larissa Mattos, M.Sc. Universidade Federal de Viçosa, abril de 2009. **Melhoramento por evolução dirigida da termoestabilidade da xilanase xynA de *Orpinomyces* sp. PC-2.** Orientadora: Valéria Monteze Guimarães. Co-orientadores: Sebastião Tavares de Rezende e Marcelo Matos Santoro.

Xilana é o polissacarídeo hemicelulósico mais comum da parede celular de plantas. Este polissacarídeo é composto por uma cadeia principal constituída exclusivamente por resíduos de xilose unidos por ligação β-1,4. Esta cadeia principal possui ramificações contendo resíduos de arabinofuranosil, acetil e glucuronosil. A hidrólise enzimática de xilanas é realizada por uma variedade de enzimas que são agrupadas pelo termo genérico de hemicelulases. Endoxilanases (EC 3.2.1.8) hidrolisam ligações β-1,4 internas da cadeia principal e geram oligossacarídeos de xilose solúveis. Uma variedade de microrganismos, incluindo bactéria, leveduras e fungos filamentosos têm sido descritos como produtores de xilanase, sendo que os mais potentes produtores são os fungos. Pesquisadores estão especialmente interessados em xilanases fúngicas porque elas são secretadas extracelularmente e sua atividade é muito maior que as xilanases provenientes de leveduras e bactérias. Dentre as aplicações biotecnológicas das xilanases, pode ser enfatizada a bioconversão de materiais lignocelulósicos em produtos fermentáveis, melhoria da digestibilidade de rações animais, clarificação de sucos, auxílio na liberação da lignina da polpa de celulose e redução da quantidade de cloro requerido para o branqueamento da polpa na indústria de papel. Nas últimas décadas, tem surgido um crescente interesse na bioconversão de lignocelulose como uma fonte de energia renovável, e xilanases podem ser efetivamente usadas, em associação com as celulases, para hidrólise de biomassa lignocelulósica e produção de bioetanol. Muitas das xilanases fúngicas mostram atividade ótima em pHs neutros ou ácidos e em temperaturas abaixo de 45 ºC. Xilanase (XynA), originalmente isolada do fungo anaeróbico *Orpinomyces* PC-2, foi testada para diversas aplicações industriais em escala piloto. Esta enzima apresentou atividade extremamente alta com o substrato xilana de ração animal, polpa de celulose e outros tipos de biomassa.

Estudos demonstraram que o uso da enzima nativa foi mais eficiente em condições de pH 5.0-5.5 e temperatura de 50-55 ºC. No entanto, os processos industriais são normalmente realizados em temperaturas mais elevadas e em pH alcalino, o que justifica o interesse por xilanases que sejam ativas nestas condições. A metodologia de evolução dirigida do DNA surgiu como uma alternativa de sucesso na engenharia genética de enzimas e tem sido empregada para melhorar as propriedades funcionais dessas moléculas. Neste trabalho, a técnica de e*rror-prone* PCR foi utilizada para aprimorar a termoestabilidade da endo-β-1,4-xylanase de *Orpinomyces* PC-2. A biblioteca de mutantes (xynA) construída foi submetida a vários ciclos de *screening.* Os transformantes foram inicialmente expostos a 60 ºC durante uma hora e os mutantes termoestáveis foram selecionados com o substrato azo-xilana-agarose 0,2% pH 6,5. Seis mutantes selecionados foram seqüenciados e suas seqüências de aminoácidos foram analisadas para identificação das mutações. Dois mutantes apresentaram maior estabilidade térmica se comparado a xilanase sem mutações (tipo selvagem). Enquanto o tipo selvagem perdeu 60% de sua atividade depois de 10 min a 60 ºC, os mutantes M4 e M6 mostraram maior termoestabilidade e mantiveram aproximadamente 50% de suas atividades depois do tratamento a 60 ºC por 60 min. Em adição, M4 manteve cerca de 40% de sua atividade inicial depois de incubação a 75 ºC por 60 min. Com o objetivo de avaliar os efeitos das mutações nas propriedades das enzimas, estas foram caracterizadas quanto à temperatura ótima, pH ótimo e especificidade por substratos. Os mutantes e a xilanase tipo selvagem apresentaram temperatura ótima de 60 ºC e pH ótimo na faixa de 5,0-7,0 para atividade de xilanase. As enzimas apresentaram atividade com os dois substratos testados. Um estudo inicial de modelagem estrutural da xilanase tipo selvagem e duas mutantes, que apresentaram maior termoestabilidade, foi realizado, visando o melhor entendimento da relação entre estrutura e função dessas xilanases.

ABSTRACT

TREVIZANO, Larissa Mattos, M.Sc. Universidade Federal de Viçosa, April, 2009. **Improvement of the thermostability of *Orpinomyces* sp. PC-2 xylanase by directed evolution.** Adviser: Valéria Monteze Guimarães. Co-advisers: Sebastião Tavares de Rezende and Marcelo Matos Santoro.

Xylan is the most common hemicellulosic polysaccharide in cell walls of land plants. This polysaccharide is composed of a main-chain polymer made up exclusively of β-1,4 xylose residues. This backbone possesses branches containing arabinofuranosyl, acetyl, and glucuronosyl residues. Enzymatic hydrolysis of xylans is brought about by a variety of enzyme activities that are grouped under the generic term hemicellulases. Endoxylanases (EC 3.2.1.8) hydrolyze internal β-1,4 bonds in the main-chain and generate soluble xylooligosaccharides. A variety of microorganisms, including bacteria, yeast and filamentous fungi, have been reported to produce xylanase, in which the most potent producers are fungi. Researchers are especially interested in fungal xylanases because they are secreted extracellularly and their activity is much higher than the xylanases from yeasts and bacteria. Among the biotechnological applications of xylanases, it can be outstanding the bioconversion of lignocellulosic materials into fermentative products, improvement of digestibility of animal feedstock, clarification of juices, facilitating the release of lignin from the pulp and reducing the amount of chlorine required for bleaching in pulp in the paper industry. Over the last few decades, there has been a growing interest in lignocellulose bioconversion as a renewable energy source and xylanases can be effectively used with cellulases to hydrolyze the lignocellulosic biomass and bioethanol production. Most of the fungal xylanases show optimal activities at neutral or acidic pH and at temperatures below 45 °C. Xylanase (XynA), originally isolated from the anaerobic fungus *Orpinomyces* PC-2, has been tested for several industrial applications, in pilot scale. This enzyme presented extremely high activity with the xylan substrate of animal ration, cellulose pulp and other biomass types. Studies demonstrated that the use of the native enzyme has been more efficient in optimal conditions as pH 5.0-5.5 and temperature of 50-55 °C. However, the

industrial process is usually accomplished at higher temperature and alkaline pH, what justifies the interest for xylanases that are active in these conditions. Directed evolution has emerged as a successful alternative in the genetic engineering of enzymes and it has been used to improve the functional properties of those molecules. In this study, error-prone PCR was used to improve the thermostability of endo-β-1,4-xylanase from *Orpinomyces* PC-2. The constructed library of xylanase (xynA) mutants was submitted to several screening cycles. The transformants were first exposed to 60 ºC during one hour and then the thermostable mutants were selected with the azo-xylan-agarose 0.2% pH 6.5 as substrate. Six mutants selected were sequenced and these amino acid sequences were analyzed to identify the mutations. Two mutants displayed higher thermal stability than the xylanase without mutations (wild-type). Whereas the wild-type lost 60% of its activity after 10 min at 60 ºC, mutants M4 and M6 showed enhanced thermostability and retained approximately 50% of its activities after treatment at 60 ºC for 60 min. In addition, M4 retained about of 40% of its initial activity after incubation at 75 ºC for 60 min. In order to evaluate the mutation effects in the enzyme properties, these ones were characterized for the optimum temperature, optimum pH and substrate specificity. The mutants and the wild-type showed an optimal temperature and pH for xylanase activity at 60 ºC and pH range of 5.0-7.0. The enzymes showed activity against the two tested substrates. An initial structural modeling study was accomplished with the wild-type and two mutants, which presented higher thermostability, seeking the best understanding of the relationship between structure and function of those xylanases.

Justificativa e objetivos

A aplicação de enzimas em diversos processos industriais e agrícolas é uma realidade e o mercado mundial dessas macromoléculas, que teve início nos anos 60, vem crescendo a cada ano em função das vantagens que estes biocatalisadores conferem. As reações catalisadas pelas enzimas frequentemente demandam processos mais econômicos quando comparadas com catalisadores químicos; a catálise é mais específica, não gerando produtos indesejáveis e consequentemente reduzem a poluição ambiental (Said e Pietro, 2004).

Muitas enzimas hidrolíticas são altamente ativas, mas muitas vezes não são adequadas para alguns processos industriais. Nos últimos anos, pesquisas em engenharia de proteínas têm sido desenvolvidas com o objetivo de obter enzimas com propriedades melhoradas, tornando-as adequadas para as condições requeridas nos processos industriais. Na última década, a tecnologia de evolução dirigida do DNA tem se tornado uma realidade. Evolução molecular dirigida foi desenvolvida visando modificar a função de produtos gênicos (Stemmer, 1994; Moore *et al.,* 1997; Yano *et al*., 1998; Shibuya *et al.*, 2000). Muitas moléculas modificadas têm sido obtidas como enzimas e anticorpos. Algumas técnicas estão disponíveis para facilitar as mutações *in vitro* de um gene específico, como mutagênese sítio-dirigida, *error-prone PCR* (Leung *et al*., 1989), *DNA shuffling* (Stemmer, 1994) ou *DNA cassette shuffling* (Short, US patent 5939250).

Error-prone PCR é um método que introduz mutações randômicas durante múltiplos ciclos de amplificação. Este método explora a propriedade de baixa fidelidade da enzima Taq polimerase, em certas condições, para incorporar nucleotídeos errados a partir do molde de DNA. Este método é melhor aplicado caso seja necessário a introdução de um pequeno número de mutações no gene e simultaneamente promover pequenas alterações nas propriedades catalíticas (Leung *et al,* 1989; Cadwell e Joyce, 1992). Com esta técnica, tenta-se preservar as propriedades interessantes das enzimas e simultaneamente aumentar sua funcionalidade de acordo com parâmetros industriais. A metodologia de evolução dirigida de enzimas poderá adequar enzimas de interesse a vários processos industriais, gerando um impacto

significativo na indústria agrícola, gerando uma alternativa mais econômica e ambientalmente responsável para os processos industriais.

O uso de xilanase em processos industriais iniciou em 1980, tendo sido primeiramente utilizada na preparação de ração animal e posteriormente nas indústrias de alimentos, têxteis e de papel. Uma das aplicações mais promissoras de xilanase em processos industriais consiste na sua utilização para branqueamento de polpa de celulose, em substituição ao uso de agentes químicos clorados. Muitos dos compostos organoclorados gerados em indústria de polpa e papel são tóxicos, difíceis de serem reciclados e resistentes a biodegradação, constituindo uma das fontes que mais contribuem para poluição da água e do ar (Techapun *et al.,* 2003).

A produção de ração animal é um importante setor do agronegócio e a adição de enzimas à ração melhora a digestão dos grãos e utilização de nutrientes, exercendo um papel importante para sustentabilidade da agroindústria. Uso de xilanases em dietas a base de milho, trigo ou sorgo promove aumento no ganho de peso do animal e conversão alimentar. Na indústria de alimentos, xilanase é utilizada para aumentar o rendimento da produção de sucos de frutas e vegetais. Também adição de xilanase à farinha de trigo melhora a textura e aumenta o volume de pães e produtos crocantes (Polizelli *et al.,* 2005).

Biomassa lignocelulósica como bagaço de cana, palha e sabugo de milho, subprodutos florestais e diversas gramíneas, poderia ser usada como fonte alternativa para futura expansão da produção de biocombustíveis, especialmente etanol (Somerville, 2007). Os açúcares nesses tipos de biomassa existem como polissacarídeos, principalmente na forma de celulose e hemiceluloses, os quais estão física e quimicamente associados e também com lignina, proteínas e amido. Para tornar esses açúcares disponíveis para fermentação, a lignocelulose precisa ser convertida em monossacarídeos através do pré-tratamento e sacarificação enzimática (Dien *et al.,* 2006). Dentre as várias hemiceluloses, arabinoxilana representa a mais abundante forma encontrada em materiais lignocelulósicos agrícolas e a enzima chave para sua degradação é a xilanase, que hidrolisa as ligações glicosídicas.

O interesse científico neste campo é refletido pelo número de publicações durante os últimos anos, descrevendo numerosas xilanases

produzidas a partir de várias fontes. Tem sido descrita a produção de xilanases a partir de bactérias, fungos, actinomicetos e leveduras (Wong *et al.,* 1988; Kuhad e Singh, 1993; Kuhad *et al*., 1997; Beg *et al.,* 2001). Entretanto, a busca por produtores mais eficientes de xilanases é um objetivo que deve ser perseguido.

Xilanase (XynA), originalmente isolada do fungo anaeróbico altamente fibrolítico *Orpinomyces* PC-2, tem sido estudada por Li *et al.* (1997a,b) e testada para várias aplicações industriais, em escala piloto. Esta enzima apresentou atividade extremamente alta com o substrato xilana de ração animal, polpa de celulose e outros tipos de biomassa. Estudos demonstraram que a utilização da enzima nativa tem sido mais eficiente em condições ótimas como pH 5,0-5,5 e temperatura de 50-55 °C. Entretanto, o processo industrial para o branqueamento da polpa é geralmente realizado em temperatura entre 70-90°C e pH em torno de 10. Para utilização da enzima, este processo precisaria ser ajustado com ácido forte e água fria, o que levaria à adição de custos extras e reduziria a capacidade de produção da indústria. No caso de aplicações em ração, a xilanase não resiste ao processamento padrão para peletização da ração, o qual ocorre a 85-100°C. Indústrias de ração poderiam diminuir a temperatura do processo para utilização da enzima com atividade máxima, entretanto, existiria maior probabilidade de contaminação bacteriana gerando um produto inferior. A xilanase ideal deveria ser mais ativa sob as condições dos processos industriais existentes. Para um processo realmente viável tanto ambiental quanto economicamente, é importante aumentar a estabilidade térmica e de pH da xilanase.

Assim, a proposta desse trabalho foi selecionar através de vários ciclos de *screening,* realizados em uma biblioteca de xynA mutante criada pela técnica de *error-prone PCR*, mutantes que apresentassem maior estabilidade térmica. Os genes que codificam para as enzimas mutantes foram sequenciados para identificação dos resíduos substituídos nas enzimas. Com o objetivo de avaliar os efeitos das mutações nas propriedades das enzimas, estas foram caracterizadas quanto à temperatura ótima, pH ótimo, termoestabilidade e especificidade por substratos. Além disso, uma análise preliminar de modelagem estrutural foi realizada para melhor entendimento da relação estrutura/função dos mutantes.

Estado da arte:

Xilanases: Aplicações biotecnológicas e melhoramento de propriedades funcionais.

1. Xylan: definition, structure and microorganisms that produce xylan-degrading enzymes

Hemicelluloses include xylan, mannan, galactan, and arabinan as the main heteropolymers. The classification of these hemicellulose fractions depends on the types of sugar moieties present. The principal monomers present in most of the hemicelluloses are D-xylose, D-mannose, D-galactose, and L-arabinose. (Bastawde, 1992). Xylan is the most common hemicellulosic polysaccharide in cell walls of land plants, representing up to 30–35% of the total dry weight (Joseleau *et al*., 1992).

This polysaccharide is composed of a main-chain polymer made up exclusively of β-1,4 xylose residues. Chemical and structural diversity in xylans arises from the side chain decorations of the polymer (Smaali *et al.,* 2008). The backbone of β-1,4-linked xylopyranose residues possesses branches containing arabinofuranosyl, acetyl, and glucuronosyl residues (Biely, 1985). Arabinose, the main substituting group, is linked to xylosyl moieties through α-1,2 and/or α-1,3 bonds. In turn, arabinose residues can be esterified at their O-5 position by phenolic acids, while the O-2 and O-3 positions of main chain xylose residues can also be esterified by acetyl moieties (Smaali *et al.,* 2008).

Enzymatic hydrolysis of xylans is brought about by a variety of enzyme activities that are grouped under the generic term hemicellulases (Smaali *et al.,* 2008). The complete hydrolysis of xylan requires orchestrated actions of various enzymes including endoxylanase, β-D-xylosidase, α-glucuronidase, acetyl esterase and α-L-arabinofuranosidase (Beg *et al.,* 2001). Depolymerizing endoxylanases (β-1,4-D-xylan xylanohydrolase , EC 3.2.1.8) hydrolyze internal β-1,4 bonds in the main chain and generate soluble xylooligosaccharides that are suitable substrates for exoacting β-xylosidases (EC 3.2.1.37) that converts xylobiose and xylooligosaccharide into xylose (Smaali *et al*., 2008). In addition, α-L-arabinofuranosidase, α-D-glucuronidase, acetyl xylan esterases, ferulic-acid esterases, and p-coumaric-acid esterases are required for the removal of the side chains (Subramaniyan and Prema, 2002). α-L-arabinofuranosidases (EC 3.2.1.55) hydrolyse α-1,2 and/or α-1,3 bonds that link arabinose to xylose and feruloyl-acid esterases (EC 3.1.1.73) break ester bonds between ferulic acid and arabinose (Smaali *et al.,* 2008).

A wide variety of microorganisms are known to produce xylan-degrading enzymes, including bacteria, yeast and filamentous fungi. The most potent xylanase producers are fungi (Haltrich *et al.,* 1996). A number of xylanases have been purified from a wide variety of microorganisms such as *Bacillus* sp. (Sa-Pereira *et al.,* 2004), *Trichoderma* sp. (Xiong *et al.,* 2004) and *Streptomyces* sp. (Wang *et al.,* 2003; Suchita *et al.,* 2007). However, xylanases are produced mainly by *Aspergillus* and *Trichoderma* sp. on an industrial scale. Microbial xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, and negligible substrate loss and side product generation (Beg *et al.,* 2001). Researchers are especially interested in fungal xylanases because they are secreted extracellularly and their activities are higher than the xylanases from yeasts and bacteria (Twomey *et al.,* 2003, Krisana *et al.,* 2005).

2. Biotechnological applications

Over the last few decades, there has been a growing interest in lignocellulose bioconversion as a renewable energy source so since their discovery, xylanases have generated considerable research interest. This is partly because of their promising biotechnological applications and because xylanases have shown an immense potential for increasing the production of several useful products in a most economical way (Kulkarni *et al.,* 1999; Beg *et al.,* 2001).

Among the biotechnological applications, it can be outstanding the bioconversion of lignocellulosic materials into fermentative products, improvement of digestibility of animal feedstock and clarification of juices, and facilitating the release of lignin from the pulp and reducing the amount of chlorine required for bleaching in pulp and paper industry (Beg *et al,* 2001, Wong *et al.,* 1993, Wong *et al.,* 1998, Twomey *et al.,* 2003, Sunna *et al.,* 1997). In addition, xylanases can be effectively used with cellulases to hydrolyze the lignocellulosic biomass in bioethanol production (Chandrakant *et al.,* 1998, Berlin *et al.,* 2006).

Depression in weight gain and feed conversion efficiency in rye-fed broiler chicks has been associated with intestinal viscosity. Incorporation of xylanase into a rye-based diet of broiler chickens results in reduced intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency (Bedford *et al.,* 1992, Vanparidon *et al.,* 1992). The enzyme has been used in the pretreatment of forage crops to improve the digestibility of ruminant feeds and to facilitate composting (Gilbert and Hazlewood, 1993).

The efficiency of xylanases in improving the quality of bread has been seen with an increase in specific bread volume. This is further enhanced when amylase is used in combination with xylanase (Maat *et al.,* 1992).

The xylanase has also been used for the extraction of coffee, plant oils, and starch (Wong and Saddler, 1992), in the improvement of nutritional properties of agricultural silage and grain feed (Kuhad and Singh, 1993), and in combination with pectinase and cellulase for clarification of fruit juices (Biely, 1985) and degumming of plant fiber sources such as flax, hemp, jute, and ramie (Kapoor *et al.,* 2001; Puchart *et al.,* 1999; Sharma, 1987).

Xylanase in synergism with several other enzymes, such as mannanase, ligninase, xylosidase, glucanase, glucosidase, etc., can be used for the generation of biological fuels, such as ethanol and xylitol, from lignocellulosic biomass (Dominguez, 1998; Kuhad and Singh, 1993; Olsson and Hahn-Hagerdal, 1996). The biological process of ethanol fuel production requires delignification of lignocellulose to liberate cellulose and hemicellulose from their complex with lignin, followed by depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars, and finally fermentation of mixed pentose and hexose sugars to produce ethanol (Lee, 1997). The hydrolysis of hemicellulose from lignocellulosic biomass in bioethanol production is important for the recovery of monosaccharides from the residual hemicellulose and for the removal of hemicellulose which otherwise restricts the access of cellulases to cellulose fiber (Berlin *et al.,* 2006).

Currently, the most effective application of xylanase is in prebleaching of kraft pulp to minimize use of harsh chemicals in the subsequent treatment stages of kraft pulp. Byproducts from using these chemicals are chlorinated organic substances, some of which are toxic, mutagenic, persistent, and

bioaccumulate, and cause numerous harmful disturbances in biological systems (Onysko, 1993). In response to government and environmental protection groups, paper industries are currently changing practices to minimize the use of chlorine-based chemicals (Beg *et al.,* 2001).

3. New developments in xylanase technologies

The availability of xylanases isolated from nature with the desired thermostability and pH characteristics is limited but the potential benefits of using these enzymes for biotechnological processes has encouraged widespread research endeavours towards producing desirable xylanases through protein engineering using techniques such as site-directed mutagenesis (Wakarchuk *et al.,* 1994; Georis *et al.,* 2000; Mesta *et al.,* 2001; Turunen *et al.,* 2001, 2002; Liu *et al.,* 2002; Fenel *et al.,* 2004) and directed evolution (Arase *et al.,* 1993; Chen *et al.,* 2001; Inami *et al.,* 2003; Palackal *et al.,* 2004).

Directed evolution has emerged as a successful alternative to rational design for genetic engineering of enzymes (Kuchner and Arnold, 1997; Williams and Berry, 2003). This methodology has been used to improve the existing properties of enzymes (Giver *et al.,* 1998). This revolutionary type of protein engineering technology mimics Darwinian evolution in nature and does not require extensive knowledge of the gene of interest, detailed information on protein structures, accurate predictions on amino acid substitutions at the proper sites and information about the catalytic mechanisms to guide the evolution of enzymes. It consists of iterative steps of random mutagenesis, screening and recombination (Arnold and Volkov, 1999, Kim *et al.,* 2003). It involves generating a vast library of the gene of interest by random mutagenesis such as error-prone polymerase chain reaction (PCR) or DNA shuffling, followed by screening mutants for desired properties. This approach has been particularly successful in improving the thermostability of proteins. The success of this strategy depends on the size, quality and diversity of the libraries and, crucially, on the sensitivity, efficiency and discriminatory power of the screening technique available (Fernandez-Gacio *et al.,* 2003; Turner, 2003).

An important step in a directed evolution experiment is to explore efficiently the sequence space through random mutagenesis. Among random

mutagenesis methods, error-prone PCR methods, based on the inaccurate amplification of genes, have been very successful and are generally used in directed evolution experiments due to their simplicity and versatility (Wong *et al.,* 2004).

Error-prone PCR is a random mutagenesis technique for introduction of alterations in the amino acid sequence. The mutations are introduced during PCR through the error-prone activity of the DNA polimerase under certain reaction conditions. The sequences of DNA are cloned in expression vectors and the mutant library is then screened for activity of the altered protein. This method has the advantage of the low fidelity of the Taq polimerase, in certain conditions, to incorporate wrong nucleotides from the DNA template. To increase the Taq DNA polymerase mistake, buffers containing Mn^{++} and no balanced concentrations of dNTPs are used in the PCR. This method is better applied if it is necessary to create moderate alterations in the gene and simultaneously to promote small alterations in the catalytic properties (Leung *et al.,* 1989; Cadwell and Joyce, 1992). The error-prone PCR technology has larger success when it is used to maintain some of the most desirable properties of a specific molecule. The use of random mutagenesis to explore the mechanism of thermostability is an attractive approach as this methodology introduces a small number of amino acid changes, which can be directly linked to increased stability.

The methodology of directed evolution can adapt enzymes of interest to several industrial processes, generating a significant impact in the agricultural industry, providing a more economical and responsible alternative for the biotechnological processes. Like the pulp-bleaching processes that are carried out at high temperature and under alkaline conditions, thermostable and alkali-tolerant xylanases are well suited for such industrial applications.

We focus our work in a xylanase (XynA) produced by the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2. Anaerobic fungi produce highly active hydrolytic enzymes (Borneman *et al.,* 1989) and that are now being recognized for their ability to effective degradation of plant biomass (Chen *et al.,* 1997).

Several enzymes of *Orpinomyces* PC-2 were shown to be superiors to the equivalent enzymes from others sources, mainly in the specific activity.

XynA from *Orpinomyces* PC-2 has a specific activity of 3.500 U/mg of protein with the xylan substrate from wood, while the specific activity of xylanases from other sources varies from 20 to 600 U/mg (Li *et al.,* 1997a,b). XynA has been studied by Li *et al.* (1997a,b) and tested for several industrial applications, in pilot scale. This enzyme presented extremely high activity with the xylan substrate of animal ration, cellulose pulp and other biomass types. Studies demonstrated that the use of the native enzyme has been more efficient in optimal conditions as pH 5.0-5.5 and temperature of 50-55 °C. However, many industrial processes are accomplished usually in elevated temperature and alkaline pH.

Directed evolution was applied to improve xylanase thermostability of *Orpinomyces* PC-2. With this purpose, a mutant library of *Orpinomyces* PC-2 xynA gene was created using error-prone PCR technique. The library, with a mutation level of 0.9%, contains mutant xynA genes cloned in a pET24b expression vector into competent *E. coli* Express Electocompetent BL21 (DE3) DUOs cells (Stratagene, USA).

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1) Melhoramento por evolução dirigida da termoestabilidade da xilanase xynA de *Orpinomyces* sp. PC-2

Abstract

Error-prone PCR has been used to improve the thermostability of enzymes with biotechnological applications and this method was applied to the endo-β-1,4-xylanase from *Orpinomyces* PC-2. The constructed library of xylanase (xynA) mutants was submitted to several screening cycles and the transformants were first exposed to 60 ºC during one hour and then the thermostable mutants were selected with the azo-xylan-agarose 0.2% pH 6.5 as substrate. Six mutants selected were sequenced and these amino acid sequences were analyzed to identify the mutations. Two mutants displayed higher stabilities than wild-type XynA. Whereas the wild-type lost 60% of its activity after 10 min at 60 ºC, mutants M4 and M6 showed enhanced thermostability and retained approximately 50% of its activities after treatment at 60 ºC for 60 min. In addition, M4 retained about of 40% of its initial activity after incubation at 75 ºC for 60 min. The mutants and the wild-type showed an optimal temperature and pH for xylanase activity at 60 ºC and pH range of 5.0-7.0.

Keywords: Error-prone PCR; *Orpinomyces*; Xylanase; Thermostability.

1. Introduction

Xylan is the most common hemicellulosic polysaccharide in cell walls of land plants, representing up to 30–35% of the total dry weight (Joseleau *et al.,* 1992). This polysaccharide is composed of a main-chain polymer made up exclusively of β-1,4 xylose residues. Chemical and structural diversity in xylans arises from the side chain decorations of the polymer (Smaali *et al.,* 2008). The backbone of β-1,4-linked xylopyranose residues possesses branches containing arabinofuranosyl, acetyl, and glucuronosyl residues (Biely, 1985).

Enzymatic hydrolysis of xylans is brought about by a variety of enzyme activities that are grouped under the generic term hemicellulases (Smaali *et al.,* 2008). The complete hydrolysis of xylan requires orchestrated actions of various enzymes including endoxylanase, β-D-xylosidase, α-glucuronidase, acetyl esterase and α-L-arabinofuranosidase (Beg *et al.,* 2001). Depolymerizing

endoxylanases (β-1,4-D-xylan xylanohydrolase , EC 3.2.1.8) hydrolyze internal β-1,4 bonds in the main-chain and generate soluble xylooligosaccharides.

A wide variety of microorganisms are known to produce xylan-degrading enzymes. A variety of microorganisms, including bacteria, yeast and filamentous fungi, have been reported to produce xylanase, in which the most potent producers are fungi (Haltrich *et al.,* 1996). Microbial xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, and negligible substrate loss and side product generation (Beg *et al.,* 2001). Researchers are especially interested in fungal xylanases because they are secreted extracellularly and their activities are higher than the xylanases from yeasts and bacteria (Twomey *et al.,* 2003, Krisana *et al.,* 2005).

Over the last few decades, there has been a growing interest in lignocellulose bioconversion as a renewable energy source so since their discovery; xylanases have generated considerable research interest. Among the biotechnological applications, it can be outstanding the 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 and reducing the amount of chlorine required for bleaching in pulp and paper industry (Beg *et al.,* 2001, Wong *et al.,* 1993, Wong *et al.,* 1998, Twomey *et al.,* 2003, Sunna *et al.,* 1997). In addition, xylanases can be effectively used with cellulases to hydrolyze the lignocellulosic biomass in bioethanol production (Chandrakant *et al.,* 1998, Berlin et al., 2006).

Most of the xylanases show optimal activities at neutral or acidic pH and at temperatures below 45 °C (Eyzaguirre *et al.,* 1992, Krisana *et al.,* 2005, Leskinen *et al.,* 2005). Xylanases that are active at higher temperatures are of great interest in industries. The availability of xylanases isolated from nature with the desired thermostability and pH characteristics is limited but the potential benefits of using these enzymes for biotechnological processes has encouraged widespread research endeavours towards producing desirable xylanases through protein engineering using techniques such as site-directed mutagenesis (Wakarchuk *et al.,* 1994; Georis *et al*., 2000; Mesta *et al.,* 2001; Turunen *et al.,* 2001, 2002; Liu *et al.,* 2002; Fenel *et al.,* 2004) and directed evolution (Arase *et al.,* 1993; Chen *et al.,* 2001; Inami *et al*., 2003; Palackal *et al.,* 2004).

Directed evolution has emerged as a successful alternative to rational design for genetic engineering of enzymes (Kuchner and Arnold, 1997; Williams and Berry, 2003). This methodology has been used to improve the existing properties of enzymes (Giver *et al.,* 1998). Among random mutagenesis methods, error-prone PCR methods, based on the inaccurate amplification of genes, have been very successful and are generally used in directed evolution experiments due to their simplicity and versatility (Wong *et al.,* 2004).

Xylanase (XynA), produced by the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2, was shown to be superior to the equivalent enzyme from others sources, mainly in the specific activity. XynA from *Orpinomyces* PC-2 has a specific activity of 3.500 U/mg of protein with the xylan substrate from wood, while the specific activity of xylanases from other sources varies from 20 to 600 U/mg (Li *et al.,* 1997a,b).

Here we have used a library constructed by the error-prone PCR methodology to identify thermostable xylanase variants from *Orpinomyces* PC-2. Mutants with improved properties were selected through several screening cycles accomplished at this xynA mutant library. This study was conducted to explore the possibility of using random mutagenesis and screening to improve both the thermostability and activity of the *Orpinomyces* xylanase to render an enzyme better suited to industrial applications.

2. Material and methods

2.1. Material

The xynA mutant library and the wild-type were given by the USDA/ARS/NCAUR, Peoria, IL, USA. The xynA library used for selection of the mutants possesses only the catalytic domain cloned in the plasmid. Oat spelt azo-xylan was purchased by Megazyme (Wicklow, Ireland). The birchwood and the oat spelt xylans were purchased by Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Screening for thermostable xylanase mutants

The xylanase activity tests on agar plates were used for screening of mutant colonies using the error-prone PCR xynA library. Top agar containing oat spelt azo-xylan 0.2% (Megazime) was used to isolate xylanase-producing clones. *E. coli* library cells were thawed on ice and diluted into up 10^{-5} with Luria Bertani (LB) broth and 100 µL of diluted library were plated onto LB medium with agar containing kanamycin (50 µg/mL). The Petri dishes (100 mm) with each plate with 10.000 colonies were incubated at 37 °C for 12 h and left at room temperature for one hour. A replicate set of dishes were made using sterilized velvet towels. The replicate set was incubated at 37 °C for 3 h and saved at 4 °C. The original set of dishes was left in a 60 °C incubator for exactly 1.0 h, transferred to a refrigerator for an additional 1.0 h and then transferred to room temperature for 30 min. Then, each dish received 15 mL solution equilibrated at 48 °C containing 1.5% agarose, 0.2% azo-xylan, and 100 mM sodium phosphate buffer, pH 6.5. After solidification, the dishes were incubated in a 37 °C incubator for between 2.0-6.0 h. A small area (~2.0 mm diameter) of the dish corresponding to a colony with a large blue zone indicated xylanase-producing clones. The colonies that showed xylanase activity were identified in the replicate set of dishes and were picked with a disposable plastic inoculation loop and transferred to microtubes containing 100 µL LB broth. The tubes containing the agar pieces were vortexed and 10 µL supernantant were streacked to a new petri dish containing kanamycin as decribed above and a secondary screening was done to purify the colonies with remained xylanase activity after 60 °C for 1 h.

2.3. Sequence analysis of xynA mutants

Well separated mutant active colonies with more thermostable xylanase and the wild-type colony were inoculated into 5 mL liquid LB/Kan broth and shaken at 180 rpm, 37 °C for 12 h (New Brunswick, Scientific Co., Edison, NJ, USA). The plasmidial DNA was purified from 5.0 mL culture (QIAprep Spin Miniprep Kit, Santa Clarita, CA) and subjected to DNA sequencing. Nucleotide sequences of insert DNA were determined with an automatic PCR sequencer

by Macrogen (Gasan-dong, Geumchun-gu, Seoul Korea). T7 promoter and T7 terminator universal primers were used to sequence both strands of the inserts. DNA sequencing data was initially processed using the Sequencher software package (version 4.1.4, Gene Codes Corporation) and both DNA strands were edited to yield the complete gene sequences. The DNA sequences were then translated into their protein counterparts and compared to the wild-type parent using the CLUSTALW (Version 1.81) alignment program (http://www.ebi.ac.uk/clustalw).

2.4. Production of xylanases

The selected thermostable colonies and wild-type colony were cultivated in 5 mL of LB broth containing kanamycin (50 µg/mL), incubated at 37 ºC, 180 rpm, overnight. Soon afterwards, 10 µL of this culture were plated in LB/Kan plates and incubated at 37 ºC, overnight, to obtain isolated colonies. One isolated colony of each mutant and from the wild-type was inoculated in 4 mL of LB/Kan broth and incubated at 37 ºC, 180 rpm, overnight. Of these 4 mL, a volume of 0.5 mL of each culture was removed and added to 5 mL of SOB medium (triptone 2%, yeast extract 0.5%, NaCl 0.05%, KCl 2.5 mM, $MgSO_4$ 0.01 M, NaOH 0.5 mM) containing IPTG (isopropyl-β-D-thiogalactopyranoside) and kanamycin (1.0 mM and 0.1 g/L respectively). The cells were induced to produce xylanase by adding IPTG to the flasks and further left in the shaker (New Brunswick) at 37 ºC, 180 rpm for 6 h. Following induction, the cultures were put on ice during 20 min and then centrifuged at 4 ºC, 4.000 rpm during 20 min (Thermo Scientific Jouan BR4i, Thermo Scientific), and the clear supernatant maintained at 4 ºC. This supernatant serves as crude xylanase preparations for measurement of levels of xylanase production and thermostability of the crude xylanase mutants.

2.5. Enzymatic assay

The supernatant was used to enzymatic assay to test the activity, effect of temperature and pH, thermostability and pH stability of the xylanase variants and the wild-type, which were analyzed in triplicate.

Xylanase activity was determined by measuring the release of reducing sugars from birchwood xylan (1% w/v, pH 6.5) by Miller method using dinitrosalicylic acid reagent (DNS) (Miller, 1959). Xylanase activity was assayed using birchwood xylan (Sigma Chemical Co) as the substrate, which was preliminarily suspended homogeneously in a 100 mM sodium phosphate buffer, pH 6.5. The reaction mixture containing the enzymatic sample and substrate was incubated at 40 °C for 30 min. After incubation 0.5 mL of DNS reagent was added to the reaction mixture, boiled for 5 min and then cooled. Absorbance was measured at 540 nm using a UV–Vis spectrophotometer (DU-65 Spectrophotometer Beckman). The absorbance was converted into μmoles of reducing sugar produced, using a standard curve generated with xylose. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per min at 40 °C.

Substrate specificities of the xylanase were determined using the following substrates: 1% birchwood xylan (w/v) and 1% oat spelt xylan (w/v) prepared in 100 mM sodium phosphate buffer (pH 6.5). The enzyme activity was estimated as described previously.

2.6. Effect of temperature, pH, thermostability and pH stability

The effect of temperature in the xylanase activity was measured under various temperatures (ranging from 20–80 °C in 5 ºC of increment), at pH 6.5, according to the standard method described earlier.

In order to monitor thermal stability, samples of the supernatant were pre-incubated for fixed period of time until 1 h at 60 °C. Aliquots were removed at timed intervals and cooled on ice before assaying to determine the residual enzyme activity using the standard assay procedure. Enzymes that were more thermostable at 60 ºC, were assayed at higher temperatures (65, 70, and 75 ºC). Mutants and the wild-type were analyzed in triplicate. To determine the

percentage residual activity after heat treatment, activities of the enzymatic samples without pre-incubation were considered as 100%, and enzyme activities were expressed as percentages of the untreated sample. The wild-type XynA served as the control. The estimated half-lifes for the mutants and the wild-type XynA were determined using the Curve Expert 1.3 program.

The pH profile of XynA was determined at 40 °C, (ranging from 2–11 in one unit of pH increment) using different buffers (sodium phosphate buffer for pH 2.0; 3.0; 7.0; 8.0; sodium citrate buffer for pH 4.0-6.0; and sodium carbonate buffer for pH 9.0-11.0, all of these at the concentration of 100 mM in the assay final volume). To analyze the effect of the pH on xylanase activity, the enzymatic sample was diluted with each buffer of different pH and then the activities of the treated enzymes were measured by the standard assay procedure. The mutants and the wild-type were analyzed in triplicate.

To investigate the pH stability of the wild-type and one mutant, samples of these enzymes were pre-incubated in different buffers ranging from 2.0 to 11.0 at 4 ºC for 1 h. Fractions were collected and the residual activity of treated enzymes was measured under standard assay.

3. Results and discussion

3.1. Screening, sequence analysis and enzyme activity

The xynA library created by the technique of error-prone PCR was submitted to several screening cycles to identify clones with increased thermostability. Initially, six positive clones were selected, which exhibited blue zone on azo-xylan-agarose-LB plates after hydrolysis of xylan. This substrate is insoluble in buffered solutions, but rapidly hydrates to form gel particles which are readily and rapidly hydrolyzed by specific endo-hydrolases releasing soluble dye-labeled fragments. Blue areas indicating xylanase activity was observed in the mutant colonies with higher thermoestability (Figure 1).

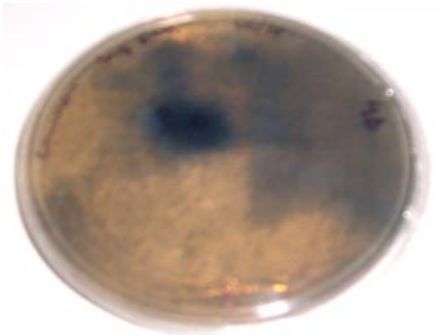

Figure 1: Agar plate containing azo-xylan-agarose. Blue areas indicate xylanase activity in the mutant colonies with higher thermoestability.

Theoretically, xylanase secreted by colonies capable of acting on azo-xylan after the incubation at 60 ºC for 1 h presented improved thermal stability, because these incubation conditions inactivated wild-type XynA. The xylanase activities of six clones (M1-M6) produced larger zones of hydrolysis than the control.

The success of the directed evolution strategy depends on the size, quality and diversity of the libraries and, crucially, on the sensitivity, efficiency and discriminatory power of the screening technique available (Fernandez-Gacio *et al.,* 2003; Turner, 2003). Therefore, it is required a quick and sensitive screening system. With the blue zone assay, the formation of a complex between the dyes and sugars enables us to detect using the naked dye enzyme-producing colonies. Using this screening method, the variants with improved thermostability compared to the parental enzyme were easily screened.

DNA sequencing was carried out to identify the mutations that were responsible for the observed changes in the thermal stabilities of some xylanase variants. The DNA sequences were translated into their protein counterparts and aligned to the wild-type parent to identify the mutations (Figure 2, Table 1).

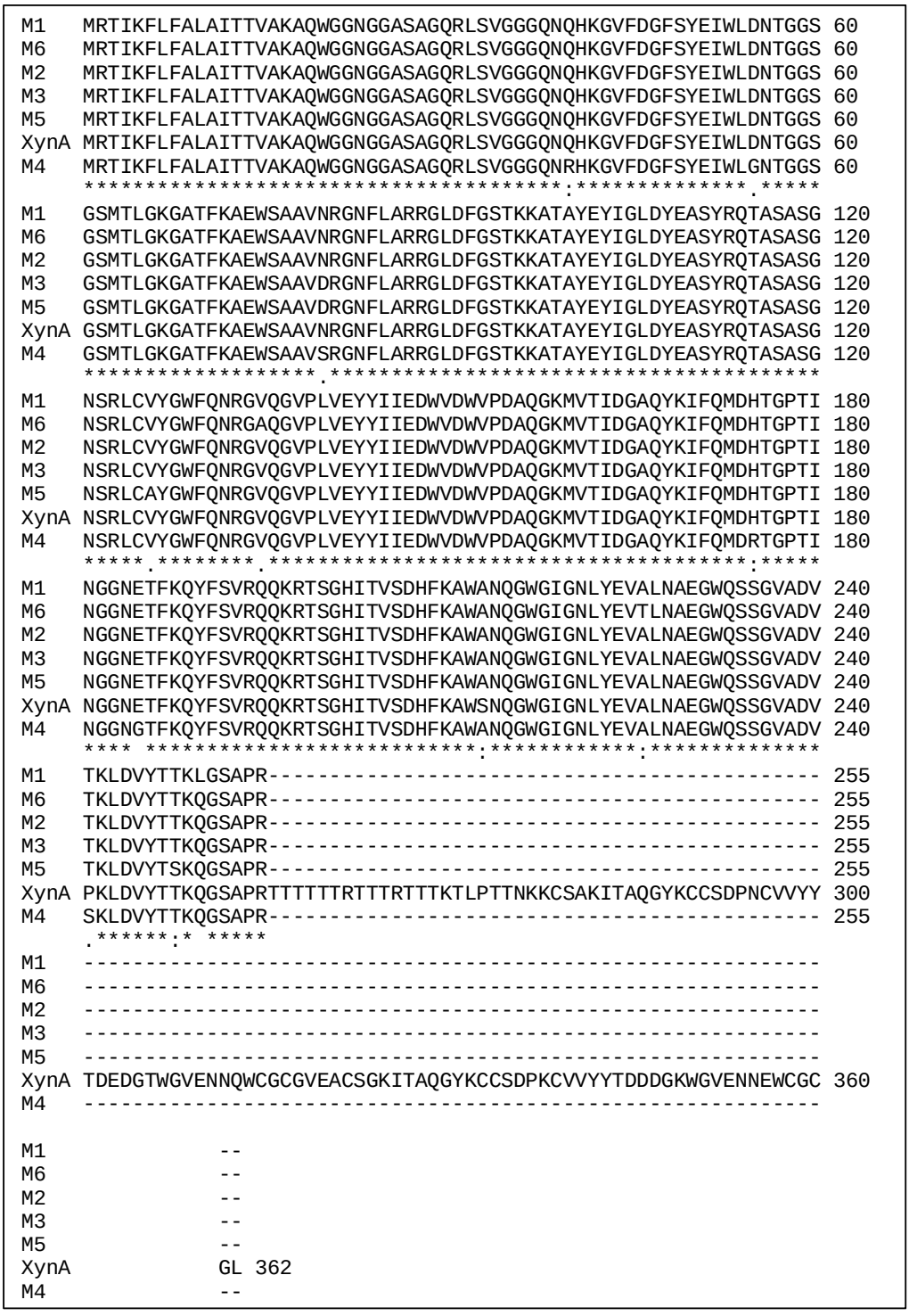

Figure 2: Protein sequence alignment of mutants (M1-M6) with each other and with the wild-type (XynA) from the data base (accession number AAD04194). The mutants possess only the catalytic domain cloned in the plasmid. The alignment was performed by using the CLUSTALW (Version 1.81) alignment program on the server (http://www.ebi.ac.uk/clustalw). Alignment sequence characters are indicated as follows: '*' indicates positions which have a single, fully conserved residue; ':' shows conservation within a strong group of amino acids; '.' indicates conservation within weaker groups of amino acids. The absence of an alignment character implies that an unrelated amino acid was substituted.

Table 1: Mutants with its changed amino acids and its respective xylanase activity.

Mutants	Number of mutations	Inicial amino acid/ Position/Changed amino acid	Xylanase Activity (U/mL ± SD)
M1	3	S/213/A P/241/T Q/250/L	0.668 ± 0,008
M2	2	S/213/A P/241/T	0.602 ± 0,002
M3	3	N/80/D S/213/A P/241/T	0.671 ± 0,004
M4	7	Q/40/R D/55/G N/80/S H/175/R E/185/G S/213/A P/241/S	0.071 ± 0,002
M5	5	N/80/D V/126/A S/213/A P/241/T T/248/S	0.574 ± 0,013
M6	4	V/135/A S/213/A A/226/T P/241/T	0.736 ± 0,011
WT	-	-	0.457 ± 0,011

Sequence analysis of the selected mutants showed that the number of amino acid substitutions varied for each mutant and the mutations were not concentrated in any particular region of the protein and exhibited much variation. M1 and M3 showed 3 mutations, M2 showed 2, M4 showed 7, M5 showed 5 and M6 showed 4 substitutions. It is interesting to note that all of the mutants possess 2 mutations in common (S213A, P241T), corresponding to the mutant M2 (Table 1).

The error-prone PCR was also used to improve the thermostability of the xylanase from *Thermomyces lanuginosus* (Stephens *et al.,* 2007). These authors observed that sequence analysis of second generation mutants revealed that mutations exhibited variation: mutant 2B7-6 sequence differed

from the wild-type in three amino acids (L18P, A193S, H201Y), whereas mutants 2B11-16 (D72G) and 2B7-10 (Y58F) had single mutations. Phytases was also submitted to directed evolution and sequence analysis of the selected mutants showed that the number of amino acid substitutions varied from one to four per mutant (Kim *et al.,* 2008).

The xylanase activities of the mutants, when assayed using xylan from birchwood as substrate, were higher than the wild-type activity. The M4 was an exception showing an activity about 6 times smaller than the control activity (Table 1).

Kim *et al.* (2008) showed that specific activity of five mutants was lower than that of the wild-type, except for one mutant. It is interesting to emphasize that the impacts of individual mutations on stability of a given protein are not necessarily additive or synergistic (LiCata and Ackers, 1995).

3.2. Thermostability

The thermostability assays showed that the mutants M1, M2, M3 and M5 lost 80% of its activities after incubation at 60 ºC for 1 h (Figure 3). At this incubation temperature, M5, M3, M2 and M1 retained about 60, 40, 40 and 30%, respectively, of its activities after 10 min of incubation and drastic reduction in enzyme activity was found after 20 min of incubation. At this point, while the others mutants present a basal activity, the M6 retained 60% and M4 retained 65% of its activities. The mutants M4 and M6 maintained about of 50% of its activities after incubation at 60°C for 1 h. The M4 and M6 residual activity values decreased slightly, about 10%, from 20 to 60 min of incubation, characterizing these mutants like the more thermostable ones. The estimated half-lifes for M1, M2, M3, M4, M5 and M6 at 60 ºC were 5.66 min; 8.58 min; 6.42 min; 50.63 min; 12.10 min and 35.09 min respectively (Figure 3).

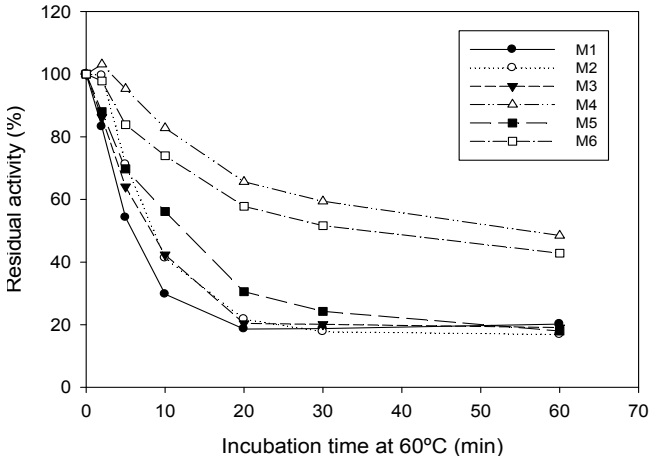

Figure 3: Thermal stability. Effect of temperature on the stability of the xylanase mutants (M1-M6). Enzyme samples were heated at 60 ºC for different period of time, collected, placed on ice, and residual xylanase activity determined using the standard assay procedure at 40 °C.

The wild-type was used as a control and lost 70% of its activity after 1h of incubation at 60 ºC and its estimated half-life at 60ºC was 8.00 min (Figure 4).

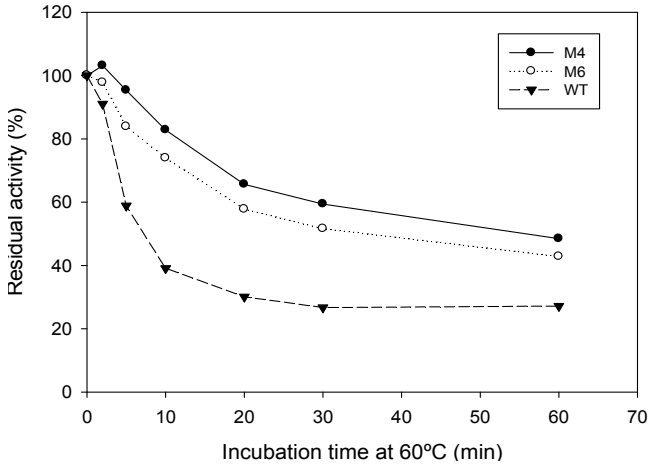

Figure 4: Thermal stability. Effect of temperature on the stability of M4, M6 and wild-type (WT). Enzyme samples were heated at 60 ºC for different period of time, collected, placed on ice, and residual xylanase activity determined using the standard assay procedure at 40 °C.

Since the mutants M4 and M6 presented higher thermostability, they were challenged to higher temperatures assays. A thermostable variant was successfully obtained. M4 was assayed at 70 ºC and 75 ºC retaining about 50% and 40% of its activity after incubation for 1 h (Figure 5). At 70 ºC an activity drop was observed at 2 min but at 10 min of incubation, M4 still shows a considerable activity value, little less of 60%, maintaining this residual activity during the limit incubation time. When the thermostability assay was conducted at 75 ºC, the M4 lost about 60% of its activity at 20 min and maintained this residual activity until 1 h of incubation (Figure 5). In this way, M4 exhibited promising features that make it a strong candidate for future industrial applications.

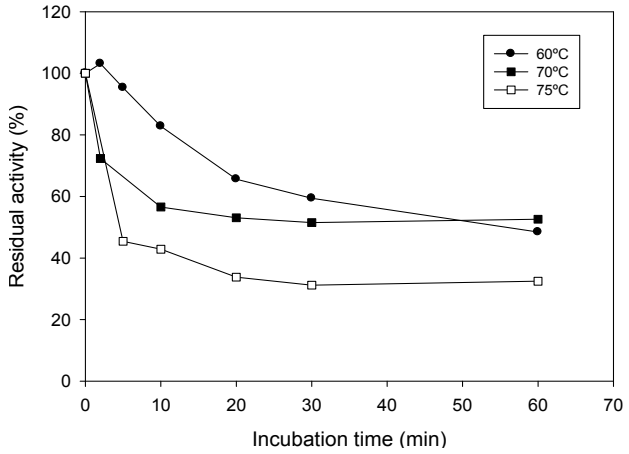

Figure 5: Thermal stability. Effect of temperature on the stability of M4. Enzyme samples were heated at the relevant temperatures for different period of time, collected, placed on ice, and residual xylanase activity determined using the standard assay procedure at 40 °C.

M6 did not support so elevated temperatures. At 65 ºC its activity dropped at the first 5 min of incubation and after 1 h, M6 retained only 20% of its activity and its estimated half-life at 65 ºC was 6.37 min (Figure 6).

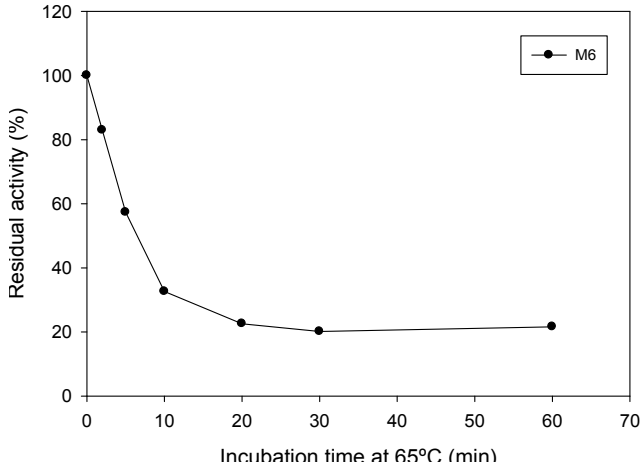

Figure 6: Thermal stability. Effect of temperature on the stability of M6. Enzyme samples were heated at the 65 ºC for different period of time, collected, placed on ice, and residual xylanase activity determined using the standard assay procedure at 40 °C.

The methodology of directed evolution was also used to enhance the thermostability of xylanase from *Bacillus subtilis*, and a thermostable variant, Xylst was identified which contained three amino acid substitutions (Q7H, N8F, S179C). At 60 °C the wild-type enzyme was inactivated within 5 min, but Xylst retained full activity for at least 2 h besides increasing the optimum reaction temperature from 55 to 65 °C (Miyazaki *et al.,* 2006).

The random mutagenesis was also used to improve the alkaline and thermostability of the already thermostable xylanase (XynA) from *Thermomyces lanuginosus*. The most stable mutant generated by initial random mutagenesis of xynA was G41 (K30E, W40R, T57A and K80R), which displayed 75% retention of its total activity after 90 min of heat treatment (Stephens *et al.,* 2009). In this case, the error-prone PCR was applied in an enzyme that it is already thermostable, to create a robust xylanase for industrial application, like paper industry. Like this, it could be shown that the thermostability of a xylanase from a thermophilic fungus can be significantly increased using error-prone PCR, which offers a fast, effective means of generating mutants with superior traits.

Of the selected mutants, the M4 and M6 were the more thermostable ones. M4 has a total of seven mutations and M6 has four mutations which

cumulatively conferred considerable thermal stability to the enzymes, albeit the poor catalytic activity of M4 (Table 1). It can be observed that the use of random mutagenesis to explore the mechanism of thermostability is an attractive approach as this methodology introduces a small number of amino acid changes, which can be directly linked to increased stability.

Researchers speculate the inverse relationship between activity and thermostability. Stephens *et al.* (2007) used the error-prone PCR to improve the thermostability of the xylanase from *T. lanuginosus* and they observed that when new mutations were introduced to a few improved first generation clones it resulted in most of the second generation mutants having very reduced xylanase activity. Previous work also agrees with these observations where improvements in thermal stability have deleteriously affected catalytic activity (Schoichet *et al.,* 1995; Palackal *et al.,* 2004; Fenel et al., 2006; Stephens *et al.,* 2007). It seems that reduced flexibility is a necessary consequence of thermostabilization (Stephens et al., 2009) and enzymes can lose their activity if they are mutated to improve stability (Arnold *et al.,* 2001). This profile was followed in our work since M4 was the mutant with the best thermostability presenting smaller activity (Table 1). This behavior is not a rule since enzymes with increased specific activity and thermostability could be found as it was observed by Stephens *et al.* (2007) where the best mutant selected (2B7-10; Y58F) showed these characteristics.

Often either thermostability or catalytic activity is impaired and it is indeed rare, but not impossible, to improve both properties simultaneously in a single enzyme. In a study conducted on the laboratory evolution of the p-nitrobenzyl esterase from *Bacillus subtilis*, there was coevolution of both activity as well as thermostability in this enzyme (Giver et al., 1998).

In a noteworthy observation, Stephens *et al.* (2009) noted that the catalytic activity of the more alkaline stable variants xylanases was comparable with wild-type (XynA), which is in complete contrast to the observations regarding thermostability in the study. G53 (A54T, the best alkali stable variant) had catalytic activity (197 $nkat.mL^{-1}$) almost comparable to XynA (216 $nkat.mL^{-1}$), while G41 (K30E, W40R, T57A and K80R, the most thermostable variant) had a much lower activity (22 $nkat.mL^{-1}$). Previous studies on the alkaline stability of other xylanases showed similar findings (Chen *et al.,* 2001; Turunen

et al., 2002; Palackal *et al.,* 2004). It appears that structural adaptation for temperature stability exerts a greater effect of lowering the catalytic activity compared with alkaline stability adaptation.

3.3. Effect of temperature

The optimum temperature for xylanase activity for each mutant (M1-M6) remained unchanged as that of wild-type XynA. This value was found to be 60 °C. Enzyme activity was reduced below 50 ºC and above 65 ºC, except for M6 that kept about 90% of its activity at 65 ºC. Drastic reduction in enzyme activity was found at 70 ºC. Except for the more thermostable mutants, M4 and M6, which retained about 40% of its activities at 70 ºC (Figure 7).

This result demonstrates that the identified mutations are not responsible for moving the temperature range where these enzymes have maximum performance. Similar results were observed for AppA2 phytase (Kim *et al.,* 2008). Error-prone PCR was applied to improve phytase thermostability and the optimum temperature between mutants and the wild-type was the same, in spite of the selected mutant enzymes had improved heat stability over the wild-type enzyme.

Most of the xylanases show optimal activities at neutral or acidic pH and at temperatures below 45 ºC (Eyzaguirre *et al.,* 1992, Krisana *et al.,* 2005, Leskinen *et al.,* 2005). The xylanase from *Aspergillus ficuum* AF-98 and *Trichoderma reesei* were most active at 45 ºC (Fengxia *et al.,* 2008; Tenkanen *et al.,* 1992). Optimum temperature for both xylanases from *Aspergillus caespitosus* was 50–55 ºC (Sandrim *et al.,* 2005). The XynA mutants from *Orpinomyces* PC-2 showed a higher optimum temperature than many xylanases from others sources, indicating a potential application of these enzymes. On the other hand, the methodology of directed evolution was capable to enhance the thermostability and also to increase the optimum reaction temperature of the xylanase from *Bacillus subtilis* from 55 to 65 ºC (Miyazaki *et al.,* 2006), showing that this methodology can improve the performance of enzymes.

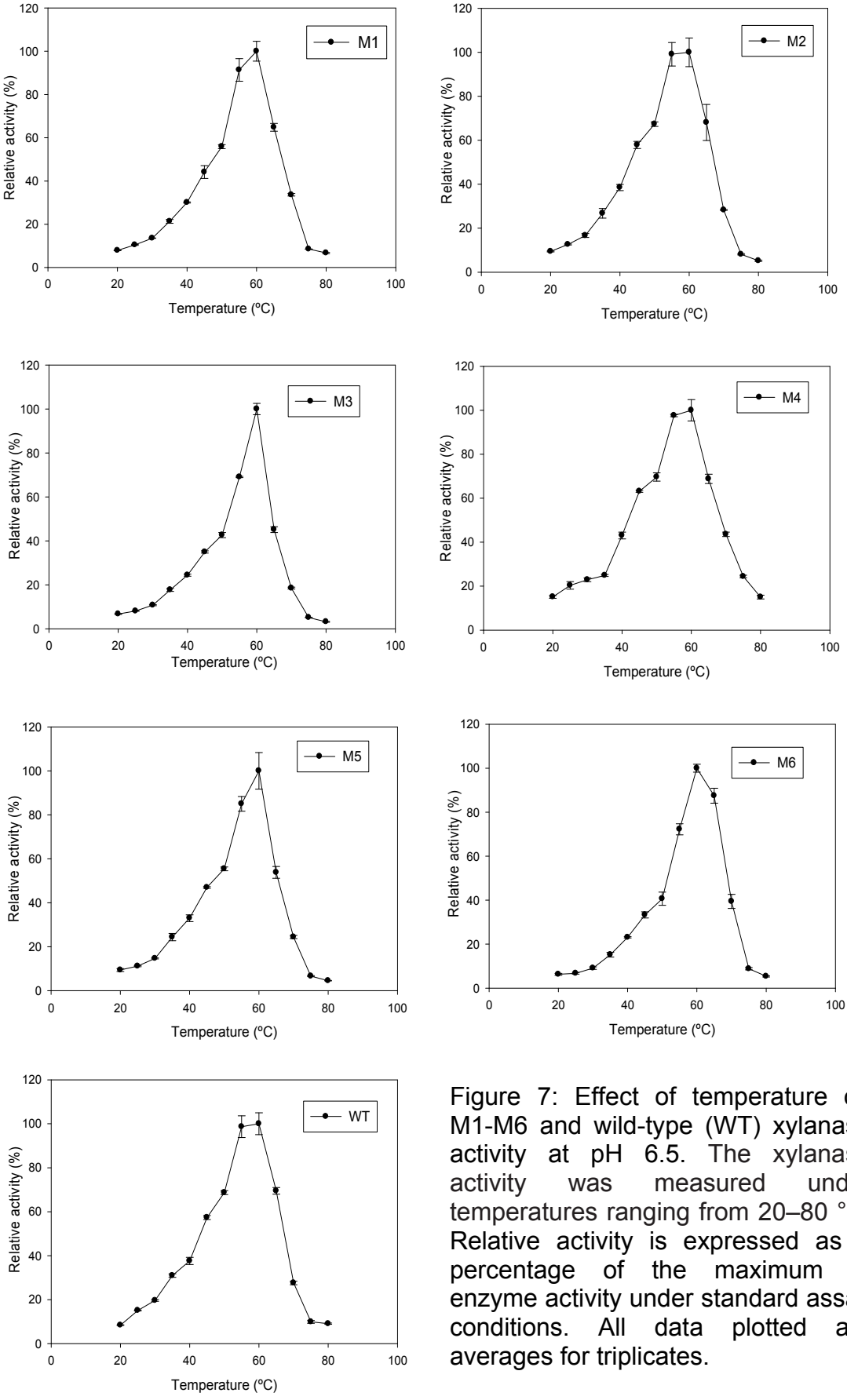

Figure 7: Effect of temperature on M1-M6 and wild-type (WT) xylanase activity at pH 6.5. The xylanase activity was measured under temperatures ranging from 20–80 °C. Relative activity is expressed as a percentage of the maximum of enzyme activity under standard assay conditions. All data plotted are averages for triplicates.

3.4. Effect of pH and stability

The mutants and the wild-type showed similar pH profiles (Figure 8).

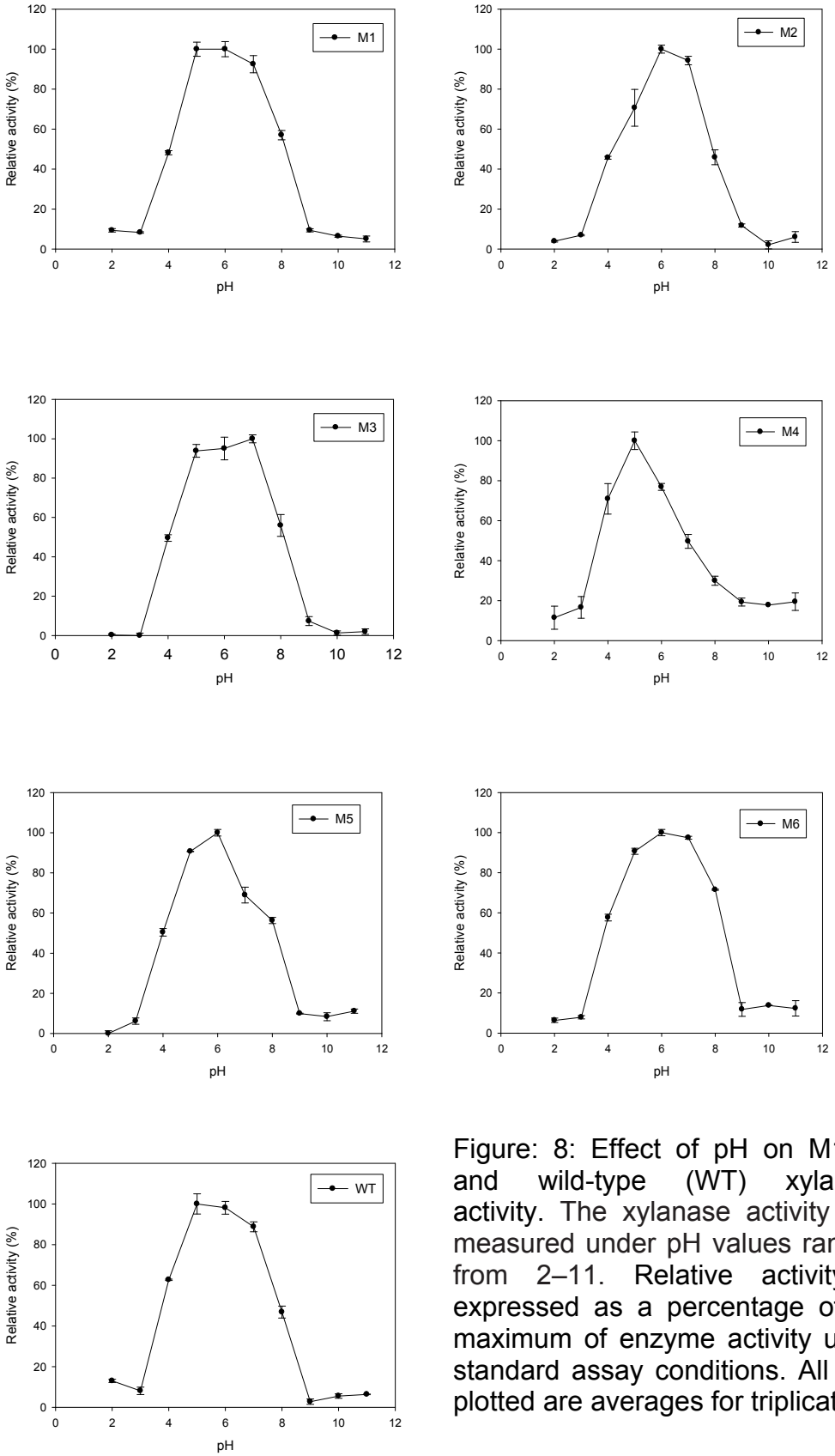

Figure: 8: Effect of pH on M1-M6 and wild-type (WT) xylanase activity. The xylanase activity was measured under pH values ranging from 2–11. Relative activity is expressed as a percentage of the maximum of enzyme activity under standard assay conditions. All data plotted are averages for triplicates.

The enzymes presented an optimum pH for xylanase activity in a pH range of 5.0-7.0. A significant decrease in enzyme activity was observed below pH 4.0 and above pH 8.0. Sudden decrease in the enzyme activity was seen at pH 9.0. M4 showed a different profile, the significant decrease in enzyme activity was observed above pH 7.0, but was observed a small activity (20%) at pH 9.0-11.0. M5 and M6 also presented about 10% of activity in a pH range of 9.0-11.0. The activity profiles suggest that these enzymes are more suitable for any application in the acidic and neutral conditions.

The xylanase from *Orpinomyces*, in agreement with most of the xylanases (Eyzaguirre *et al.,* 1992, Krisana *et al.,* 2005, Leskinen *et al.,* 2005), show optimum activity at neutral or acidic pH. The favorable pH range for xylanase activity of *A. foetidus* MTCC 4898 was 4.6–5.6, with optimum pH 5.3 (Shah *et al.,* 2005). Costa Ferreira *et al*. (1994) also reported optimum pH around 5.0 for xylanases of *A. niger*, with favorable pH range between 4.0 and 6.0. The optimum pH of *A nidulans, A. kawachii* and *A. usamii* are 6.0, 5.5 and 4.6 respectively (Fengxia *et al.,* 2008). In another investigation, *A. ochraceus* NG-13 mutant strain had relatively broader pH range (4.5–7.5), with optimum pH 6.0 (Biswas *et al.,* 1990).

Preliminary assays to investigate the pH stability of the WT and M6 enzymes were done. In the assay conditions, both enzymes, the wild-type and the mutant, maintained its original activities when assayed in optimum pH, after pre-incubation in several pH values, suggesting a possible renaturation of the enzymes.

The results about the effects of pH in the enzymatic activity suggest that the condition used for screening on plate (pre-incubation at 60 ºC during 1 h) probably favored the isolation of mutants with only higher thermostability.

It has been told that is not an easy work to identify a mutant that is thermostable and alkali tolerant. Stephens *et al*. (2009) emphasized that after screening the entire mutant library, only mutant G41 (K30E, W40R, T57A and K80R) was sufficiently thermostable while mutant G53 (A54T) was extremely alkali tolerant. In contrast, G41 was found to have low alkaline stability at pH 10 and G53 displayed dismal thermal tolerance at 80 ºC.

3.5. Substrate specificity

The mutants and the wild-type showed a discrete higher activity when assayed against the oat spelt xylan. M1, M3 and M6 presented higher activity values against the oat spelt xylan while M2 and M5 showed practically the same activity against the two analyzed substrates. M4 was an exception because its activity was higher with the birchwood xylan but the activity difference among the two substrates was very small. So, due to this small activity difference, we can conclude that these enzymes are effective against both substrates.

The activities of the xylanase mutants against both substrates were higher than the wild-type. The M4, the more thermostable variant, was an exception, showing an inferior activity value, if compared to the control activity. M5 also showed a smaller activity but very close to the wild-type activity, when assayed with oat spelt xylan (Table 2).

Table 2: Xylanase activities of wild-type and mutant enzymes against birchwood and oat spelt xylans.

Xylanases	Activity (U/mL ± SD) against birchwood xylan	Activity (U/mL ± SD) against oat spelt xylan
M1	0.416 ± 0,013	0.542 ± 0,002
M2	0.420 ± 0,006	0.423 ± 0,021
M3	0.480 ± 0,005	0.557 ± 0,005
M4	0.022 ± 0,001	0.010 ± 0,005
M5	0.370 ± 0,006	0.382 ± 0,027
M6	0.452 ± 0,013	0.548 ± 0,010
WT	0.271 ± 0,007	0.407 ± 0,011

The key factors that influence the rate of xylan hydrolysis are chain length and degree of substitution. There is also little information about the effect that xylan structure has on the activities of different xylanases. An understanding of the key factors that influence the rate of xylan hydrolysis by xylanase enzymes will lead to a better choice of enzymes in a given application (Li *et al.,* 2000).

Over 90% of birchwood xylan is composed of xylose and small amounts of glucose and galactose. The oat spelt xylan contains xylose and arabinose

with minor amounts of glucose and galactose. Birchwood xylan was shown to have 2,4-linkages as well as 1,4-linkages between xyloses (Li *et al.,* 2000).

Lee *et al*. (2009) showed that the purified *L. sulphureus* xylanase presented highest activity on birchwood xylan and that the relative xylanase activity on oat spelt xylan was only 22.3%. Among the substrates tested, purified *L. sulphureus* xylanase efficiently hydrolyzed the birchwood xylan that was composed mainly of xylose. The efficiency of hydrolysis reduced with decreasing amounts of xylose, as in oat spelt xylan.

Two family 11 xylanases, *Orpinomyces* PC-2 xylanase and *Trichoderma longibrachiatum* xylanase, were studied by Li *et al.* (2000). It was observed that these two xylanases can rapidly hydrolyze xylans that have a chain length greater than 8 xylose residues, and their hydrolytic rates are not sensitive to substituents on the xylan backbone. These enzymes behave similarly showing roughly the same activity among the birchwood and oat spelt substrates.

Different xylanases have different activities against various xylan structures. Li *et al. (*2000) also analyzed a family 11 xylanase from *Aureobasidium pullulans* and concluded that this enzyme is most effective on xylans that have a long chain (greater than 19 xylose residues), and also is effective against substituent groups. Although *Thermatoga maritima* xylanase is also more active on a long xylan chain (greater than 19 xylose residues), its hydrolytic rate is greatly reduced by substituents on xylan backbones.

Thus, the mutants and the wild-type xylanases from *Orpinomyces* appeared to efficiently hydrolyze 1,4- linkage of xylose in xylan molecules and they are also effective against xylans that possess substituents such as arabinose, glucose, and galactose, showing that these enzymes are effective against the birchowood and the oat spelt substrates.

With this work it can be emphasized the efficiency of the error-prone PCR technique and the importance of accomplishing new cycles of mutations in the mutants already selected, mainly in M4 and M6, in the attempt to achieve more appropriate xylanases to biotechnological processes.

It is also interesting the use of the site-directed mutagenesis to obtain more thermostable mutants. Sequences and crystal structure alignments studies have been showing that highly thermostable xylanases have an increased number of charged residues, especially arginine, resulting in

enhanced polar interactions (Hakulinen *et al.,* 2003). For this reason, it is intended to introduce in M2, the mutant that possesses the mutations in common S213A and P241T, through site-directed mutagenesis, the identified arginines in M4 (Q40R and H175R). As the mutant M6 also presented considerable thermostability, it is interesting that the new mutant possesses the alteration A226T, that it is suspected that it contributes for the highest thermal stability of the molecule. It is also planned the introduction of the arginines (Q40R and H175R) in the mutant M6, the second mutant with higher thermostability.

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2) Predição da estrutura tridimensional de XynA e dos mutantes mais termoestáveis

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1. Introduction

Model building by homology is a useful technique for predicting the structure of a target protein of known sequence, when the target protein is related to at least one other homologous protein of known sequence and structure. If the proteins are closely related, the known protein structures -called the parents- can serve as the basis for a model of the target. If a protein of unknown structure has homologues of known structure with 40% identical residues or higher in an optimum alignment, homology modeling methods are likely to produce a nearly complete structural model and the model will have sufficient accuracy to be useful for many applications. The quality of the model is likely to be good enough to interpret the protein's function. (Lesk, 2008).

At this work we studied the xylanase (XynA) produced by the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2. Mutants with improved properties were selected through several screening cycles accomplished at a library of xynA mutant created by the error-prone PCR technique. This study was conducted to explore the possibility of using random mutagenesis and screening to improve the thermostability of the *Orpinomyces* xylanase to render an enzyme better suited for industrial applications.

The selected thermostable mutants and the wild-type were submitted to the structural modeling in order to create a comparative modeling of three-dimensional structures to study structure/function relationship to identify possible mutations that confer thermostability and to predict the structural basis for the biochemical properties of xylanase.

2. Material and method

2.1. Structural modeling

To investigate the structure/function relationship between the mutants and the wild-type an analysis of homology or comparative modeling of three-dimensional structures was accomplished. Initially, to predict the structure of the wild-type xylanase and the mutants from *Orpinomyces,* a search in a database (http://www.rcsb.org – PDB – protein data bank) was done to identify a related

xylanase, one of the same family of the xylanase from *Orpinomyces* (hydrolase family-11, previously family G), that presented its three-dimensional structure described and an amino acid identity about 50% with the XynA of interest. Once identified the xylanase with this characteristics, this one was used as basis for the prediction of the structures. An alignment, based on a dynamic programming algorithm, of the sequences to be modeled and the sequence with known related structure, provides to the program one way to automatically calculates a 3D model. It is different from standard sequence-sequence alignment methods because it takes into account structural information from the base-protein in the PBD when constructing the alignment. The Modeller program (9v6, http://salilab.org/modeller/) was the tool to execute the modeling. Modeller program accept the amino acid sequence of a target protein, determine whether a suitable parent or parents for homology exist, and, if so, deliver a set of coordinates for the target.

To visualize the modeled structures it was used the Pymol Viewer (http://www.pymol.org/funding.html) and then to analyze the interactions between the residues of amino acids was used the Blue Star Sting program (http://www.cbi.cnptia.embrapa.br/SMS/). All alignments were done by using the CLUSTALW (Version 1.81) alignment program on the server (http://www.ebi.ac.uk/clustalw)

3. Results and discussion

3.1. Structural modeling

The identities of the *Orpinomyces* XynA catalytic domain with the two catalytic domains of *Neocallimastx patriciarum* XylA (accession number P29127) were 86 to 90% (Gilbert *et al.,* 1992). The xylanase from the fungus *N. patriciarum* was used as basis for the structural modeling because of its high percentage of identical residues in their sequence alignment with the catalytic domain of the studied xylanase and because its structure is known and available at the PDB (PBD ID: 2C1F). With such a close degree of similarity, it is expected that the model fits the experimental result very closely and is also

expected that the homology modeling method give a very reasonable estimation of the structure.

The xylanase cDNA (*xynA*) was 1.2 kb long with an ORF encoding a polypeptide of 362 amino acids (Li *et al.,* 1997a,b). XynA contain in addition to a catalytic domain, a noncatalytic repeated peptide domain (NCRPD) which are not involved in catalysis but have been postulated to be involved in the interaction of polypeptides. Most hydrolytic enzymes of anaerobic fungi reported so far have a noncatalytic repeated peptide domain (Fanutti *et al.,* 1995, Gilbert *et al.,* 1992, Li *et al.,* 1997a,b). Enzymes from anaerobic fungi with the NCRPDs are now considered to be associated with large multienzyme cellulosome-like complexes. Several hydrolytic enzymes from the monocentric *Neocallimastix* species have more than one catalytic domain in a single protein (Fanutti *et al.,* 1995, Gilbert *et al.,* 1992), a feature not found in any of the enzymes cloned and sequenced from the polycentric *Orpinomyces* (Li *et al.,* 1997a,b).

These two domains, catalytic and noncatalytic, are together by a linker region. XynA still possesses a putative signal peptide containing all features associated with secretion (Li *et al.,* 1997a,b). The xynA library used for selection of the mutants possesses only the catalytic domain cloned in the plasmid. A protein sequence alignment between the *Orpinomyces* XynA from data base (accession number AAD04194) and one mutant selected (M4) show these domains. The XynA cloned include only the catalytic domain that has 255 amino acids. The whole protein possesses 362 amino acids (Figure 1).

Figure 1: Protein alignment between XynA from data base and mutant M4. The region aligned corresponds to the catalytic domain, the repeated peptide and linker regions are underlined and double-underlined respectively.

The *Orpinomyces* xylanase (XynA) contain one catalytic domain and thus differed from the *Neocallimastix* xylanase (XylA), which had two similar catalytic domains, so the size of *Orpinomyces* XynA is about half of that of XylAs of *Neocallimastix* (Gilbert *et al.,* 1992) (Figure 2).

Figure 2: Protein alingment of the *Orpinomyces* strain PC-2 xylanase (X*ynA*) (accession number AAD04194) and *N. patriciarum* xylanase (XylA) (accession number P29127). At the top is shown the alignment between the catalytic domain from the XynA and the first catalytic domain from XylA. The linker and the repeated peptide domain are shown at the bottom. The second catalytic domain from XylA is not aligned.

The alignment between one mutant (M4) and the second catalytic domain from XylA (PDB ID 2C1F, residue 275-495) evidences what region from the basis protein with known structure was used for structural modeling (Figure 3).

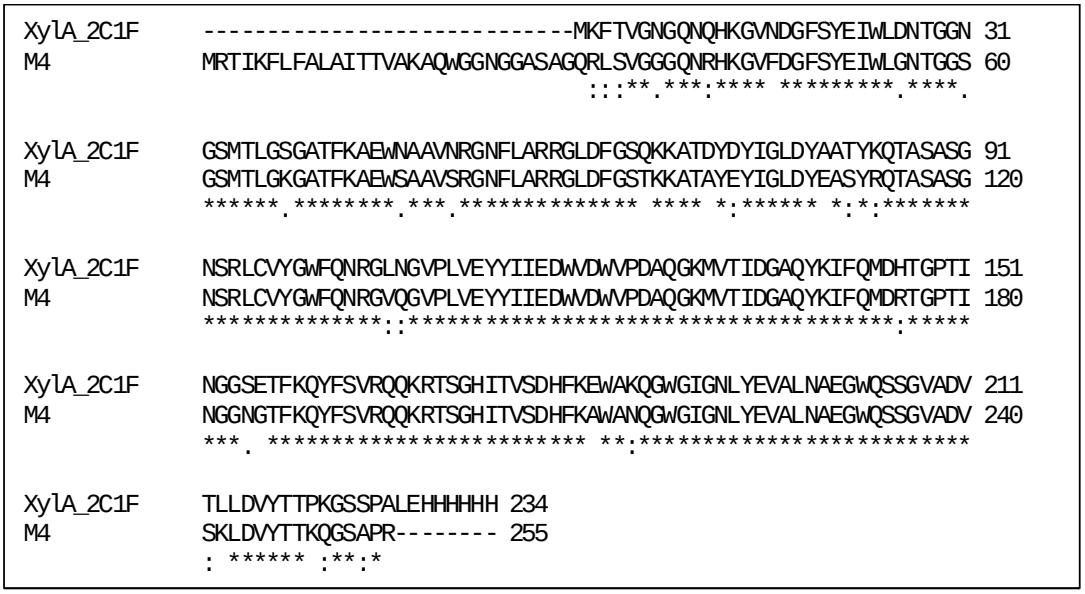

Figure 3: Protein alingment of the M4 and the second catalytic domain from *N. patriciarum* xylanase (XylA).

Many efforts have been made to improve the properties of the glycosyl hydrolase family-11 xylanase to handle industrial tasks. In particular, thermostability is a major target of such modifications (Wakarchuk *et al.,* 1994; Georis *et al.,* 2000; Turunen *et al.,* 2001, 2002; Xiong *et al.,* 2004). Understanding the structural basis for protein thermostability is of considerable biological and biotechnological importance (Dumon *et al.,* 2008). So, the structural modeling was done to study the structure/function relationship in order to identify positions that confer thermostability.

A mutant library of xynA was generated by error-prone PCR. Directed evolution was applied to improve XynA thermostability for lowering its heat inactivation during industrial applications. Compared with the wild-type enzyme, two selected variants showed enhanced resistance to relatively high temperatures. In particular, the M4 showed about 50% of residual activity after incubation at 70 ºC and about 40% of residual activity after incubation at 75 ºC during 1 h. M6 presented 50% of its activity in a temperature of 60ºC during incubation time of 1 h.

This result suggests that substitutions in M4 and M6 might introduce additional hydrogen bonds with adjacent residues, improving the enzyme

thermostability by stabilizing local interactions and it emphasizes our interest in infer its three-dimensional structure.

To investigate the structural basis for the biochemical properties of XynA, the structural modeling of this enzyme was done using the XylA xylanase from *Neocallimastx patriciarum* (PDB ID 2C1F) as the model.

The enzyme XylA displays the β jelly-roll fold typical of GH11 xylanases. The concave antiparallel β-sheet comprises nine β strands in the order β2, β3, β6, β14, β8, β9, β12, β11, β10, while the order of the β strands in the antiparallel convex sheet is β1, β4, β5, β15, β7, β13. An α-helix is found in the loop connecting β strands 13 and 14, and a 3_{10} helix in the loop between β6 and β7 (Vardakou *et al.,* 2008). The structure of GH11 xylanases has been compared with the shape of a right hand (Torronen *et al.,* 1994). By comparison with other GH11 xylanases, the concave larger β-sheet of XylA is predicted to comprise the substrate-binding cleft. In the centre of the active site are the two predicted catalytic residues, Glu113 (catalytic nucleophile) and Glu201 (catalytic acid–base) which are invariant in GH11 enzymes (Vardakou *et al.,* 2008).

In the figure 4, it can be seen the predicted structures of M4, M6 and the wild-type with the substitutions and the catalytic glutamates identified. The positions of the catalytic glutamates in the XynA, according the alignment between XynA and XylA, are at positions 142 and 230.

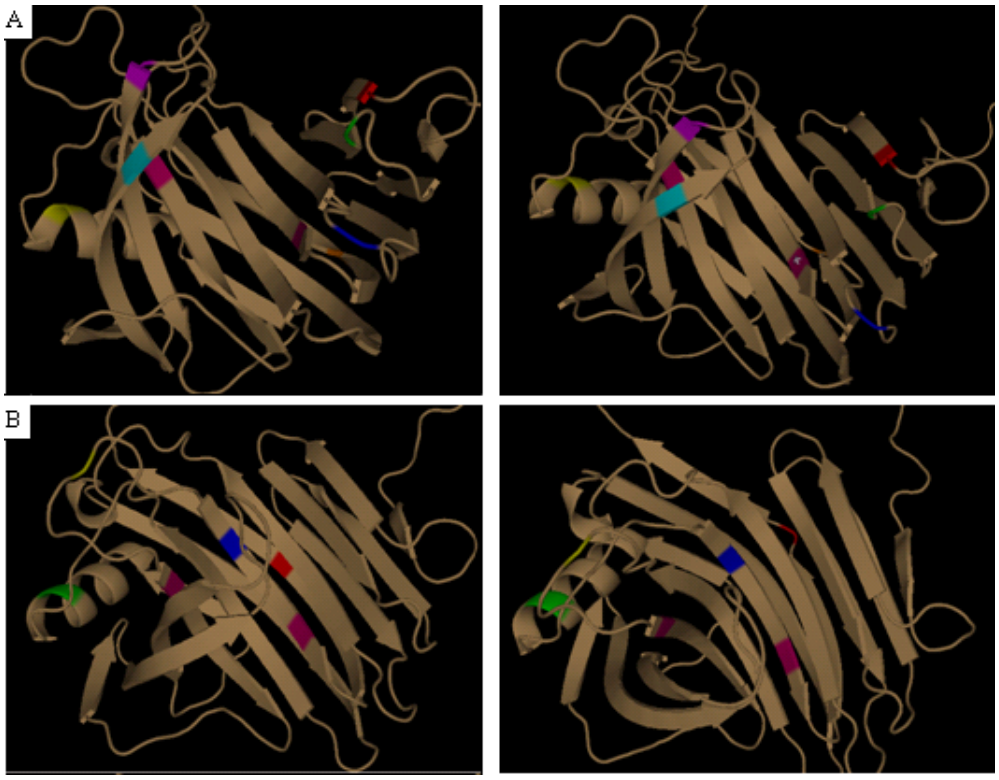

Figure 4: Predicted three-dimensional structures. Modeller program predicts the structure of wild-type xylanase and the mutants from *Orpinomyces* from a closely related protein (xylanase form *N. patriciarum*). The prediction was done automatically. The positions of amino acids substitutions and the catalytic glutamates are represented and coloured in the structures. A – Predicted structure of M4 (left) and wild-type XynA (right). Q40R is shown in red, D55G in green, N80S in blue, H175R in cyan, E185G in magenta, S213A in yellow and P241S in orange. B - Predicted structure of M6 (left) and wild-type XynA (right). V135A is shown in yellow, S213A in green, A226T in blue and P241T in red. The Glu142 and the Glu230 are represented in pink in all the structures. The Modeller program (9v6, http://salilab.org/modeller/) was used to execute the modeling. This Figure and subsequent structural representations were all done with PyMol (http://www.pymol.org/funding.html). The second catalytic domain from XylA (PDB ID 2C1F, residue 275-495) was used for structural modeling.

The identified amino acid substitutions in the selected M4 and M6 mutants are distributed throughout the structure and most of mutations are found in loops or surface regions. The only exception is the substitution A226T in M6 that is located on the β-strand near the catalytic residues (Figure 4B).

The interaction study between the residues of amino acids showed that many of the new random mutations inserted by the error-prone PCR technique were responsible for decreased the number of stabilizing interactions that could harm its thermostability (Tables 1 and 2, see table list).

Based on the structural predictions to analyze the molecular basis for thermostability, the substitution of A226T in M6 introduces two new stabilizing interactions. Treonine establishes one additional hydrogen bond with Tyr127 whereas Ala226 forms only two hydrogen bonds. One additional hydrophobic interaction with Glu224 is also introduced (Table 2).

However, analyzing the new interactions between the catalytic glutamates (Glu142 and Glu230) and others residues in the catalytic core, we can observe an increase in the stabilizing interactions. The Glu142 in the M4 mutant introduced one hydrophobic interaction with the Tyr144 besides one hydrogen bond that already existed. In addition, the total of hydrogen bonds and hydrophobic interactions established by the Glu142 increased in the nucleus of the molecule. The other catalytic residue, Glu230, also introduced another hydrophobic interaction with the Arg123 besides two charge attractive interaction and two hydrogen bonds that already existed.

In the M6 mutant, the Glu142 also introduced new interactions that stabilize the molecule. Glu142 interacted with Tyr144 establishing two more hydrophobic interactions besides the only one hydrogen bond. The number of representative interactions was also improved in the catalytic core of the M6 (Table 3 and 4, see table list).

Based on these results, an analysis of interactions between internal amino acids was done to investigate if the number of stabilizing interactions increased in the thermostable mutants (Tables 5, 6 and 7).

Table 5: List of amino acids with highest number of internal contacts in M4

Residue	**Number of contacts**
Arg 088	23
Phe 191	20
Arg 194	19
Tyr 144	18
Tyr 127	18
Tyr 050	18
Tyr 190	17
Trp 129	17
Phe 209	16
Tyr 143	16
Total of interactions	182

Table 6: List of amino acids with highest number of internal contacts in M6

Residue	Number of contacts
Trp 212	21
Phe 191	21
Arg 087	21
Tyr 190	19
Tyr 144	19
Glu 142	19
Arg 194	18
Trp 129	18
Phe 092	18
Phe 209	17
Total of interactions	191

Table 7: List of amino acids with highest number of internal contacts in XynA

Residue	Number of contacts
Arg 194	24
Glu 147	20
Phe 209	19
Arg 198	18
Phe 191	17
Tyr 190	17
Arg 087	17
Trp 149	16
Tyr 144	16
Phe 130	16
Total of interactions	180

Thus, this study suggested that the mutations in M4 and M6 resulted in a higher number of internal interactions that could contribute to the stability of these molecules. Analyzing the amino acids that establish the most internal contacts, M4 and M6 accomplished 182 and 191 internal interactions, respectively, while the wild-type accomplished 180 interactions.

Based on these results we can infer that the isolated study of the mutations do not explain the higher thermostability of the M4 and M6 mutants, but the overall structural rearrangement created by these mutations increased the number of interactions, mainly in the catalytic core, improving the packing of the hydrophobic core and possible justifying the greater resistance of these

mutants to elevated temperatures. Wang *et al.* (2008) used the directed evolution to enhance the activity and the alkaline pH stability of *Thermobifida fusca* xylanase A and concluded that, apparently, the greater activity of chimeras depends not on the single site substitution, but on the directed evolution of this change in concert with the appropriate context provided by the rest of the protein.

These data also suggest that mutations inside the molecule should generate more representative alterations in the conformation of the molecule than the substitutions located on the surface and significantly improve the desired characteristics. Irwin *et al.* (1994) observed that 3 substitutions (V87P, I91T, and G217L) that are located in the vicinity of the catalytic triad (Glu88 and Glu216) of TfxA (xylanase from *Thermobifida fusca*) were responsible for the high activity toward alkaline pH of this enzyme.

Analyses of structural basis for protein stability have illustrated general factors governing the stability of proteins (Kumar *et al.,* 2000a; Querol *et al.,* 1996; Ragone, 2001; Vieille and Zeikus, 1996, 2001; Yip *et al.,* 1995). They include increase in hydrogen bonds and ionic interactions, reduction of conformational strain, improvement of the packing of the hydrophobic core, and enhanced secondary structure propensity.

Previous studies reveled several minor modifications responsible for the increased thermostability of family 11 xylanases: (a) a higher Thr:Ser ratio (b) an increased number of charged residues, especially Arg, resulting in enhanced polar interactions, and (c) an improved stabilization of secondary structures due to an increased number of residues in the β-strands and stabilization of the α-helix region. Some members of family 11 xylanases have a unique feature to improve their stability, such as a higher number of ion pairs or aromatic residues on protein surface, a more compact structure, tighter packing, and insertions at some regions resulting in enhanced interactions. These changes increase protein rigidity, a property associated with enhanced stability. Several studies have demonstrated that the highly thermophilic xylanases have a great number of side chain-side chain polar interactions and several salt bridges. There could be a trend in xylanase structures that acidophilic xylanases have few salt bridges than alkalophilic xylanases. It is possible that alkaline and

thermal adaptation use similar mechanisms for improving stability (Wang *et al.,* 2008).

Some reports show that the molecular basis for the increased thermostability is extraordinarily subtle. The crystal structures of *Ev*Xyn11 (wild-type xylanase) and *Ev*Xyn11TS (hyperthermostable variant) revealed an absence of substantial changes to identifiable intramolecular interactions. The only explicable mutations are T13F, which increases hydrophobic interactions, and S9P that apparently locks the conformation of a surface loop (Dumon *et al.,* 2008).

The structural modeling and the analysis of internal interactions took us to identify important residues that contribute to the molecule stability. Tyr144 and Tyr190 establish a large number of contacts, as in the mutants as the wild-type, and in this way, they are residues that contribute to the stability of the xylanase. This finding suggests that these ones are possible residues to be submitted to site-directed mutagenesis trying to alter them to residues that maximize the internal interactions and then improving the thermostability of the XynA.

It is interesting to mention that two of the mutations in M4 are arginine substitutions. Sequences and crystal structure alignments studies have been showing that highly thermostable xylanases have an increased number of charged residues, especially arginine, resulting in enhanced polar interactions (Hakulinen *et al.,* 2003). Lysine-arginine mutations in a number of enzymes lead to enhanced thermostability (Mrabet *et al.,* 1992). Shallowly buried arginines are likely to influence the overall electrostatic potential of the protein surface. Several studies indicate that there is a correlation between protein stability and the number of arginines on the protein surface. The comparison of mesophilic proteins and their thermophilic counterparts has revealed that thermophilic proteins have, on average, higher arginine content on the protein surface (Argos *et al.,* 1979; Vogt et al., 1997). A change in polarity might also contribute to the thermostabilization of these variant xylanases by rearrangement of interactions such as those with hydrogen bonds and/or salt bridges (Murashima *et al.,* 2002).

In spite of the interaction prediction study have evidenced that the added arginines can have a destabilizing power for the molecule, these residues

continue being the focus of intense research for more refined bioinformatics studies and for crystallography analysis (Table 1 and 2, see table list).

Sequence comparisons of thermostable enzymes and their mesophilic counterparts have been useful in identifying “thermostabilizing” residues or motifs that suggest evolutionary adaptations to heat challenge (Mueller *et al.,* 2000; Perl *et al.,* 2000). Comparisons of the three-dimensional structure of thermophilic and mesophilic enzymes have been used in efforts to develop general rules that can be applied to improving the stability of industrially useful biocatalysts. Disulphide bridges and salt bridges have been suggested as reasons for the enhanced thermal stability of thermophilic xylanases (Gruber *et al.,* 1998; *Kumar et al.,* 2000b) and can be a good tentative, using the site-directed methodology, in improving the thermostability of interest enzymes.

It is important to mention that this is a preliminary study based in predictions and that a more complete and robust bioinformatics analysis and crystallography studies should be executed.

Wang *et al.* (2008) mention that the structural basis for the thermostability of family 11 xylanases is not well understood and much investigation should be developed. Further study of the mutant enzymes should expand our understanding of the structure/function relationship for the valuable biocatalyst.

Table list

Table 1: Comparison of interaction profile of M4 and wild-type (XynA).

M4				XynA			
Residue	**Residue Interaction**	**Interaction Type**	**Amount**	**Residue**	**Residue Interaction**	**Interaction Type**	**Amount**
40R	53W	Hydrophobic Interaction	1	40Q	51E	Hydrogen Bond (SC-SC)	1
		Aromatic Stacking	1				
	42K	Charge Repulsive Interaction	2				
55G	83N	Hydrogen Bond (MC-MC)	1	55D	83N	Hydrogen Bond (MC-MC)	1
					81R	Charge Attractive Interaction	1
					53W	Hydrophobic Interaction	2
80S	82G	Hydrogen Bond (MC-MC)	1	80N	82G	Hydrogen Bond (MC-MC)	1
					233Q	Hydrogen Bond (SC-SC)	1
					57T	Hydrophobic Interaction	1
175R	186T	Hydrogen Bond (SC-MC)	1	175H	186T	Hydrogen Bond (SC-MC)	1
	187F	Hydrogen Bond (MC-MC)	2		187F	Hydrogen Bond (MC-MC)	2
	191F	Aromatic Stacking	1		142E	Charge Attractive Interaction	1
	189Q	Hydrophobic Interaction	2		189Q	Hydrophobic Interaction	1
	123R	Charge Repulsive Interaction	1				
185G	177G	Hydrogen Bond (MC-MC)	2	185E	177G	Hydrogen Bond (MC-MC)	1
					183G	Hydrogen Bond (MC-MC)	2
213A	216G	Hydrogen Bond (MC-MC)	1	213S	216G	Hydrogen Bond (MC-MC)	1
	215Q	Hydrogen Bond (MC-MC)	1		215Q	Hydrogen Bond (MC-MC)	1
	210K	Hydrogen Bond (MC-MC)	1		210K	Hydrogen Bond (MC-MC)	1
	209F	Hydrogen Bond (MC-MC)	1		209F	Hydrogen Bond (MC-MC)	1
						Hydrogen Bond (SC-MC)	1
					219I	Hydrophobic Interaction	1
241S	109E	Hydrogen Bond (MC-MC)	1	241P	109E	Hydrogen Bond (MC-MC)	1
					239D	Hydrophobic Interaction	1
					110A	Hydrophobic Interaction	1
					70T	Hydrophobic Interaction	1

* The amount of interactions is approximated.

Table 2: Comparison of interaction profile of M6 and wild-type (XynA).

M6				XynA			
Residue	**Residue Interaction**	**Interaction Type**	**Amount**	**Residue**	**Residue Interaction**	**Interaction Type**	**Amount**
135A	132N	Hydrogen Bond (MC-MC)	1	135V	132N	Hydrogen Bond (MC-MC)	1
					138V	Hydrophobic Interaction	2
213A	216G	Hydrogen Bond (MC-MC)	1	213S	216G	Hydrogen Bond (MC-MC)	1
	215Q	Hydrogen Bond (MC-MC)	1		215Q	Hydrogen Bond (MC-MC)	1
	210K	Hydrogen Bond (MC-MC)	1		210K	Hydrogen Bond (MC-MC)	1
	209F	Hydrogen Bond (MC-MC)	1		209F	Hydrogen Bond (MC-MC)	1
	219I	Hydrophobic Interaction	2			Hydrogen Bond (SC-MC)	1
					219I	Hydrophobic Interaction	1
226T	127Y	Hydrogen Bond (MC-MC)	2	226A	127Y	Hydrogen Bond (MC-MC)	2
		Hydrogen Bond (SC-MC)	1		89G	Hydrophobic Interaction	1
	88R	Hydrophobic Interaction	1		88R	Hydrophobic Interaction	1
	89G	Hydrophobic Interaction	2				
	224E	Hydrophobic Interaction	1				
241T	109E	Hydrogen Bond (MC-MC)	1	241P	109E	Hydrogen Bond (MC-MC)	1
	70T	Hydrophobic Interaction	1		70T	Hydrophobic Interaction	1
					110A	Hydrophobic Interaction	1
					239D	Hydrophobic Interaction	1

* The amount of interactions is approximated.

Table 3: Comparison of interaction profile of M4 and wild-type (XynA) involving the catalytic residues.

M4				XynA			
Catalitic Residue	**Residue Interaction**	**Interaction Type**	**Amount**	**Catalitic Residue**	**Residue Interaction**	**Interaction Type**	**Amount**
142E	189Q	Hydrogen Bond (SC-SC)	2	142E	189Q	Hydrogen Bond (SC-SC)	1
	190Y	Hydrogen Bond (MC-MC)	1		190Y	Hydrogen Bond (MC-MC)	1
	188K	Hydrogen Bond (MC-MC)	1		188K	Hydrogen Bond (MC-MC)	1
	87R	Charge Attractive Interaction	1		87R	Charge Attractive Interaction	1
	144Y	Hydrogen Bond (SC-SC)	1		144Y	Hydrogen Bond (SC-SC)	1
		Hydrophobic Interaction	1				
	129W	Hydrophobic Interaction	5		129W	Hydrophobic Interaction	3
	127Y	Hydrophobic Interaction	3		127Y	Hydrophobic Interaction	2
		Hydrogen Bond (SC-SC)	1			Hydrogen Bond (SC-SC)	1
					175H	Charge Attractive Interaction	1
230E	228N	Hydrogen Bond (SC-SC)	1	230E	228N	Hydrogen Bond (SC-SC)	1
	87R	Charge Attractive Interaction	3		87R	Charge Attractive Interaction	4
	123R	Charge Attractive Interaction	2		123R	Charge Attractive Interaction	2
		Hydrogen Bond (MC-MC)	2			Hydrogen Bond (MC-MC)	2
		Hydrophobic Interaction	1				

* The amount of interactions is approximated.

Table 4: Comparison of interaction profile of M6 and wild-type (XynA) involving the catalytic residues.

M6				XynA			
Catalytic Residue	**Residue Interaction**	**Interaction Type**	**Amount**	**Catalitic Residue**	**Residue Interaction**	**Interaction Type**	**Amount**
142E	189Q	Hydrogen Bond (SC-SC)	1	142E	189Q	Hydrogen Bond (SC-SC)	1
	190Y	Hydrogen Bond (MC-MC)	1		190Y	Hydrogen Bond (MC-MC)	1
	188K	Hydrogen Bond (MC-MC)	1		188K	Hydrogen Bond (MC-MC)	1
	175H	Charge Attractive Interaction	1		175H	Charge Attractive Interaction	1
	87R	Charge Attractive Interaction	2		87R	Charge Attractive Interaction	1
	144Y	Hydrogen Bond (SC-SC)	1		144Y	Hydrogen Bond (SC-SC)	1
		Hydrophobic Interaction	2				
	129W	Hydrophobic Interaction	7		129W	Hydrophobic Interaction	3
	127Y	Hydrophobic Interaction	2		127Y	Hydrophobic Interaction	2
		Hydrogen Bond (SC-SC)	1			Hydrogen Bond (SC-SC)	1
230E	228N	Hydrogen Bond (SC-SC)	1	230E	228N	Hydrogen Bond (SC-SC)	1
	123R	Hydrogen Bond (MC-MC)	2		123R	Hydrogen Bond (MC-MC)	2
		Charge Attractive Interaction	2			Charge Attractive Interaction	2
	87R	Charge Attractive Interaction	4		87R	Charge Attractive Interaction	4

* The amount of interactions is approximated.

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Perspectives

As perspectives, considering the increased thermostability of the M4 and M6 mutants, these enzymes will be super-expressed and used for further purification and characterization to compare the kinetic parameters between them and the wild-type. We also intend to submit these two improved first generation clones to a second round of mutations, by error-prone PCR, trying to introduce new mutations that have the capacity to generate a xylanase with enhanced thermostability to produce a better xylanase for industrial application.

The homology modeling showed to be an important tool in the identification of interesting positions that could be altered using the site-directed methodology and this study prompted us, in future experiments, to employ this technique to generate more thermostable *Orpinomyces* XynA variants. So, we intend to combine the bioinformatics study and the site-directed mutagenesis to evaluate the contribution of each amino acid change in increasing catalytic activity and thermostability. We also intend to crystallize the thermostable enzymes to provide better understanding in the functional properties differences between the wild-type and the more thermostable xylanases. It is also planned to determine the melting temperatures (T_m) for the more thermostable xylanases (M4 and M6) and XynA besides completing the structural studies with circular dicroism.

Conclusões Gerais

- O protocolo de *screening* em placa, utilizando azo-xilana-agarose, foi eficiente na seleção de seis xilanases mutantes, a partir de uma biblioteca mutante de xynA de *Orpinomyces* sp. PC-2, criada pela técnica de *error-prone-PCR*.

- Duas xilanases mutantes, M4 e M6, apresentando quatro e sete resíduos substituídos, respectivamente, foram mais termoestáveis, mantendo aproximadamente 50% de suas atividades originais, após pré-incubação a 60°C por 1 h.

- A xilanase mutante M4, mais termoestável, apresentou menor atividade (U/mL), confirmando a existência da relação inversa entre atividade e termoestabilidade de enzimas.

- Os valores de temperatura e pH ótimos dos mutantes não foram alterados.

- As xilanases mutantes e a xilanase tipo selvagem foram eficientes na hidrólise dos dois substratos testados não sendo sensibilizadas pela presença de grupos substituintes na cadeia principal de xilose.

- Duas substituições em M4 são por resíduos de arginina e muitos estudos têm mostrado que xilanases termoestáveis possuem um número elevado de resíduos carregados. Sendo assim, estes resíduos continuam sendo alvos de intensa pesquisa em bioinformática e cristalografia.

- A modelagem estrutural foi uma importante ferramenta na inferência de possíveis mecanismos que justifiquem a maior termoestabilidade dos mutantes M4 e M6, sugerindo um rearranjo estrutural final da molécula

- A modelagem por homologia foi importante na identificação de resíduos importantes para a estabilidade da xilanase devido ao grande número de interações estabelecidas, tornando-os alvos de estudo.

- Análise estrutural preliminar necessitando de estudos de bioinformática mais completos.