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PRODUÇÃO, PURIFICAÇÃO E CARACTERIZAÇÃO DE XILANASES (EC 3.2.1.8) EXCRETADAS POR ISOLADOS AMAZÔNICOS DE *Bacillus* EM CULTIVO SEMI-SÓLIDO

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PRODUÇÃO, PURIFICAÇÃO E CARACTERIZAÇÃO DE XILANASES (EC 3.2.1.8) EXCRETADAS POR ISOLADOS AMAZÔNICOS DE *Bacillus* EM CULTIVO SEMI-SÓLIDO [1]

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RESUMO

A xilanase (1,4-β-xilana-xilanahidrolase, EC 2.2.1.8) é a principal constituinte do sistema enzimático xilanolítico microbiano. Ela tem sido extensivamente estudada e empregada em vários processos biotecnológicos, podendo ser produzida tanto em cultivo semi-sólido (CSS) quanto em cultivo submerso (CSm), com prevalência deste último. No entanto, nos últimos anos o interesse no CSS para produção de enzimas tem aumentado, em virtude das inúmeras vantagens econômicas e de engenharia que este oferece. Neste trabalho, objetivou-se aumentar o conhecimento existente sobre a produção e as aplicações de xilanases bacterianas. Uma xilanase livre de celulases produzida por um isolado amazônico de *Bacillus coagulans* em CSS utilizando um abundante resíduo fibroso de soja foi identificada e o seu potencial para biobranqueamento de polpa Kraft foi demonstrado. Outra xilanase produzida em CSS pelo *Bacillus circulans* BL 53 também foi identificada. Através do emprego de ferramentas de planejamento experimental determinou-se que as melhores condições para a produção dessa enzima são tempos de cultivo e aerações moderadas (5 dias e 500 $mL.min^{-1}$) e temperaturas baixas (25°C), com as quais foi possível aumentar a produção da enzima em 2,5 vezes, em relação ao que era obtido nas condições anteriormente empregadas. Também investigou-se, através de metodologias de planejamento experimental, as melhores condições para extração da xilanase produzida em CSS. Os resultados indicam que a extração da enzima foi máxima quando utilizou-se água a 7°C como solvente, por 40 minutos, 150 rpm e uma relação de sólidos/líquidos de 1:6. A enzima foi purificada à homogeneidade por precipitação fracionada com sulfato de amônio, cromatografia de troca catiônica e gel filtração. Uma purificação de 428 vezes foi alcançada, apresentando uma atividade específica de aproximadamente 37 $U.mg^{-1}$ de proteína. O peso molecular da enzima foi determinado por SDS-PAGE, sendo de aproximadamente 38 KDa. A máxima atividade enzimática foi determinada usando um planejamento fatorial 2^2 e a enzima apresentou um ótimo de temperatura entre 40 e 80°C e de pH entre 5,0 e 8,0. Os resultados obtidos sugerem que as xilanases produzidas neste trabalho apresentam algumas propriedades interessantes para futuras aplicações industriais.

[1]/Tese de Doutorado em Biologia Celular e Molecular (área de concentração Biotecnologia), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (146p.), maio de 2005.

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF XYLANASES (EC 3.2.1.8) FROM AMAZON ISOLATED *Bacillus* ON SOLID-STATE CULTIVATION[1]

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ABSTRACT

Xylanase (1,4-β-xylan xylanohydrolases, EC 3.2.1.8) is the main constituent of microbial xylanolytic enzyme systems. They are extensively used in many biotechnological applications and have been produced in either solid-state (SSC) or submerged cultivations with the prevalence of the last one. However, SSC has gained renewed interest in recent years and has often been employed for the production of many enzymes due to a number of economical and engineering advantages. In this work, an effort has been made in order to increase available knowledge about bacterial xylanases. A cellulase-free xylanase produced by an amazon *Bacillus coagulans* strain on solid-state cultivation using an industrial fibrous soybean residue as substrate was identified and their biobleaching potential was showed. Another xylanase, produced by *Bacillus circulans* BL 53 also was identified. A 2^3 central composite design (CCD) was applied to determine the optimal conditions of cultivation time, aeration and temperature to xylanase production by *Bacillus circulans* BL53. The results suggest that xylanase production by this strain is higher at a moderate cultivation time and aeration (5 days and 500 $mL.min^{-1}$, respectively) and at low temperatures (25°C) and following CCD modeled conditions, it was possible to increase 2.5 fold enzyme activities previously obtained and published by our group. The present work also dealt with the extraction optimization of xylanases produced in solid state cultivations of *Bacillus circulans,* with the purpose of reducing enzyme losses in order to obtain crude extracts as concentrated as possible. A 2^3 factorial design was performed to find the best conditions of time, agitation and solid/liquid ratio. Maximum recovery was obtained by extracting in water at 7 °C, 40 minutes, 150 rpm and 1:6 solid/liquid ratio. This xylanase was purified to apparent homogeneity by ammonium sulfate precipitation, cation-exchange chromatography and gel filtration. A 428-fold purification was achieved, with the purified xylanase having a specific activity of about 37 $IU.mg^{-1}$ protein. The molecular weight of the enzyme is about 38 Kda, as determined by SDS-PAGE. The maximum activity was obtained using a 2^2 factorial design over a large range of temperature (40 - 80°C) and pH (5.0 – 8.0). Results strongly suggest that the xylanases produced in this work exhibit some interesting properties for industrial applications.

[1]/ Doctor of Science Thesis on Cellular and Molecular Biology (Area of Biotechnology), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre (146p.), May, 2005.

ÍNDICE

LISTA DE FIGURAS

LISTA DE TABELAS

A produção de proteína isolada de soja em uma grande indústria da região metropolitana de Porto Alegre, por exemplo, gera cerca de 20 toneladas por dia de um resíduo composto de hemicelulose, celulose, proteínas e açúcares (23, 15, 26 e 14%, respectivamente). Este abundante material não recebe, normalmente, um destino que agregue valor a ele, sendo descartado no ambiente ou subutilizado com aproveitamento parcial como ração para suínos. A necessidade de agregar valor a este subproduto agrícola e de aumentar o leque de possíveis aplicações dos materiais lignocelulósicos têm estimulado o interesse no desenvolvimento de processos industriais que visem o aproveitamento deste resíduo. Em países como o nosso, altamente dependente de tecnologias externas, este tipo de solução é condição *sine qua non* para romper com as amarras econômicas e tecnológicas que impedem o nosso desenvolvimento verdadeiramente independente e soberano. Ademais, contamos com uma variabilidade única de recursos genéticos distribuída em nossos sistemas naturais, como a Amazônia, que certamente podem trazer inúmeros benefícios aos vários segmentos da sociedade. Uma exploração comercial racional destes recursos pode não apenas proporcionar o surgimento de novos produtos e processos amparados nesta biodiversidade, mas também provocar uma nova postura de preservação e conservação.

Neste contexto, os sistemas de cultivo semi-sólido (CSS) podem ser empregados satisfatoriamente, em virtude da baixa solubilidade dos substratos e dos baixos níveis de investimento exigidos pelo processo. Além disso, este sistema constitui-se em uma alternativa nova e promissora, que vem ganhando espaço a cada ano, principalmente em países de menor desenvolvimento científico e tecnológico, apresentando-se como uma alternativa viável no intuito de aproveitar resíduos agroindustriais e produzir, a partir deles, metabólitos de interesse comercial. Além disso, já está comprovado que o emprego em larga escala dos processos de CSS é perfeitamente possível, desde que seja seguido um planejamento racional de equipamentos e processos.

O potencial biotecnológico para a aplicação das xilanases (EC 3.2.1.8) nos diversos segmentos industriais é promissor. As indústrias de celulose e papel, por exemplo, estão modificando a maneira de tratar a matéria-prima, buscando alternativas que possam reduzir a carga tóxica de seus efluentes. Nestas indústrias, o cloro é usado como agente químico branqueador, o que acaba por gerar produtos residuais nocivos ao ambiente. Neste caso, o emprego de xilanases surge como uma alternativa ao cloro no processo de branqueamento da polpa de papel (polpa Kraft), permitindo reduções de até 20% na quantidade utilizada para o branqueamento da polpa. Além disso, as xilanases

podem ser empregadas na hidrólise de materiais ricos em xilana e o produto desta hidrólise, a xilose e outros xilooligassacarídeos, ser convertido, biotecnologicamente, a xilitol, um adoçante muito empregado na indústria química, farmacêutica e de alimentos. As xilanases podem, ainda, ser aplicadas na indústria de panificação, hidrolisando polissacarídeos insolúveis da farinha e que prejudicam o desenvolvimento do glúten, e em processos de maceração enzimática, hidrolisando a parede celular de frutas e vegetais e melhorando os processos de extração da polpa.

Outro aspecto amplamente abordado neste trabalho é o emprego de ferramentas estatísticas para planejamento e análise dos resultados, pois a falta de planejamento muitas vezes é a causa do insucesso de uma investigação. Usando planejamentos experimentais baseados em princípios estatísticos, os pesquisadores podem extrair do sistema em estudo o máximo de informações úteis, fazendo um número mínimo de experimentos. Os métodos empregados nos planejamentos experimentais independem da natureza do problema a que são aplicados. Servem tanto para estudar reações químicas, sistemas biológicos, processos mecânicos ou qualquer outro sistema. E também abrangem todas as escalas possíveis de interesse, desde uma única reação numa bancada até um processo industrial operando em larga escala.

Este trabalho teve a intenção de ampliar o montante de conhecimentos sobre xilanases bacterianas. Em um primeiro momento, identificou-se uma xilanase livre de celulases produzida por um *Bacillus coagulans* isolado de ambientes amazônicos e demonstrou-se o potencial de aplicação da enzima produzida por este microrganismo em processos de branqueamento de papel. Em uma segunda etapa, identificou-se outra xilanase, produzida pelo isolado *B. circulans* BL53 em CSS e estudou-se a produção, a extração, a purificação e a caracterização da enzima, abrindo perspectivas para empregos futuros dessa enzima em processos biotecnológicos e demonstrando a aplicabilidade real dos processos de cultivo semi-sólido.

2. REVISÃO BIBLIOGRÁFICA

2.1. Hemiceluloses e xilanas

A hemicelulose é o segundo polissacarídeo mais abundante na natureza, representando cerca de 20-35% da biomassa lignocelulósica (HALTRICH *et al.*, 1996). É um polímero de estocagem e também um componente estrutural nas paredes celulares das plantas (BASTAWDE, 1992). Segundo SAHA (2003), a fração lignocelulósica inclui vários resíduos agrícolas (cascas, palhas, bagaços, sementes), madeiras, resíduos sólidos municipais e resíduos de indústria de papel. Apesar de a composição dessa biomassa variar muito, geralmente a celulose está presente em maiores quantidades (35-50%), seguida pela hemicelulose (20- 35%) e pela lignina (10%). A tabela 1 mostra a composição de alguns compostos lignocelulósicos.

Tabela 1. Composição aproximada de alguns resíduos lignocelulósicos. Adaptado de SAHA (2003).

Material	Composição (% de peso seco)			Referência
	Celulose	Hemicelulose	Lignina	
Farelo de milho	15	35	8	Saha, 2003
Palha de arroz	35	25	12	Saha, 2003
Farelo de Trigo	30	50	20	Saha, 2003
Bagaço de cana-de-açúcar	40	24	25	Saha, 2003
Fibra de soja	16	23	ND	Heck *et al.*, 2002

ND- Não Determinado.

Quimicamente, a hemicelulose é um polímero heterogêneo de pentoses (xilose, arabinose), hexoses (manose, galactose, glicose) e açúcares ácidos unidos entre si por ligações glicosídicas do tipo β-1,4 (BASTAWDE, 1992). Ao contrário da celulose, as hemiceluloses não são quimicamente homogêneas. As hemiceluloses provenientes de *hardwoods* contêm principalmente xilana, enquanto as provenientes de *softwoods* são compostas principalmente de galactomananas (MCMILLAN, 1993).

SUN e CHENG (2002) referem-se ao fato de que nos últimos anos as bioconversões da hemicelulose têm recebido especial atenção em virtude, principalmente, de suas potenciais aplicações em vários processos de conversão da biomassa hemicelulósica em combustíveis e outros produtos de interesse. Ressaltam ainda que a eficiente hidrólise da hemicelulose é fundamental para a consolidação do uso de biomassas vegetais para a produção de etanol. E são vários os resíduos agrícolas lignocelulósicos que podem servir como substratos baratos para a produção de etanol combustível. Qualquer hemicelulose hidrolisada gera uma mistura de açúcares após um tratamento eficiente, que pode ser químico ou enzimático (BOTHAST e SAHA, 1997). A mistura contém xilose, arabinose, glicose, galactose, manose, fucose e ramnose.

A xilana, por sua vez, é o principal constituinte da hemicelulose e apresenta um grande potencial para ser convertida em produtos de interesse comercial (CORTEZ e PESSOA, 1999). Nas plantas, ocupa um importante papel na integridade estrutural da parede celular, formando interações covalentes e não covalentes (SAHA, 2000). Possui uma cadeia linear compreendida por ligações do tipo β-1,4 formada por resíduos de xilopiranose, que, dependendo da origem, podem ser substituídos por ramificações contendo principalmente resíduos acetil, arabinosil e gluconosil (FILHO *et al.*, 1997). Xilanas não ramificadas já foram isoladas, principalmente da semente de guar e do talo do tabaco. (KULKARNI *et al.*, 1999). O grau de polimerização da xilana é maior nas *hardwoods* do que nas *softwoods* (TOMA *et al.*, 1989; MACKENZIE, 1989) A cadeia lateral é determinante para a solubilidade, a conformação física e a reatividade da molécula de xilana com os outros componentes hemicelulósicos, além de influenciar enormemente o modo e a extensão da clivagem enzimática (KULKARNI *et al.*, 1999). A Figura 1 mostra a estrutura química básica de uma heteroxilana.

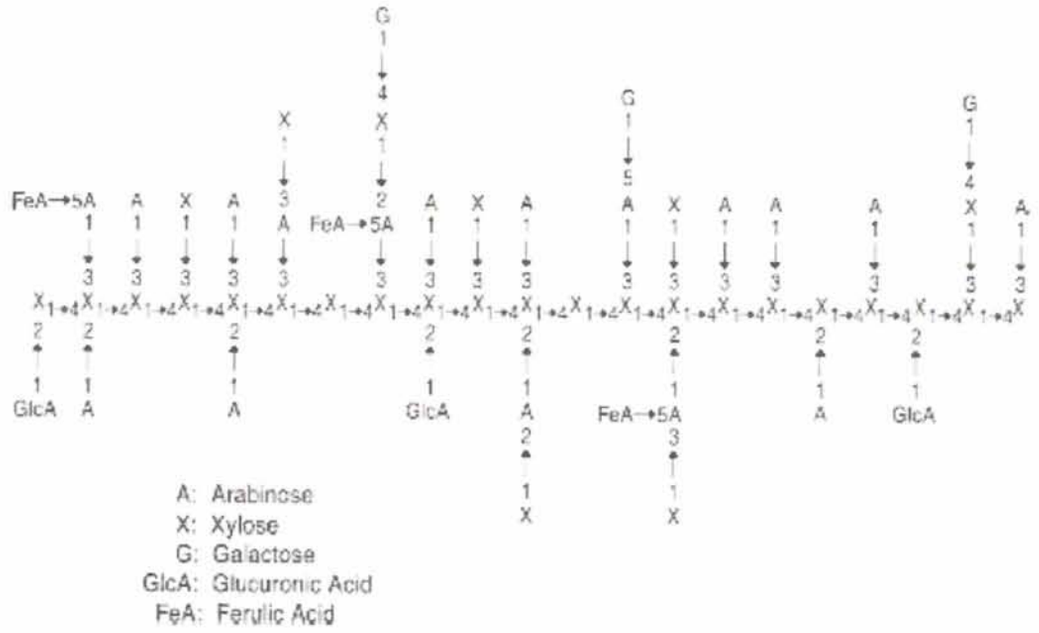

Figura 1. Estrutura química simplificada de uma heteroxilana (SAHA,2003).

Na maioria das plantas as xilanas são, de fato, heteropolissacarídeos. A freqüência e a composição das ramificações presentes dependem unicamente da fonte da xilana (SAHA, 2003). A xilana de Birch wood (Bétula), por exemplo, contém aproximadamente 89% de xilose, 1% de arabinose, 1,5% de glicose e 8,3% de ácido anidrourônico (KORMELINK e VORAGEN, 1993); xilanas de palha de arroz contêm aproximadamente 46% de xilose, 44% de arabinose, 6,1% de galactose, 2% glicose e 1% de ácido anidrourônico (SHIBUYA e IWASAKI, 1985); e as xilanas de trigo contêm cerca de 66% de xilose, 34,5% de arabinose, 0,1% de manose, 0,1% de galactose e 0,3% de glicose (GRUPPEN *et al.*, 1992).

2.1.1. Sacarificação enzimática da hemicelulose

Os polissacarídeos apresentam uma notável diversidade, como conseqüência da ampla variedade de monossacarídeos existentes na natureza e das diferentes ligações glicosídicas possíveis entre eles (HALTRICH *et al.*, 1996). Portanto, não é surpresa que a degradação microbiana dos polissacarídeos abranja uma diversidade de hidrolases glicosídicas com diferentes especificidades e modos de ação. Estes polissacarídeos se apresentam na natureza sob a forma insolúvel. Por isso, os microrganismos que os utilizam devem possuir enzimas extracelulares, livres ou associadas à parede celular,

para converter os polissacarídeos para produtos solúveis que possam ser transportados para o interior da célula (WARREN, 1991).

As hidrolases glicosídicas são classificadas em mais de 50 famílias de diferentes composições, em aminoácidos, do sítio catalítico (HENRISSAT e BAIROCH, 1993). E mesmo dentro da mesma família existem enzimas com diferentes especificidades, sugerindo que novas especificidades são adquiridas por divergências evolutivas (DAVIES e HENRISSAT, 1995). As exo-hidrolases glicosídicas removem unidade de um ou mais açúcares no final da cadeia polissacarídica, enquanto as endo-hidrolases agem randomicamente dentro da cadeia, produzindo mais extremidades para a ação das exo-enzimas (WARREN, 1991).

As celulases e as hemicelulases (das quais a xilanase é a enzima mais importante) são consideradas componentes do sistema de hidrólise de paredes celulares de plantas; e os organismos que degradam a celulose normalmente degradam a hemicelulose também (BASTAWDE, 1992). A biodegradação total das xilanas, por exemplo, requer endo-β-1,4-xilanases, β-xilosidases e muitas outras enzimas acessórias, como arabinofuranosidase, glicuronidases e acetil esterases. A tabela 2 apresenta as enzimas necessárias para a completa degradação de uma heteroxilana.

Muitas xilanases (EC. 3.2.1.8) não clivam ligações glicosídicas entre unidades de xilose que estejam substituídas. Nestes casos, a cadeia lateral deve ser clivada antes da cadeia de xilana poder ser completamente hidrolisada (BASTAWDE, 1992). Por outro lado, muitas enzimas acessórias removem apenas a cadeia lateral dos xilooligossacarídeos e requerem uma hidrólise parcial da xilana antes da cadeia lateral ser clivada (FILHO *et al.*, 1993). Ainda que a estrutura da xilana seja mais complexa que a da celulose e requeira uma quantidade maior de enzimas e com diferentes especificidades para a hidrólise completa, estes polissacarídeos não formam estruturas cristalinas tão firmes como a celulose e, portanto, são mais acessíveis ao ataque enzimático (GERBER *et al.*, 1997).

Tabela 2. Enzimas envolvidas na degradação completa das heteroxilanas (Adaptado de SAHA, 2003 e WARREN, 1996).

Enzima	Modo de ação
Endo-xilanase	Hidrolisa principalmente ligações β,1-4 entre xiloses no interior da molécula da xilana
Exo-xilanase	Hidrolisa ligações β-1,4 liberando xilobiose
β-xilosidase	Libera xilose a partir da xilobiose e outras cadeias curtas de xilooligossacarídeos
α-arabionofuranosidase	Hidrolisa extremidades terminais não-redutoras de α-arabionofuranosídeos a partir das arabionoxilanas
α--glicuronidase	Libera ácido glicurônico das glicuronoxilanas
Acetilxilana esterase	Hidrolisa ligações acetil-éster em acetil xilanas
Ácido ferúlico esterase	Hidrolisa ligações ferúlico-éster em xilanas
ρ-ácido cumárico esterase	Hidrolisa ligações ρ–cumárico-estér em xilanas

As xilanases microbianas são proteínas com apenas uma cadeia polipetídica, com massas molares variando entre 8 e 145 KDa (SUNNA e ANTRANIKIAN, 1997). A temperatura ótima para as endo-xilanases bacterianas e fúngicas varia entre 40 e 60°C. As enzimas fúngicas são geralmente menos termoestáveis que as bacterianas. As xilanases são usualmente estáveis em pHs entre 3 e 10, possuindo um pH ótimo entre 4 e 7 (ITO *et al.*, 1992). O ponto isoelétrico das xilanases varia de 3 a 10. Normalmente as bactérias são conhecidas por produzirem xilanases que possuem alta massa molecular e baixo ponto isoelétrico ou baixa massa molecular e alto ponto isoelétrico. No entanto, esta relação não é observada em fungos, sendo as xilanases fúngicas, em sua maioria, de baixo peso molecular. A composição de aminoácidos das xilanases indica principalmente a presença de ácido aspártico, ácido glutâmico, glicina, serina e treonina (HALTRICH *et al.*, 1996).

As xilanases de fontes procarióticas, como *Clostridium stercorarium*, *Streptomyces* sp. e o termofílico *Bacillus* sp. são glicoproteínas (DEY *et al.*, 1992). Os carboidratos estão ligados covalentemente à proteína ou estão presentes como complexos dissociáveis das xilanases. A glicosilação parece estar implicada na estabilização da enzima em ambientes extremos (pH e temperatura, por exemplo). A xilanase recombinante do termofílico *Bacillus* sp expressa em *Escherichia coli* mostra

uma menor estabilidade em altas temperaturas quando comparada com a linhagem selvagem, e isto foi atribuído a uma deglicosilação da proteína (KULKARNI *et al.*, 1999).

As xilanases também variam na sua atividade diante de vários substratos celulósicos. As xilanases da família 11 são altamente específicas e hidrolisam apenas xilana, enquanto que as xilanases da família 10, que são maioria, hidrolisam xilana e também celulose, ainda que as atividades xilanolíticas sejam muitas vezes maiores que as celulolíticas (WARREN, 1996).

2.1.2. Produção de xilanases e purificação

Poucos são os microrganismos que possuem o sistema xilanolítico completo, ou seja, todas as enzimas necessárias para a completa degradação das heteroxilanas. Os fungos filamentosos *Penicillum capsulatum* e o *Talaromyces emersonni* são dois exemplos destes microrganismos (GERBER *et al.*, 1997). No entanto, as xilanases são produzidas por uma grande variedade de microrganismos, como bactérias, fungos, algas e protozoários (HALTRICH *et al.*, 1996). A maioria dos fungos e bactérias secreta xilanases extracelulares que agem no material hemicelulósico liberando xilose como um produto assimilável, permitindo o crescimento do microrganismo (GERBER *et al.*, 1997). As xilanases microbianas são as preferidas para os processos de hidrólise em virtude da sua especificidade, das condições suaves de reação, das poucas perdas de substrato e da sua biodegradabilidade. No entanto, o custo da hidrólise enzimática ainda é um importante fator limitante do processo (BARAZNENOK *et al.*, 1999).

Para aplicações comerciais as xilanases devem ser produzidas rapidamente e em grandes quantidades, usando substratos simples e baratos (LONSANE e GHILDYAL, 1999). Neste aspecto, a técnica de cultivo semi-sólido, que será discutida mais adiante, oferece algumas vantagens em relação ao cultivo submerso, incluindo economia de espaço físico e, principalmente, a reprodução do ambiente natural dos microrganismos (GAWANDE e KAMAT, 1999) Além disso, o controle de contaminações é facilitado, devido ao baixo teor de umidade do sistema, e não requer máquina e equipamentos complexos e caros (RIMBAULT, 1998).

O fator básico para a produção de xilanases é a escolha de um substrato apropriado. Substratos hemicelulósicos baratos e abundantes, como palha de trigo, palha de arroz e outros bagaços têm se mostrado extremamente interessantes para a produção dessa enzima (PANAGIOTOU *et al.*, 2003). A atividade xilanolítica é normalmente

maior em fungos do que em bactérias (GERBER *et al.*, 1997) e o maior número de trabalhos sobre produção de xilanases é com este tipo de microrganismo. Entre os fungos a máxima atividade reportada é de 3350 U/mL, em *Trichoderma reesei* (HAAPALA *et al.*, 1994). No entanto, a produção de xilanases já foi extensivamente reportada em bactérias (TECHAPUN *et al.*, 2002; BOCCHINI *et al.*, 2002; SÁ-PEREIRA *et al.*, 2002; NASCIMENTO *et al.*, 2002, HECK *et al.*, 2002). O *Bacillus* sp. JB-99 foi estudado para a produção de uma xilanase celulase-free termoestável em CSS. Dentre os substratos testados, a produção de xilanases foi maior em farelo de arroz, e a impregnação do substrato com meio mineral favoreceu a produção (VIRUPAKSHI *et al.*, 2005). As bactérias exigem um pH próximo da neutralidade para crescimento e produção da enzima (BALL e MCCARTHY, 1989), em contraste com os fungos, que geralmente exigem condições ácidas.

De acordo com TECHAPUN *et al.* (2003), a produção de xilanases em cultivos é regida por poucos parâmetros-chaves, além daqueles normalmente envolvidos no processo. Quando o cultivo é conduzido empregando-se substratos heterogêneos, vários fatores apresentam um efeito combinado no nível de expressão da enzima. Entre eles incluem-se a acessibilidade ao substrato, taxa e quantidade de liberação de xilooligossacarídeos e sua natureza química e a quantidade de xilose liberada, a qual atua como fonte de carbono para o microrganismo e como inibidor da síntese de enzima na maioria dos casos (KULKARNI *et al.*, 1999).

GOKHALE *et al.* (1998) reporta que as xilanases tendem a ligar-se fortemente ao substrato. Nesses casos, uma parte da enzima produzida durante o cultivo é perdida na forma de enzima ligada ao substrato insolúvel. Além disso, outras enzimas metabólicas, como proteases e transglicosidases, também agem sobre a enzima, diminuindo a produtividade. Estas enzimas são normalmente expressas ao final da fase exponencial de crescimento do microrganismo.

Um grande número de publicações nos últimos anos descreve o isolamento de xilanases de novas fontes, sua produção, purificação, clonagem, sequenciamento, mutagênese e análises cristalográficas. A maioria dessas publicações trata do uso das xilanases como agente branqueador de polpa Kraft na indústria de papel (PRADE, 1995).

NASCIMENTO *et al.* (2002) isolaram diversas linhagens de *Streptomyces* sp. produtores de xilanases de solos do cerrado brasileiro. MEDEIROS *et al.* (2003)

produziram xilanases com fungos isolados da floresta amazônica. SÁ-PEREIRA *et al.* (2002) relataram a produção de xilanases em meio submerso por um *Bacillus subtillis* isolado de águas termais em Portugal. Neste trabalho, a máxima produção foi alcançada em 18 horas de cultivo com uma temperatura de 60°C. ARCHANA e SATYNARAYANA (1997) relataram a produção de xilanases em cultivo semi-sólido por um *B. licheniformis* A99 usando farelo de trigo como substrato. Os mesmos pesquisadores relataram posteriormente a purificação desta xilanase, a qual possui uma massa molecular de 45 KDa, determinado por SDS-PAGE, e um ótimo de pH entre 6,0 e 7.5 e de temperatura de 60°C (ARCHANA e SATYNARAYANA, 2003).

Uma endo-xilanase foi produzida por *Rhizopus oryzae* utilizando diversos resíduos agroindustriais, como palha de trigo, bagaço de algodão e farelo de milho como fontes de carbono e bagaço de soja, como fonte de nitrogênio. Uma purificação de 55 vezes foi alcançada e o peso molecular determinado foi de 22 kDa, determinado por SDS-PAGE. Os valores ótimos de pH e temperatura foram 4,5 e 55°C, respectivamente. (BAKIR *et al.*, 2001).

MAGNUSON e CRAWFORD (1997) relataram que uma linhagem de *Streptomyces viridosporus* T7A produziu uma alta atividade de xilanase. A enzima foi purificada empregando-se troca iônica (Q-Sepharose) e gel filtração (G-75). A enzima apresentou um peso molecular de 59 kDa, determinado por eletroforese. A temperatura ótima ficou entre 65 e 70°C e o de pH entre 7 e 8.

O *Bacillus thermantarcticus*, uma bactéria termofílica, produziu uma xilanase extracelular, que foi purificada à homogeneidade por gel filtração (Sephacryl S-200). A temperatura ótima de atividade foi de 80°C e o pH de 5,5 (LAMA *et al.*, 2004).

2.1.3. Mecanismos de ação das xilanases e domínios de ligação à celulose

Frequentemente é sugerido que o mecanismo catalítico das glicosidases assemelha-se ao da lisozima. A reação hidrolítica catalisada pelas xilanases, como pelas celulases, ocorre através de um mecanismo ácido-base envolvendo dois resíduos. O primeiro resíduo age como um catalisador geral e protona o oxigênio da ligação osídica. O segundo resíduo age como um nucleófilo, o qual (a) interage com o oxocarbono intermediário ou (b) promove a formação de um íon OH^-, a partir de uma molécula de água (KULKARNI *et al.*, 1999).

Muitas xilanases e celulases são conhecidas por possuírem uma estrutura modular, compreendida por um domínio catalítico associado a um domínio de ligação à

celulose, não catalítico (Cellulose Binding Domain – CBD) (MANGALA *et al.*, 2003). Os CBDs são encontrados não apenas em celulases e xilanases (SADLER *et al.*, 1995) mas também em arabinofuranosidases, acetilxilana esterases (FERREIRA *et al.*, 1993) e mannanase (STALBRAND *et al.*, 1995).

A presença de CBDs confere um aumento da atividade das celulases e xilanases contra substratos recalcitrantes insolúveis, como as paredes celulares de plantas (BLACK *et al.*, 1997). E esta deve ser a razão para as xilanases conservarem esses CBDs durante a evolução (KULKARNI *et al.*, 1999). Ainda que o mecanismo de ação dos CBDs das celulases seja bem conhecido (HALL *et al.*, 1995; COUTINHO *et al.*, 1993), o seu mecanismo em outras hidrolases de plantas, como xilanases, arabinofuranosidases e mananases, ainda não está bem compreendido. Uma hipótese sugerida por FERREIRA *et al.*, (1990), é de que os CBDs possam potencializar a atividade catalítica das hemicelulases mediante o aumento do contato entre a enzima e a parece celular da planta.

A presença de CBDs em xilanases já foi relatada em *Streptomyces olivaceoviridis* por FUJIMOTO *et al.* (2002), em *Clostridium Stercorarium* por SUN *et al.*, (1998), em *Pseudomonas fluorescens* por FERREIRA *et al.* (1993), além de outros microrganismos.

2.1.4. Aplicações das xilanases

Durante a última década as potenciais aplicações biotecnológicas das xilanases têm atraído especial atenção dos pesquisadores e a maioria das publicações trata do uso das xilanases como agente branqueador de polpa Kraft na indústria de papel (GERBER *et al.*, 1997; DHILLON *et al.*, 2000).

SUBRAMANIYAN *et al.* (2001) apontam as xilanases como importantes agentes para os processos de branqueamento da polpa Kraft, uma vez que a hidrólise da xilana facilita a liberação da lignina da polpa e reduz a quantidade de cloro utilizada nos processos tradicionais. Xilanases produzidas por *Trichoderma longibrachiatum* e por *T. harzianum* E 58, têm sido extensivamente testadas para branqueamento de polpa de eucalipto (SUURNAKKI *et al.*, 1997). Os resultados indicam uma significativa diminuição na quantidade de cloro e de peróxido de hidrogênio usados no processo. Os estudos com xilanases fúngicas para branqueamento de papel têm resultado na diminuição da quantidade de cloro; entretanto, acontece também, invariavelmente, uma inaceitável diminuição da viscosidade da polpa, possivelmente devido a contaminações

das preparações de xilanases com celulases (SRINIVASAN e RELE, 1995). Por isso a necessidade de obter-se xilanases livres de celulases para este tipo de aplicação. DHILLON *et al.* (2000) empregaram uma xilanase livre de celulase produzida pelo *Bacillus circulans* AB16 em CSS para branqueamento de papel e reduziram em até 20% a quantidade de cloro empregada, sem perdas perceptíveis na qualidade do papel.

No entanto, as xilanases também encontram aplicações na maceração de matérias vegetais, na liquefação da mucilagem do café, na recuperação de óleo de minas subterrâneas, na extração de aromas e pigmentos (COUGHLAN e HAZLEWOOD, 1993) e para aumentar a eficiência das ensilagens agrícola (SÁ-PEREIRA *et al.*, 2002). SIMS *et al.* (1993) relataram que a aplicação de uma mistura de xilanases e pectinases resultou em uma melhora da cor do suco de cenoura, sem afetar a turbidez típica. A melhora da cor foi atribuída à maior extração de carotenos durante a prensagem.

As xilanases também podem ser aplicadas em panificação, uma vez que as pentosanas insolúveis presentes na farinha de trigo podem impedir o desenvolvimento do glúten e, consequentemente, reduzir o volume e prejudicar a textura do pão. DA-SILVA *et al.* (1994) aplicaram uma mistura de xilanases e arabinosidases à farinha e perceberam uma degradação considerável das pentosanas, melhorando o manuseio da massa e dando um produto final com maior volume e melhor estrutura do miolo.

BAYLEY *et al.* (1995) relataram que uma xilanase purificada de *Trichoderma viride* foi capaz de induzir a biossíntese de etileno e de duas outras proteínas relacionadas à patogênese no tabaco, sugerindo que as xilanases devem ter um papel na indução dos mecanismos de defesa em plantas.

Em muitas aplicações das xilanases, a presença de celulases na mistura não afeta o processo e nem o produto, e em alguns casos, as celulases têm um efeito sinérgico, aumentando a conversão aos produtos de interesse (GERBER *et al.*, 1997), como é o caso da hidrólise para a produção de etanol (BOTHAST e SAHA, 1997), do processamento de alimentos (MONFORT *et al.*, 1997) e no aumento da digestibilidade de alimentos animais (WONG *et al.*, 1988).

GURGEL *et al.* (1995) afirmaram que os produtos da hidrólise da xilana (xilose e xilooligossacarídeos) também têm potenciais aplicações na indústria de alimentos, principalmente como substrato para produção de xilitol, um adoçante de baixo poder calórico e com demanda crescente na indústria alimentícia moderna. Para esse fim, as xilanas obtidas de madeiras são ainda as mais utilizadas e a hidrólise é normalmente feita com o emprego de ácidos, mesmo que esse tipo de hidrólise gere mais compostos

tóxicos para as células e para o ambiente (MEYERHOFF *et al.*, 1997). Isso se deve ao fato de ainda não terem sido encontradas enzimas que possam realizar o processo de forma eficiente e econômica (HALTRICH *et al.*, 1996).

Eventualmente, em um futuro próximo, a xilana e celulose poderão suprir a demanda mundial por matérias-primas. As leveduras *S. cerevisiae* e *Zymomonas mobilis*, por exemplo, podem utilizar a glicose proveniente da hidrólise da celulose para produzir etanol. E as leveduras *Pichia stipitis* e *Cândida shehate* possuem a capacidade de converter xilose em etanol eficientemente (SAHA, 2003). SUN e CHENG (2002) acreditam que não é tão irreal imaginar que dentro de 50 anos o carvão e o óleo possam ser substituídos por biomassa vegetal, desde que se desenvolvam tecnologias eficientes para estas conversões. GOKHALE *et al.* (1998) trataram resíduos agroindustriais (bagaço de cana e farelo de milho) com uma xilanase produzida por levedura e obtiveram uma hidrólise de 19,4%, sugerindo que estes resíduos possam ser utilizados na produção de etanol.

2.2. O cultivo semi- sólido

O cultivo semi-sólido (CSS) e seus diversos sinônimos – cultivo em estado sólido, cultivo em substrato sólido, fermentação semi-sólida ou fermentação em estado sólido - pode ser definido como o crescimento de microrganismos em substratos insolúveis na ausência de água livre (PANDEY, 1992).

A origem do CSS remonta à produção de pães no antigo Egito. O CSS sempre foi largamente empregado para a produção de alimentos tradicionais no Oriente e para a produção de bebidas alcoólicas: tempeh na Indonésia, licores na China e o tradicional saquê no Japão. Em virtude dessas aplicações, o CSS sempre ocupou um lugar de destaque na indústria biotecnológica oriental (RIMBAULT, 1998).

No ocidente, o CSS foi completamente ignorado por muito tempo, devido, principalmente, ao grande desenvolvimento das tecnologias de cultivo submerso (CSm) para a produção de penicilina e a enorme importância que este antibiótico alcançou durante a Segunda Guerra Mundial. Por isso o CSm tornou-se a tecnologia quase que única para a produção de compostos por cultivo de microrganismos. Poucos trabalhos com CSS foram realizados nesses períodos (PANDEY, 2003).

No entanto, felizmente, nas duas últimas décadas do século passado houve uma revigoração dos trabalhos com CSS e um grande número de publicações e patentes têm surgido, bem como um grande desenvolvimento na construção de biorreatores,

industriais e de laboratório. A tabela 3 mostra a evolução do número de publicações com CSS. A grande maioria desses trabalhos refere-se a escalas de laboratório. E muito poucos autores tratam dos problemas relacionados à engenharia do cultivo.

Tabela 3. Número de publicações com os termos "solid-state fermentation and/or cultivation" Fonte: Base de dados Science Direct on Line (aproximadamente 1800 títulos),

Ano	nº de publicações
1950 - 1979	0
1980 – 1989	22
1990 – 1999	132
2000	39
2001	23
2002	28
2003	46
2004	34
2005 (até abril)	38

2.2.1. Substratos para CSS

RIMBAULT (1998) afirma que todos os substratos usados em CSS têm um aspecto em comum: possuem estrutura macromolecular (celulose, amido, pectina e lignocelulose, por exemplo). É essa estrutura macromolecular que confere a característica de substrato para CSS. Em geral, substratos para CSS apresentam uma composição heterogênea e são, normalmente, resíduos agroindustriais. Os substratos sólidos são normalmente obtidos a baixos preços e ainda fornecem uma quantidade grande de nutrientes para o crescimento microbiano e conseqüente produção de metabólitos (TENGERDY e SZAKACS, 2003).

O Brasil é uma das mais importantes economias agrícolas do mundo, produzindo café, cana-de-açúcar, mandioca, soja, milho, frutas, entre outros. Praticamente todos os produtos são exportados, contribuindo enormemente para o desenvolvimento da economia (SOCCOL e VANDENBERGHE, 2003). No entanto, esta grande produção é

responsável pela geração de uma grande quantidade de resíduos, que podem causar problemas ambientais (PANDEY e SOCCOL, 1998). Nos últimos anos, tem aumentado a preocupação em agregar valor a estes resíduos agroindustriais (PANDEY *et al.*, 2000). Para a sua eficiente utilização, os resíduos agroindustriais devem ser convertidos por processos químicos e/ou biológicos. Neste contexto, a aplicação de técnicas de cultivo semi-sólido ocupa um importante papel. Resíduos agrícolas (cascas, bagaços, palhas) são particularmente interessantes para este propósito, uma vez que estão disponíveis em grandes quantidades (TENGERDY e SZAKACS, 2003).

Vários resíduos agroindustriais, como bagaço de mandioca, borra e polpa de café, casca de maçã, resíduos de soja e batata têm sido usados em processos de CSS com diferentes microrganismos (SOCCOL *et al.*, 1994a; SOCCOL *et al.*, 1994b; VANDENBERGHE *et al.*, 2000).

2.2.2. Características e vantagens do CSS

Existem muitos aspectos importantes, os quais devem ser considerados para o desenvolvimento de qualquer processo de CSS. Estes aspectos incluem a seleção de microrganismos e substratos, a otimização dos parâmetros de cultivo e de extração dos produtos, bem como as formas de purificação dos metabólitos produzidos (BANERJEE e BHATTACHARYYA, 2003).

Durante o CSS uma grande quantidade calor é gerada, o qual é diretamente proporcional à atividade do microrganismo. Os materiais sólidos usados em CSS possuem baixa condutividade térmica, o que faz com que a remoção do calor seja muito lenta (MITCHELL e LONSANE, 1992). Algumas vezes o acúmulo de calor pode desnaturar o produto formado e acumulado no leito. Segundo MITCHELL *et al.* (2003) a temperatura, em alguns lugares do biorreator, pode ser até 20°C maior que a temperatura de incubação. Por tudo isso, a temperatura do substrato é o aspecto mais crítico no CSS, afetando diretamente o crescimento do microrganismo, a formação e germinação de esporos e a formação do produto.

Altos teores de umidade resultam numa diminuição da porosidade do substrato, o que impede a penetração do oxigênio. Isso pode favorecer a contaminação por bactérias. Por outro lado, uma baixa umidade pode levar a uma menor disponibilidade dos nutrientes, resultando em um menor crescimento microbiano (CEN e CHIA, 1998).

A atividade de água (a_w) do substrato também tem influência determinante na atividade microbiana. Em geral, o tipo de microrganismo que pode ser empregado em

CSS é determinado pela sua capacidade de suportar baixas *aw* (CEN e CHIA, 1998; PANDEY, 2003). Embora a baixa a_w do CSS indique pela não utilização de culturas bacterianas, vários trabalhos mostram que bactérias podem ser empregadas em CSS eficientemente e com vantagens, em alguns casos, em relação a culturas fúngicas (ADINARAYANA *et al.*, 2003; TECHAPUN *et al.*, 2002; HECK *et al.*, 2002; NAMPOOTHIRI e PANDEY, 1996). Trabalhos com enzimas hidrolíticas bacterianas produzidas em CSS, no entanto, estão preliminarmente limitadas principalmente a *Bacillus* sp., o que pode ser devido a sua habilidade de aderir às partículas do substrato para produzir células filamentosas para penetração e sua necessidade específica por atividades de água mais baixas (ARCHANA e SATYNARAYANA, 1997).

Devido à natureza do substrato, a aeração tende a requerer menores pressões que aquelas necessárias às culturas líquidas, e agitações vigorosas não são requeridas nos CSS (SOUZA *et al.*, 1999).

O CSS oferece muitas vantagens em relação aos cultivos submersos (CSm), incluindo a economia de espaços necessários para o cultivo, a simplicidade do meio de cultivo, o não requerimento de equipamentos complexos, menores volumes de água, reduzida demanda de energia, menor capital, menores volumes de solvente para extração, ausência de formação de espuma, maior facilidade de controle de contaminações, devido à menor atividade de água do sistema (VINIEGRA-GONZÁLEZ *et al.*, 2003). Além disso, o impacto ambiental dos sistemas de CSS é usualmente menor que o do CSm, uma vez que os resíduos usados como substratos são exauridos pela biomassa (CONTI *et al.*, 2001).

Normalmente é destacado por alguns autores que as produtividades tendem a ser maiores em CSS do que em CSm. No entanto, isso não pode ser considerado uma verdade absoluta, uma vez que uma comparação em termos volumétricos não é possível entre esses dois tipos de cultivo. Cada caso deve ser analisado independentemente, apesar de alguns estudos fazerem comparações. SANDHYA *et al.* (2005), por exemplo, realizaram um estudo para a produção de proteases neutras usando resíduos agroindustriais como substrato para CSS e para CSm. Foram utilizadas sete culturas fúngicas de *Aspergillus* e *Penicillum* para a produção da enzima e diferentes resíduos agroindustriais (palha de trigo, casca de arroz, palha de milho). A produção da enzima em CSS foi, em média, 3,5 vezes maior que em cultivo submerso e a palha de trigo foi o melhor substrato.

Outro aspecto importante em CSS é a forma com que se conduz a recuperação dos metabólitos a partir dos sólidos cultivados (CASTILHO *et al.*, 2000) e no que se refere a isso poucos estudos sobre a extração de enzimas em CSS têm sido conduzidos. Segundo CASTILHO *et al.* (1999), na produção comercial de enzimas microbianas, a recuperação do produto constitui cerca de 80% dos custos totais. Como em CSS os metabólitos produzidos são, via de regra, mais concentrados que os produzidos em CSm, os custos de recuperação dos produto tendem a ser menores (SINGH *et al.*, 1999). Assim sendo, se as operações de recuperação forem bem feitas, é possível obter um extrato bruto concentrado, reduzindo, portanto, os custos dos processos de *downstream*.

IKASARI e MITCHELL (1996) estudaram a extração de proteases sob várias condições de processo. A temperatura e o tipo de solvente são conhecidos como os mais importantes parâmetros na extração de solutos a partir de sólidos. No entanto, em se tratando de enzimas, é necessário considerar a estabilidade térmica da mesma, a qual é imensamente afetada pelo tempo de exposição. Portanto, neste caso, três são os fatores fundamentais: tipo de solvente, temperatura e tempo de contato, além da inter-relação entre eles.

Para otimizar as operações de recuperação em CSS, a melhor combinação de temperatura de extração, tempo de contato e solvente foram investigados por CASTILHO *et al.* (2000). O tampão acetato (pH 4,4), a temperatura de 35°C e o tempo de 30 minutos permitiram uma melhor recuperação. Os resultados demonstram que a otimização do processo de extração de enzimas produzidas em CSS é uma forma simples de obter extratos enzimáticos mais concentrados.

De acordo com KUMAR e LONSANE (1987) e RAMADAS *et al.* (1995), é necessário desenvolver técnicas eficientes de extração para tornar o CSS aplicável para a produção de enzimas com alto grau de pureza e capazes de permitir a sua exploração comercial.

2.2.3. Aplicações do CSS

O CSS é amplamente utilizado para a produção de um largo espectro de produtos, incluindo enzimas, ácidos orgânicos, antibióticos, alcalóides e também no tratamento de resíduos orgânicos tóxicos (PANDEY, 1992). A tabela 4 mostra algumas das aplicações atuais das técnicas de CSS.

Tabela 4. Aplicações modernas do Cultivo Semi-sólido. Adaptado de RIMBAULT (1998) e PANDEY *et al.* (2000).

Produto ou processo	Microrganismo	Substrato
Amilase e glicoamilase	*Aspergillus niger* e *A. orizae*	Farelo de arroz e de trigo
Arabinofuranosidase	*Trichoderma reesei*	Polpa de beterraba
Celulase e β-glicosidase	*Aspergillus* sp, *Neurospora* sp.	Bagaços, farelo de trigo, farelo de arroz
Inulinase	*Staphylococcus* sp.	Farelo de trigo, chicória.
Lipases	*Candida* sp.	Farelo de arroz
Proteases	*Aspergillus* sp., *Bacillus* sp.	Casca de arroz, farelo de arroz, farelo de milho, farelo de trigo
Xilanase	*Aspergillus* sp, *Bacillus* sp.	Farelo de trigo, farelo de soja
Biorremediação	*Lentimula edodes*	Solo contaminado com pentaclorofenol
Etanol	*Saccharomyces sake*	Milho
Goma xantana	*Xanthomonas campestris*	Resíduos de maltaria
Penicilina	*Penicillium chrysogenum*	Bagaços

Atualmente as enzimas lignocelulósicas são produzidas principalmente por CSS, sendo as principais enzimas as celulases e as xilanases (PANAGIOTOU *et al.*, 2003; HECK *et al.*, 2002; HALTRICH *et al.*, 1996). O CSS tem apresentado um enorme potencial na produção destas enzimas, especialmente quando o extrato bruto obtido pode ser usado diretamente como fonte de enzimas (TENGERDY, 1998).

2.2.4. Biorreatores e escalonamento

A aplicação do CSS é limitada pela falta de critérios e tecnologias de escalonamento. Em virtude disso, muitos processos industriais são realizados em escalas intermediárias e envolvem reatores do tipo bandeja, os quais apresentam uma manipulação extremamente trabalhosa (MITCHELL *et al.*, 2003).

Os problemas de escalonamento sempre receberam atenção cuidadosa em se tratando de CSm. Infelizmente esse conhecimento é pequeno quando se trata de CSS, em virtude, principalmente, dos fenômenos limitantes serem diferentes. Para CSms aerados o principal aspecto limitante é a transferência de oxigênio através da interface líquido-gás. Já para CSS, o crescimento é limitado pela transferência de calor e pelas transferências de massa (oxigênio e nutrientes), dependendo da localização do substrato no leito, do estágio do cultivo e do desenho e operação do biorreator. Como resultado disso não existem critérios quantitativos para o escalonamento de processos de CSS (RAGHAVARAO *et al.*, 2003).

No entanto, nas últimas décadas, um significativo aporte de novos biorreatoes para CSS tem acontecido. A chave para esse avanço é a aplicação de técnicas de modelagem matemática para descrever os fenômenos físico-químicos e bioquímicos envolvidos no CSS (MITCHELL *et al.*, 2000). Estes modelos podem ser usados como uma orientação para escalonamentos. No entanto, ainda não existem trabalhos demonstrando a aplicabilidade prática desses modelos. Um modelo matemático para o crescimento de microrganismos em CSS para produção de tanase foi desenvolvido e testado experimentalmente por LAGEMAAT e PYLE (2005). O modelo descreveu corretamente a cinética de crescimento do fungo *Penicillium glabrum*.

Na maioria dos casos os processos de CSS são aeróbicos e um biorreator eficiente não deve apresentar as dificuldades de transferência de calor e massa e facilitar a difusão e a extração dos metabólitos. Reatores do tipo bandeja e tambor são os mais estudados ao longo dos anos; no entanto, ultimamente tem crescido o número de trabalhos com reatores do tipo leito fixo, que podem ser mais baratos e de mais fácil manipulação PANDEY (2003). Um novo e eficiente biorreator para CSS com duas opções para entrada de ar (ar pressurizado pulsado e circulação interna de ar) foi desenvolvido e testado por CHEN *et al.* (2005). Estes sistemas aumentaram a transferência de calor e o efetivo controle de temperatura do processo de CSS para produção de celulases.

2.3. Emprego de metodologias de planejamento experimental e superfície de resposta em Biotecnologia

Ao contrário do que se pensa normalmente, a estatística não é apenas a análise dos dados obtidos. Ela é também o planejamento dos experimentos em que esses dados são coletados. E talvez possa até se dizer que ela é principalmente o planejamento,

porque sem um planejamento adequado nunca se sabe se o experimento servirá para o que se deseja, por mais sofisticada que seja a análise que se faça depois.

O planejamento fatorial e a Metodologia de Superfície de Resposta (Response Surface Methodology- RSM) permitem considerar simultaneamente vários fatores em diferentes níveis e as interações entre eles, utilizando um pequeno número de experimentos. E ainda, técnicas de inferência estatística podem ser aplicadas para estimar a importância de fatores individuais, a sensibilidade da resposta para cada fator e a magnitude do erro experimental (NETO *et al.*, 1995).

A Metodologia de Superfície de Resposta tem sido amplamente utilizada para resolver problemas multivariados e otimizar várias respostas em diferentes tipos de experimentos e tem como base o método do planejamento fatorial, que consiste num grupo de técnicas utilizadas para o estudo das relações entre uma ou mais respostas medidas analiticamente e um número de variáveis de entrada que possam ser controladas (FRANCIS *et al.*, 2003). Recentemente, um número grande de métodos de desenho estatístico experimental tem sido empregado para aumentar a produção e a atividade de enzimas e outros metabólitos (SENTHILKUMAR *et al.*, 2005; GOMES *et al.*, 1994 CHEN *et al.*, 1992).

Muitas estratégias têm sido eficientemente empregadas para aumentar a produção de enzimas pelos microrganismos. Entre elas, a otimização das condições de cultivo parece ser uma das mais promissoras (PHAM *et al.*, 1998; BOCCHINI *et al.*, 2002; TECHAPUN *et al.*, 2002). No entanto, a quantidade de informações disponível na literatura sobre a completa otimização dos processos ainda é pequena.

Em geral, a otimização dessas condições é feita pelo tradicional método de variar-uma-variável-por-vez. Este método não é apenas demorado, mas também leva a um incompleto entendimento do comportamento do experimento, resultando em dados confusos e que não terão a capacidade de prever comportamentos futuros (WEJSE *et al.*, 2003; RAO *et al.*, 2000). BEG *et al.* (2003) defendem que parte desses problemas pode ser evitada com a aplicação de desenhos experimentais e adequados modelos multifatoriais.

ADINARAYANA *et al.* (2003) utilizaram a RSM para otimizar as concentrações dos nutrientes necessários para a produção de neomicina por *Streptomyces marinensis* em CSS. WEJSE *et al.* (2003) relataram um aumento de 20 vezes na produção de xilanases bacterianas empregando planejamentos experimentais. A produção de xilanases por *Bacillus* sp. I-1018 foi aumentada em 135% quando o

microrganismo foi cultivado em um meio que foi desenvolvido por desenhos estatísticos (PHAM *et al.*, 1998). Um considerável aumento na produtividade de xilanases também foi reportado por GUPTA *et al.* (2001) e por BOCCHINI *et al.* (2002) através da realização de experimentos multifatoriais com os constituintes do meio de cultura.

3. OBJETIVOS

Este trabalho teve por objetivo ampliar o montante de conhecimentos sobre a produção, purificação e caracterização de xilanases bacterianas produzidas em cultivo semi-sólido utilizando um abundante resíduo industrial fibroso de soja como substrato.

3.1. Objetivos Específicos:

- Explorar recursos microbianos isolados de ambientes da Amazônia para a produção de uma xilanase bacteriana.

- Desenvolver metodologias reprodutíveis de cultivo semi-sólido, incluindo a otimização do processo de extração das enzimas produzidas e o desenvolvimento de novos biorreatores para CSS.

- Estudar a viabilidade e a eficiência da produção de xilanases em cultivo semi-sólido usando como substrato um abundante resíduo agroindustrial, avaliando-se a influência dos parâmetros de cultivo do microrganismo, como temperatura, aeração e tempo de cultivo, visando otimizar a produção da xilanase produzida.

- Desenvolver metodologias para hidrólise de resíduos agroindustriais e para processos de branqueamento de polpa de papel (polpa Kraft) utilizando extratos enzimáticos brutos e purificados.

- Desenvolver metodologias eficientes e reprodutíveis para a purificação de uma xilanase excretada pelo isolado *Bacillus circulans* BL53 cultivado em CSS.

- Caracterizar a enzima produzida, no que ser refere ao pH e temperatura de atuação, estabilidade térmica, inibidores, ativadores, especificidade e peso molecular.

4. MATERIAIS E MÉTODOS

4.1. Instalações e reagentes

O trabalho foi realizado em sua maior parte nos laboratórios de Biotecnologia I (Laboratório 212) e Biotecnologia II (Laboratório 216), do Instituto de Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul.

Os reagentes químicos utilizados no decorrer do trabalho eram do melhor padrão analítico disponível nos laboratórios e a água utilizada na preparação de tampões e soluções era de qualidade ultra pura proveniente de um sistema de purificação da marca TKA. modelo LAB – UPW.

4.2. Instrumentação e Equipamentos

Todos os equipamentos, meios de cultura e vidrarias foram esterilizados em autoclave vertical (Phoenix Equipamentos / mod. AV. 75/Brasil) por 15 min, a 121°C. O cultivo dos microrganismos em frascos Erlenmeyer realizou-se em incubadora do tipo "shaker" (Nova Técnica Ind. e Com. Ltda/Brasil) com agitação orbital e temperatura controlada.

As leituras dos ensaios espectrofotométricos foram feitas em aparelho "Hitachi" modelo U-1100 (Japão).

As determinações de pH foram realizadas em potenciômetro da marca "Nova Técnica" modelo NT pH/Brasil.

A concentração das amostras se deu em um sistema de liofilização (marca HETO, modelo FD 1,0). Também se fez uso de microcentrífuga com velocidade fixa, marca "Eppendorf" modelo 5410, que atinge 14.000 rpm, o que corresponde a 12.800 *g*. Para volumes maiores foi usada a centrífuga "Hitachi" modelo Himaq CR21E, sempre a

15.000 *g*, 4°C e 15 minutos. Os rotores empregados foram: R12A3 (6 x 250 mL), R22A2 (6 x 35 mL) e R21A (10 x 12 mL).

4.3. Microrganismos

As linhagens de microrganismos utilizadas neste trabalho - *Bacillus circulans* BL53 *e Bacillus coagulans* BL69 - fazem parte de uma coleção de microrganismos isolados em ambientes da bacia amazônica pelo Professor Spartaco Astolfi-Filho, do Instituto de Ciências Biológicas da Universidade Federal do Amazonas. Ambos os isolados foram selecionados em trabalho anterior (HECK *et al.*, 2002), em virtude de suas atividades xilanolíticas.

As culturas foram mantidas em meio sólido com goma tragacante (0,2%) em Meio Mineral Buschnell-Hass (MMBH) e repicadas uma vez por semana. Também foram preservadas em glicerol e sob a forma liofilizada.

4.4. Substrato para cultivo

Empregou-se como substrato básico de cultivo dos microrganismos o resíduo industrial fibroso de soja (RIFS), caracterizado em trabalho anterior (HECK, 2001). Durante o período de realização deste trabalho foram empregados três lotes diferentes de RIFS. Cada lote foi analisado quanto aos teores de açúcares redutores e totais, proteína, celulose, hemicelulose e umidade. Estes lotes apresentaram pequenas diferenças na sua composição centesimal, apresentando a seguinte composição média aproximada: açúcares redutores (1,5 ± 0,5%), açúcares totais (15,5 ± 1%), proteína (29 ± 5%), celulose (16 ± 1%), hemicelulose (23 ± 4%) e umidade (10 ± 5%). No entanto, os lotes apresentavam diferenças na capacidade de retenção de água, o que fez com que houvesse algumas diferenças nas quantidades de meio mineral empregadas nos cultivos.

4.5. Determinação de atividade de xilanase

A atividade enzimática foi determinada pelo método de liberação de açúcares redutores baseado em metodologia sugerida por TAVARES *et al.* (1997). A 1,0 mL da solução contendo o substrato (xilana 10mg/mL em tampão acetato 100 mM, pH 5,0) adicionou-se 1,0 mL de sobrenadante de cultura filtrada. A mistura foi incubada por 30 minutos a 55°C e os açúcares redutores liberados foram medidos colorimetricamente pelo método DNS. A atividade de xilanase (U) foi expressa como μmol de açúcares

redutores liberados por minuto por ml de extrato enzimático. A atividade enzimática específica foi calculada em função da concentração total de proteína no extrato e expressada em $U.mg^{1}$ de proteína.

A curva padrão para açúcares redutores foi elaborada empregando-se diferentes concentrações de xilose.

4.6. Determinação de atividade de celulase

Utilizou-se a técnica do papel filtro (Filter Paper Activity – FPA), desenvolvida por MANDELS *et al.* (1976) e recomendada pela Comissão de Biotecnologia da International Union of Pure and Applicated Chemistry (IUPAC). Neste método, utiliza-se como substrato da reação enzimática papel filtro Whatman nº1, aceito como tendo 95% de α-celulose (GODFREY e WEST, 1996).

A 50 mg (1 X 6cm) de papel filtro Whatman nº1 adicionou-se 1 mL de tampão citrato de sódio 50 mM, pH 5,0 e 1 mL de sobrenadante de cultura filtrado. A mistura foi incubada por 1 hora a 55ºC e os açúcares redutores liberados foram dosados como equivalentes em glicose, pelo método do DNS. A atividade de celulase (UI) foi expressa como μmol de açúcares redutores liberados por minuto por mL de extrato enzimático. A atividade enzimática específica foi calculada em função da concentração total de proteína no extrato e expressada em $UI.mg^{-1}$ de proteína.

4.7. Determinação da atividade proteolítica

Para analisar a atividade proteolítica dos microrganismos durante o CSS, realizaram-se ensaios com azocaseína, adaptado de SARATH *et al.* (1989).

Em tubos de microcentrífuga contendo 250 μL de uma solução-substrato com 2% de azocaseína em tampão fosfato de sódio 50 mM e pH 7,0 adicionaram-se 150 μL de extrato enzimático. A mistura foi incubada a 40ºC por 40 minutos e no final deste período adicionou-se ácido tricloroacético (TCA) a 10% (Synth/Brasil) em cada tubo. Para cada amostra enzimática preparou-se um branco, misturando, nesta ordem, enzima, TCA e substrato azocaseína. Preparou-se, também, um reagente branco substituindo a enzima por solução tampão. Após 15 minutos, necessários para assegurar completa precipitação de fragmentos maiores de azocaseína, centrifugou-se os tubos a 14000 g por 5 minutos. Transferiu-se 1 mL do sobrenadante para um tubo de ensaio contendo 1

mL de hidróxido de sódio 1,0 M (Reagen/Brasil). A absorbância desta solução foi determinada a 440 nm em espectrofotômetro.

Uma unidade de atividade enzimática (U) foi definida como a quantidade de enzima necessária para produzir, em cubeta de 1 cm, a variação de uma unidade na absorbância, sob as condições do método.

4.8. Determinação de açúcares redutores totais

Os açúcares redutores totais foram quantificados pelo método do ácido dinitrosalicilico – DNS, conforme CHAPLIN (1986).

4.9. Determinação de proteína solúvel

A proteína solúvel em todas as amostras foi determinada pelo método proposto por LOWRY *et al.* (1951).

4.10. Cultivo Semi-sólido

4.10.1. Cultivo em Biorreatores Cilíndricos Verticais Estáticos

Para a maior parte dos experimentos foram utilizados Biorreatores Cilíndricos Verticais Estáticos (BCVE) de fluxo ascendente com capacidade de 500 mL (60 mm de diâmetro X 170 mm de altura), desenhados em nosso laboratório. O corpo do biorreator, conforme figura 2, é feito de vidro e o fundo é composto de vidro sinterizado (de porosidade G1). Na parte superior encaixa-se uma rolha de borracha com filtro acoplado para saída do ar e dos gases formados durante o cultivo. Foram montados em conjuntos de quatro unidades com distribuidor de ar central, que recebe ar umidificado e estéril com vazão controlada por rotâmetro. Os biorreatores foram colocados em estufa para permitir o controle de temperatura. A figura 3 mostra um conjunto de BCVEs. As zonas claras dentro do biorreator são células bacterianas.

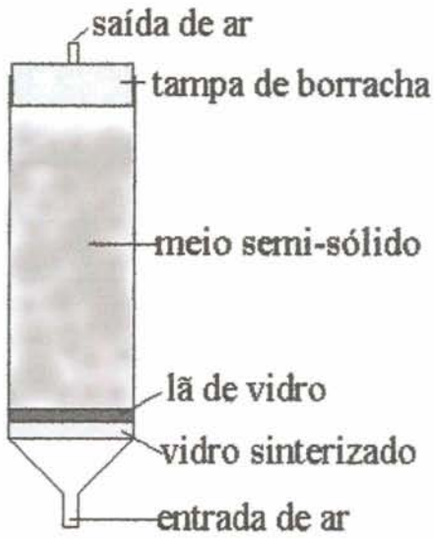

Figura 2. Desenho simplificado de um Biorreator Cilíndrico Vertical Estático.

Figura 3. Biorreatores Cilíndricos Verticais Estáticos.

- Pré-inóculo

A frascos Erlenmeyer de 250 mL adicionaram-se 40 mL de Meio Mineral Bushnell-Hass e 1,2 gramas de RIFS. Esterilizou-se em autoclave por 15 minutos a 121°C. Inoculou-se a partir das culturas em placa de goma tragacante. O crescimento foi conduzido em incubadora rotatória orbital por 18-20 horas, a de 37°C e 100 rpm de agitação.

- Preparo do Substrato

De uma forma geral o material foi preparado conforme HECK *et al* (2002): em um copo de Becker pesavam-se 20 g de RIFS e 120 mL de meio mineral Bushnell-Hass. Este material, após ser esterilizado em autoclave por 15 minutos a 121°C, recebe finalmente 40 mL do pré-inóculo, com densidade ótica (A_{620}) ajustada com água destilada estéril para obter-se uma D.O. de 1,000. Por último, a mistura é transferida assepticamente para os biorreatores.

- Extração da enzima

A recuperação das xilanases produzidas foi feita adicionando-se um dado volume de água destilada (esse volume variou no decorrer do trabalho, em virtude do que será apresentado no item Resultados III) ao conteúdo total do biorreator. Esta mistura foi transferida para frascos Erlenmeyer e agitada em placa agitadora orbital. Em seguida o material sólido foi separado da fase líquida por centrifugação a 10000 *g*, 4°C e 15 minutos. O sobrenadante foi recolhido e utilizado como extrato enzimático.

4.10.2. Cultivo em Biorreatores Cilíndricos Horizontais Agitados

Foram realizados também alguns experimentos em Biorreatores Cilíndricos Horizontais Agitados (BCHA), desenhados no nosso laboratório exclusivamente para este trabalho e construídos por uma metalúrgica da região metropolitana de Porto Alegre. Os reatores foram construídos em aço inoxidável e tem volume total de 12 L (20 cm de diâmetro X 40 cm de comprimento). A aeração foi realizada através da injeção de ar úmido estéril pelo eixo interno do biorreator e a agitação se deu por pás conectadas ao eixo interno em movimentos radiais, de forma intermitente ou contínua, em velocidades que variaram entre 2 e 10 rpm. A temperatura era controlada pela circulação de água, proveniente de um banho-maria, pela camisa do biorreator. Na Figura 3 pode-se visualizar um dos BCHAs usados neste trabalho.

Figura 4. Biorreator Cilíndrico Horizontal Agitado.

- Pré-inóculo

A frascos Erlenmeyer de 1L adicionaram-se 400 mL MMBH e 10 g de RIFS. Esterilizou-se em autoclave por 15 minutos a 121°C. Inoculou-se a partir das culturas em placa de goma tragacante. O crescimento foi conduzido em incubadora rotatória orbital por 24 horas, a de 37°C e 100 rpm de agitação.

- Preparo do Substrato

Ao biorreator eram adicionados 790 g de RIFS e 4400 mL de MMBH. Este material, após ser esterilizado em autoclave por 15 minutos a 121°C, recebeu os 400 mL do pré-inóculo,

- Extração da enzima

A recuperação das xilanases produzidas foi feita adicionando-se um volume de água destilada a uma massa conhecida de material cultivado retirado do biorreator. Esta mistura foi transferida para frascos Erlenmeyer e agitada em placa agitadora orbital. Em seguida o material sólido foi separado da fase líquida por centrifugação a 10000 *g*, 4°C e 15 minutos. O sobrenadante foi recolhido e utilizado como extrato enzimático.

4.11. Purificação da xilanase

O processo de purificação da enzima envolveu as seguintes etapas: precipitação fracionada com sulfato de amônio ("salting-out"), seguida por uma etapa de cromatografia de troca iônica e outra de gel filtração.

- Precipitação fracionada com sulfato de amônio

O extrato bruto era filtrado em papel Whatman nº42. A precipitação (SCOPES, 1993) foi realizada em temperatura próxima a 0ºC. Após cada adição de sal, feita lentamente e sob agitação, o material era deixado por 30 minutos sob agitação lenta antes de ser centrifugado a 10000 g, por 25 minutos, a 4ºC. O "pellet" era ressuspendido em tampão acetato de sódio 50 mM pH 5,5 e dialisado contra o mesmo tampão até que não mais houvesse sulfato de amônio no mesmo (3-4 trocas, com 2 litros de volume).

- Cromatografia de Troca iônica

A cromatografia de troca iônica foi realizada em coluna de 1 x 20 cm (Sigma-Aldrich Corporation) empacotada com a resina CM-Sepharose Fast Flow (carboxymethyl - trocadora fraca de cátions – Amersham Bioscience) e equilibrada com tampão acetato de sódio 50 mM pH 5,5. A amostra era oriunda diretamente da precipitação de 200 mL de extrato bruto com sulfato de amônio, dialisada. A eluição da coluna foi realizada com um gradiente linear crescente de zero a 0,5 M de NaCl com um fluxo de 0,8 $mL.min^{-1}$. As frações que apresentaram atividade enzimática foram agrupadas e liofilizadas.

- Gel filtração

A etapa de gel filtração foi realizada em coluna de 1,5 x 55 cm (Sigma-Aldrich Corporation) empacotada com a resina Sephacryl S-200 HR (Pharmacia Biotech) e equilibrada com tampão acetato de sódio 50 mM pH 5,5. A amostra aplicada na coluna era constituída pelas frações com atividade e liofilizadas da etapa anterior, ressuspendidas no mesmo tampão acetato. A eluição foi feita, por gravidade, a um fluxo de 0,4 $mL.min^{-1}$.

Nas etapas de cromatografia líquida utilizou-se um sistema de purificação composto por bomba peristáltica modelo "P-1 Peristaltic Pump" (Amersham Pharmacia Biotech) e um coletor de frações modelo "2128 Fraction Collector", da Bio-Rad

Laboratories. A medida das concentrações de proteína (A_{280}) foi realizada em espectrofotômetro, cujas especificações já foram citadas anteriormente.

4.12. Eletroforese em gel de poliacrilamida (SDS-PAGE)

A separação de proteínas por eletroforese em géis de poliacrilamida (SDS-PAGE) deu-se em um sistema descontínuo de acordo com a técnica proposta por LAEMLI (1970). As amostras foram separadas em géis com 10% de poliacrilamida. Utilizou-se minigéis de 0,1 x 10 x 10,5 cm em um sistema Hoefer miniVE Vertical Eletrophoresis e a corrente necessária foi fornecida por fonte EPS 301 (Pharmacia Biotech).

Para determinação do peso molecular da enzima foi utilizado o kit de calibração "Bio-Rad Brod Range" contendo 7 proteínas de 6.500 a 97.400 Daltons, em tamanho (Amersham Bioscience). A coloração das bandas de proteína foi realizada através de impregnação com prata.

4.13. Cinética Enzimática

Os ensaios de cinética enzimática foram realizados em triplicata, planejados e analisados de acordo com PRICE e STEVENS (1989).

4.14. Análises Estatísticas

Nas análises estatísticas dos experimentos empregaram-se os softwares Statistic 5.0 e SANEST.

5. RESULTADOS

Os resultados deste trabalho serão apresentados na forma artigos científicos, formato este que é altamente incentivado pelo PPGBCM para organização da tese. Os referidos artigos estão apresentados no item RESULTADOS - Resultados I, II, III, IV e V - nos moldes em que normalmente são submetidos aos periódicos.

No primeiro artigo (Resultados I - "Optimization of cellulase-free xylanase activity produced by Amazon *Bacillus coagulans* BL69 strain on solid-state cultivation"), foram determinadas as melhores condições para atividade e as especificidades de ação do extrato xilanolítico livre de celulases produzido pelo isolado *Bacillus coagulans* BL69 em cultivo semi-sólido. Este artigo já está publicado - *Process Biochemistry*, 40:107-112. 2004.

No segundo artigo (Resultados II - "Statistical optimization of thermo-tolerant xylanase activity from Amazon Isolated *Bacillus circulans* on Solid-state cultivation"), foram determinadas as melhores condições para atividade xilanolítica e as especificidades de ação do extrato produzido pelo isolado *Bacillus circulans* BL53 em cultivo semi-sólido, demonstrando as potenciais aplicações deste extrato na hidrólise de diversos resíduos agroindustriais. Este artigo foi submetido para publicação no periódico Bioresource Technology em maio de 2004 e encontra-se em revisão.

O terceiro artigo (Resultados III - "Extraction optimization of xylanases obtained by solid-state cultivation of *Bacillus circulans* BL53") trata do estudo das condições de extração de xilanases produzidas em cultivo semi-sólido pelo *B. circulans* BL53. Este é um dos primeiros trabalhos da literatura a abordar a otimização da extração de enzimas produzidas em CSS e já foi publicado – *Process Biochemistry*, 40:2891-2895, 2005.

No quarto artigo (Resultados IV - "Optimization of xylanase and mannanase production by *Bacillus circulans* strain BL53 on solid-state cultivation") foram determinadas as melhores condições de produção (temperatura, pH e aeração) de xilanases e mananases, outra enzima hidrolítica de interesse, pelo *Bacillus circulans* BL53 em cultivo semi-sólido. Este artigo está aceito para publicação – *Enzyme and Microbial Technology*, no prelo.

O quinto artigo (Resultados V - "Purification and properties of a xylanase produced by *Bacillus circulans* BL53 on solid-state cultivation") trata da purificação da xilanase produzida pelo isolado BL53 e da caracterização desta enzima, no que se refere à temperatura, pH, ativadores e inibidores, substratos de ação, estabilidade térmica, parâmetros cinéticos e massa molecular. Este artigo foi submetido para publicação no periódico Enzyme and Microbial Technology em maio de 2005.

5.1. RESULTADOS I. "Optimization of cellulase-free xylanase activity produced by Amazon *Bacillus coagulans* BL69 strain on solid-state cultivation" - *Process Biochemistry*, 40:107-112. 2004

Optimization of cellulase-free xylanase activity produced by Amazon *Bacillus coagulans* BL69 strain on solid-state cultivation

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Abstract

A 2^2 factorial design was performed to find the best pH and temperature for xylanolytic activity of cellulase-free *Bacillus coagulans* isolated from the Amazon environment. Solid-state cultivation was carried out on an inexpensive, abundant agro-industrial soybean residue. The central composite design (CCD) used for the analysis of treatment combinations showed that a second order polynomial regression model was in good agreement with experimental results, with R^2=0.8319 ($P<0.05$). The maximum activity was obtained over a large range of temperature (45-75°C) and pH (4.5-10.0). Enzymatic activity was maintained in heated extracts up to 50°C, suggesting that the xylanases of *B. coagulans* are thermo-tolerant biocatalysts, being of interests for biobleaching processes. The crude enzyme extract hydrolyzed Kraft pulp cellulose and its activity was stimulated by Co^{+2}, Mn^{+2} and β-mercaptoethanol but inhibited by Fe^{+3}, Cu^{+2}, Ca^{+2}, Zn^{+2}, Ba^{+2}, Mg^{+2} and by EDTA.

Keywords: Cellulase-free xylanase, solid-state cultivation, bioprocess optimization, central composite design, *Bacillus coagulans*.

1. Introduction

Large amounts of agro-industrial residues are generated every year from diverse economic activities. These residues represent one of the most energy-rich resources available on the planet and when not properly discharged or used, add to the environmental pollution [1]. Xylan is the main hemicellulolytic polysaccharide found in

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plant cell walls and represents a significant renewable biomass comprising up to 20–35% dry weight of agricultural wastes. In particular, end-processes of soybean generates a residue composed of approximately 23% hemicelluloses, 16% celluloses, and 28% insoluble proteins. It has been estimated that approximately 10,000 tons/year of this residue are currently being produced and discharged [2]. Considering that approximately 30 to 40% of production inputs of many industrial enzymes are attributed to the substrate [3], the use of simple, low-cost substrates, could significantly contribute to cost reductions [4].

Xylanase (endo-1,4-β-xylanase) and β-xylosidase (β-D-xyloside xylohydrolase) are the main constituents of microbial xylanolytic enzyme systems [5]. Interest in xylanases increased in recent years, mainly due their applications in paper industries for pulp treatment, improving the effectiveness of conventional bleaching [6,7]. They are also extensively used as food and beverage modifiers, in bakery and in the clarification of fruit juices and wines [8]. Most of the literature concerning xylanases are dealt with their purification and characterization [9], with very few studies regarding their production optimization.

Xylanases have been produced in either solid-state (SSC) or submerged cultivations with the prevalence of the last one [8]. However, SSC has gained renewed interest in recent years and has often been employed for the production of many enzymes due to a number of economical and engineering advantages [10]. For instance, productivity in SSC is usually much higher than that of submerged cultures [11], with lower operation costs, simpler plant and equipment projects and lower energy requirements [12].

In order to improve enzyme production, conventional methods based on the "change-one-factor-at-a-time" in which one independent variable is studied while fixing all others at a specific level, may lead to unreliable results and inaccurate conclusions [13]. This experimental procedure is also expensive and time-consuming for large numbers of variables. To overcome these limitations, response surface methodology (RSM) and experimental factorial design can be employed to optimize enzyme activity [14], performing a minimum number of experiments [9].

In this work, we employed RSM for the planned statistical optimization of xylanase activity by an Amazon environment isolated strain of *B. coagulans* grown in SSC, using an industrial fibrous soybean residue as substrate.

2. Materials and methods

2.1. Microorganism

Bacillus coagulans BL69 was isolated from the aquatic ambient of the Amazon environment, in Brazil. It has been selected due to its xylan-degrading properties [2]. Its identification was performed by biochemical and morphological methods by two independent certified laboratories (the Microbiology Department of The Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, and The Tropical Foundation, Campinas, SP, Brazil). Isolates were maintained at 4°C on selective agar plate containing 0.1% (w/v) Tragacanth gum in basic liquid medium.

2.2. Substrate, medium and pre-inoculum

Industrial fibrous soy residue (IFSR), chemically defined elsewhere [2], was used as solid substrate for cultures. Basic liquid medium, used as pre-inoculum, had the following composition ($g.L^{-1}$): 0.2 $MgSO_4$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.02 $CaCl_2$, and 0.05 $FeCl_2$. Pre-inocula for all experiments in SSC bioreactor were prepared in 250 mL Erlenmeyer flasks containing 1.2 g of IFSR and 40 mL of basic liquid medium. Flasks were inoculated from a single colony from agar plates and incubated for 18 h, 37°C and 125 rpm.

2.3. Bioreactor and cultivation conditions

Bioreactor experiments were carried out in a 500 mL cylindrical bioreactor (60 mm diameter; 170 mm height) designed and constructed in our laboratory. Bioreactors were loaded with 18.8 g of dry IFSR soaked in 120 mL of basic liquid medium and 50 g of irregular stones with a mean diameter of 5 mm, as inert support. Cultures were run at 37°C for 72 hours. During cultivation, wet sterile air was supplied at a constant flow of 500 $mL.min^{-1}$.

2.4. Enzyme extraction

Enzymes extraction from cultivated medium was performed by the addition of 160 mL of distilled water with agitation at 250 rpm, 45 min. The enzymatic extract was then centrifuged at 10,600 g, 20 min. The supernatant was used as the source of enzymes.

2.5. Xylanase activity assay

Xylanase activity was assayed hydrolyzing Birchwood xylan (Sigma, USA) as substrate. Liberated reducing sugars were quantified by the dinitrossalicylic method [15]. A unit of enzymatic activity was defined as amount of enzyme producing 1 μmol the xylose per minute. Specific activity was expressed as IU/mg protein.

2.6. Protein determination

Protein concentration was determined according to the Lowry assay [16], against a standard curve of bovine serum albumin, fraction V (Sigma, USA).

2.7. Thermal stability of the enzyme extract

The temperature stability of enzyme extract was determined by incubation at 50, 60, 70 and 80°C. Aliquots were withdrawn at intervals of 0, 5, 10, 20 and 30 min, and the residual enzyme activity measured.

2.8. Effects of inhibitors on enzyme activity

Xylanolytic activity inhibition was tested by incubating enzymatic extracts in the presence of 1mM solutions of Fe^{+3}, Cu^{+2}, Co^{+2}, Mn^{+2}, Ca^{+2}, Zn^{+2}, Ba^{+2}, Mg^{+2}, EDTA and β-mercaptoethanol for 15 min before the reaction with the substrate.

2.9. Experimental design

Central composite design (CCD), with k=2, was used in order to generate 11 treatment combinations, with pH and temperature as independent variables. Five levels of each variable were chosen, the upper and lower limits of them, set to be in the range described in the literature. In the statistical model, Y denotes units of xylanase activity and the coded settings were defined as follows: X_1= (temperature – 60)/20; X_2= (pH – 7.0)/3.0.

Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. This design is represented by a second order polynomial regression model, equation 1, to generate contour plots:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1^2 + b_{22} X_2^2 + b_{12} X_1 X_2 + \varepsilon \qquad (1)$$

The test factors were coded according to the following regression equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \quad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the *i*th independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. Statistic 5.0 software (Statsoft, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's t-test; the second order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination, R^2.

Table 1. Process variables used in the CCD, showing the treatment combinations and the mean experimental responses.

Treatment	Coded setting levels X_1= T; X_2= pH		Actual levels X_1= T; X_2= pH		Xylanase activity (IU/mg protein)
	X_1	X_2	X_1	X_2	
1	-1	-1	40	4	0.78
2	1	-1	80	4	0.48
3	-1	1	40	10	0.59
4	1	1	80	10	0.58
5	0	0	60	7	1.28
6	0	0	60	7	1.47
7	0	0	60	7	1.45
8	-1.41	0	32	7	0.61
9	0	-1.41	60	3	0.50
10	1.41	0	88	7	1.26
11	0	1.41	60	11	0.60

3. Results and discussion

3.1. Xylanase activity optimization

Based on previous studies, temperature and pH were identified as the major factors affecting xylanase activity of *Bacillus* spp. [9]. Therefore, CCD was used to obtain the

best conditions for xylanase activity from *Bacillus coagulans* BL69, a previously not studied strain. The experimental design and results for enzyme activity are shown in Table 1. Treatments 5 to 7 (central points) and 10 showed the highest levels of xylanase activity (1.28, 1.47, 1.45, and 1.26 IU/mg protein, respectively). These results suggest that the xylanase from *B. coagulans* BL69 has a higher enzyme active at a relatively high temperature and neutral pH. The highest activity (1.47 IU/mg of protein, obtained at 60°C, pH 7.0), experimentally obtained according to the CCD, was 2.2 fold higher than the activity obtained under conditions usually described in the literature (0.68 IU/mg at 55°C and pH 5.0). Cellulase activity in the free extracts was never detected (results not shown), therefore, *B. coagulans* BL69 must be a cellulase-free strain.

The analysis of variance (ANOVA) was employed for the determination of significant parameters and to estimate the specific xylanase activity as a function of temperature and pH. Data are shown in Table 2. The computed F-value (11.56) was 2.7 fold higher than the F-value in statistic tables [17]. indicating that the model was significant at high confidence level (95%), with R^2=0.8319, thus 83.19% of the total variation is explained by the model. This suggests a satisfactory representation of the process model [1]. Also, there was a good correlation between the experimental and predict values.

Table 2. Analysis of variance (ANOVA) for the model regression.

Source	SS	DF	MS	F-value	F-value in Statistic table
Model	1.3466	3	0.4488	11.56	4.35
Residual	0.27205	7	0.0388		
Lack of fit	0.2508	5	0.0501	4.72	19.30
Pure Error	0.02125	2	0.0106		
Total	1.6187	10			

R^2 = 0.8319; Standard error of estimate = 0.010; SS, Sum of Squares; DF, Degrees of Freedom; MS, Mean Square. Significance level = 95%.

Table 3 shows the significance of coefficients determined by Student's t-test and P-values. Significance of coefficients have been reported to be directly proportional to t-value and inversely to P-value [18]. In our work, T and pH, are significant only at second-order model ($P_{T.T}$<0.02 and $P_{pH..pH}$<0.008), which indicates that they can act as

limiting factors and even small variations in their values will alter xylanase activity to a considerable extent [14], but the interaction between them have an insignificant effect on xylanase activity. Whenever possible, the model was simplified by the elimination of statistically insignificant terms. However, the term T (PT<0.168) was maintained in the model because of its magnitude. We proposed then that the quadratic model should be reduce to:

$$Y = 1.4046 + 0.07710\,T - 0.2665\,T^2 - 0.4598\,pH^2 \qquad (3)$$

where Y is the xylanase activity (UI/mg of protein), T is the temperature and pH is the pH as coded settings. To confirm the applicability of the model, xylanase activity was determined at 60°C, pH 7.0, which are the optimum values suggested by it. In this case, the coded settings of the tested variables were T=0, pH=0, respectively, with the model predicting enzymatic activity of 1.40 IU/mg. Experimentally, 1.11 IU/mg of enzymatic activity was obtained, confirming the closeness of the model to the experimental results.

Table 3. Coefficient estimates by the regression model.

Independent variables (parameter)	Coefficient (β)	Standard error (β)	t-value	Significant value (p-value)
Intercept	1.404	0.059	23.60	0.001
T	0.077	0.036	2.11	0.168
pH	0.005	0.036	0.14	0.896
T.T**	-0.265	0.043	-6.12	0.025
T. pH	0.071	0.051	1.38	0.300
pH . pH*	-0.459	0.043	-10.60	0.008

* Statistically significant at 99% of confidence level.

** Statistically significant at 95% of confidence level.

Figure 1 shows that the shapes of contour curves are independent of the others. Contour plot indicates an optimum enzyme activity in the range of pH 4.5 to 10.0 and T 45 to 75°C. This is extremely interesting because for use in biobleaching processes, xylanases should not only be cellulase-free, but also active at high temperatures. They also should be thermostable and alkalophilic [19]. The use of xylanases for

delignification in the paper industry has been prevented by the lack of large-scale availability of enzymes that are active at around 60°C and above pH 8, which are the prevalent conditions in many biobleaching processes [20].

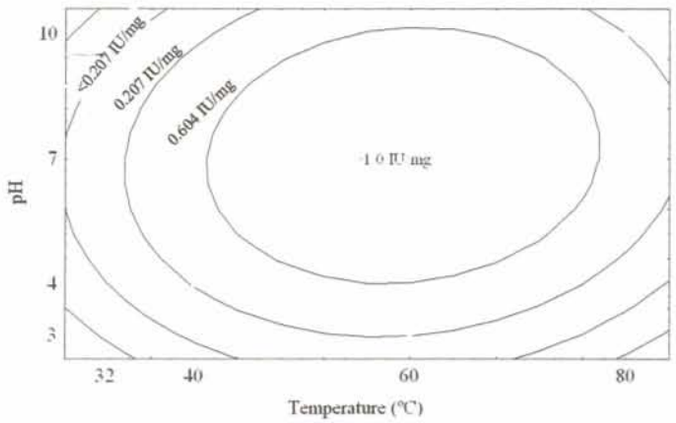

Figure 1. Contour plot for the effects of T and pH on xylanolytic activity of *B. coagulans* BL69.

Under optimized conditions (60°C and pH 7.0), xylanases from *B. coagulans* BL69 presented good ability to hydrolyze kraft pulp, liberating 2.5 mg of reducing sugars from 10 mg of this substrate. It also converted 1.7 mg of xylan (from 10 mg) to reducing sugars. This conversion indicates an extensive depolymerization of hemicellulose, condition that is needed to alter the interface between cellulose and lignin, facilitating the removal of the lignin-associated hemicellulolytic fraction with minimal damage to the pulp [8].

3.2. Effect of various additives on xylanase extract activities

Xylanase activity was assayed in the presence or not of 1mM solutions of several cations, EDTA and β-mercaptoethanol, a protein dissulphide reducing agent. As can be seen in Table 4, a considerable decrease on activities were observed in the presence of Ca^{+2}, Zn^{+2}, Ba^{+2}, Mg^{+2} and EDTA, while Fe^{+3} and Cu^{+2} produced discrete inhibitions. This effect precludes the use of this enzyme in industrial processes that present these chemicals in relevant concentrations. Stimulatory effects were, however, obtained with Co^{+2} and Mn^{+2}. The high increase on xylanase activities by these cations were also reported by Saha [21] and Panagioutou et al. [22]. These results suggest that the xylanases from *B. coagulans* are metalloproteins, probably with Co^{+2} or Mn^{+2} at the

active site. β-mercaptoethanol enhanced activity, presumably by counteracting the oxidation effects of S-S linkage of cysteine residues, thus stabilizing xylanases. This effect has been previously reported for other microbial xylanases [23].

Table 4. Effect of the addition of chemicals upon xylanase activity. The final concentration in the reaction mixture was 1 mM. Relative activity is expressed as a percentage of control.

Compound	Relative activity (%)
Control	100.0
Fe^{+3}	83.7
Ca^{+2}	9.9
Mn^{+2}	124.2
Co^{+2}	159.3
Cu^{+2}	74.7
Zn^{+2}	36.9
Ba^{+2}	54.9
Mg^{+2}	44.1
EDTA	43.2
β-mercaptoethanol	108.0

3.3. Thermal stability of enzymes

Thermal stability is a very important aspect when intending the industrial application of enzymes. The profiles of thermal stability of xylanase at 50, 60, 70 and 80°C are represented in figure 2. Xylanase from *B. coagulans* BL69 is relatively stable at 50 °C, decreasing sharply above 60°C. It kept approximately 34% of its activity when incubated at 70°C, 5 min and 20% at 80°C. Under storage conditions of 7°C for 7 days and –20°C for 30 days, no detectable loss of activities were observed (results not shown).

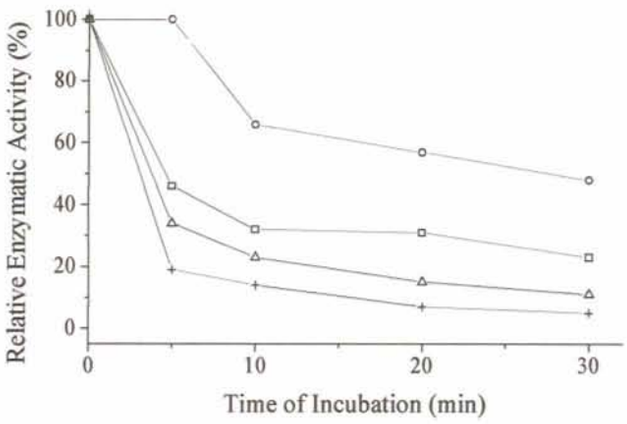

Figure 2. Thermal stability of *B. coagulans* BL69 xylanases. (°) 50°C, (□) 60°C, (Δ) 70°C, (+) 80°C. Results are the mean of 3 experiments.

4. Conclusions

Although many researches regarding xylanase production have been reported, little information on the optimization of this enzyme activity is available. In this work, we demonstrated that the CCD and regression analysis methods were effective to find optimized temperature and pH conditions for xylanase extract activity of *B. coagulans* BL69, a newly isolated strain, growing over a not previously used, cheap and abundant, substrate. The maximum activity was obtained over a large range of temperature (45 to 75°C) and pH (4.5 to 10.0). At optimal conditions, the activity predicted by the model agreed very well with experimental data, confirming the validity of it, with a 2.2-fold increase on xylanase activity achieved by optimization of the parameters. Our results strongly suggest that the cellulase-free xylanase extract from *B. coagulans* BL69 may present interesting properties for industrial applications on pulping and bleaching process. Further studies are under way to characterize individually these enzymes and optimize SSC bioreactor production.

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5.1.1. Considerações finais sobre os trabalhos com o isolado *B. coagulans* BL69

Apesar de os resultados obtidos com esse isolado serem extremamente interessantes, conforme apresentado no manuscrito, e acenarem com uma possibilidade concreta de aplicação da enzima produzida, os trabalhos com o isolado BL69 foram prematuramente suspensos (ao final do primeiro semestre de Doutorado) em virtude de algumas dificuldades apresentadas:

- Este microrganismo apresentou um crescimento consideravelmente mais lento que os outros isolados estudados anteriormente. Para se ter uma idéia, com o isolado *Bacillus circulans* BL53 atinge-se uma densidade ótica (A_{620}) de 1,000 em 6 - 8 horas, quando cultivado em meio nutritivo. Já com o isolado BL69 são necessárias aproximadamente 24 horas para atingir-se esta mesma concentração celular. Quando cultivados em meio líquido com fibra de soja como única fonte de carbono (como acontece nos pré-inóculos do CSS) a duplicação celular parece ser ainda mais lenta. Isso faz com que a concentração celular no pré-inóculo seja baixa e, como é sabido, é fundamental em CSS o emprego de pré-inóculos vigorosos.

- Quando inoculado nos biorreatores de CSS este comportamento se repete. O tempo necessário para visualizar-se crescimento é longo (mais de 48 horas), contra 12 horas do isolado BL53. Isso faz com que a probabilidade de contaminação do cultivo seja aumentada, uma vez que as condições de manipulação do CSS não são completamente assépticas. Para cada dez biorreatores inoculados com o isolado BL69, cerca de oito acabavam contaminados.

- Um dos objetivos do trabalho era um escalonamento gradativo dos biorreatores empregados, visando aumentar o volume de extrato enzimático produzido e, também, contribuir para o desenvolvimento de novas tecnologias e processos de CSS. Para isso, foram desenhados e construídos dois biorreatores do tipo cilíndrico horizontal agitado. Neste tipo de biorreator o isolado BL69 apresentou crescimento e produção de xilanases insignificantes. Em vários experimentos, com diferentes condições, não foi verificada a produção da enzima. Possivelmente a configuração do biorreator, que permite uma maior aeração, melhor transferência de calor e massa e uma distribuição do substrato mais homogênea não foram satisfatórias para este isolado.

5.2. RESULTADOS II. "Statistical optimization of thermo-tolerant xylanase activity from Amazon Isolated *Bacillus circulans* on Solid-state cultivation" submetido para publicação no periódico Bioresource Technology em maio de 2004.

Statistical optimization of thermo-tolerant xylanase activity from Amazon Isolated *Bacillus circulans* on Solid-state cultivation

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Abstract

A 2^2 factorial design was performed to find the best conditions of pH and temperature for xylonolityc activity of *Bacillus circulans* BL53 isolated from the Amazon environment. Solid-state cultivation was carried out on an inexpensive, abundant agro-industrial soybean residue. The central composite design (CCD) used for the analysis of treatment combinations showed that a second order polynomial regression model was in good agreement with experimental results, with R^2=0.9369 ($P<0.05$). The maximum activity was obtained at a high temperature (80 °C) and over a large pH range (4.0 – 7.0). Enzymatic activity was maintained in heated extracts up to 50°C, suggesting that the xylanases of *B. circulans* BL53 are thermo-tolerant biocatalysts, being of interests for industrial processes. The crude enzyme extract hydrolyzed rice straw, sugar cane bagasse and soybean fiber and its activity was stimulated by Co^{+2}, Fe^{+3}, and β-mercaptoethanol but inhibited by Mn^{+2}, Cu^{+2}, Ca^{+2}, Zn^{+2}, Ba^{+2} and Mg^{+2}.

Keywords: xylanase, enzyme technology, optimization, bioconversion, solid-state cultivation, bioprocess design.

1. Introduction

Large amounts of agro-industrial residues are generated every year from diverse economic activities. These residues represent one of the most energy-rich resources available on the planet and when not properly discharged or used, add to the

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environmental pollution (Francis et al., 2001). Xylan is the main hemicellulosic polysaccharide found in plant cell walls. It is composed of a backbone chain of β-1,4-linked xylosyl residues and short side chains of arabionosyl, glucoronosyl and acetyl residues (Bocchini et al., 2002). In particular, end-processes of soybean generates residues composed of approximately 23% hemicelluloses, 16% celluloses, and 28% insoluble proteins. It has been estimated that approximately 10,000 tons/year of this residue are currently being produced and discharged with low or no added value (Heck et al., 2002).

Xylanase (endo-1,4-β-xylanase) and β-xylosidase (β-D-xyloside xylohydrolase) are the main enzymes of microbial xylanolytic systems (Ghosh et al., 1993). These enzymes are useful in several industrial applications. They are extensively used in pre-treatment of forage crops and other lignocellulosic biomass; added to swine and poultry cereal-based diets to improve nutrient utilization; flour modification for bakery products; saccharification of agricultural, industrial and municipal wastes (Sá-Pereira et al., 2002). Most of the literature concerning xylanases are dealt with their purification and characterization, with very few studies regarding their production optimization (Bocchini et al., 2002).

Xylanases have been produced in either solid-state (SSC) or submerged cultivations with the prevalence of the last one (Techapun et al., 2003). However, SSC has gained renewed interest in recent years and has often been employed for the production of many enzymes due to a number of economical and engineering advantages (Pandey et al., 1999).

The classical method of *one-variable-at-a time* bioprocess design may be effective in some situations, but fails to consider the combined effects of all involved factors (Silva and Roberto, 2001). Factorial design optimization and response surface analysis fulfills this requirement. Response surface methodology (RSM) is a collection of mathematical and statistical techniques widely used to determine the effects of several variables and to optimize different biotechnological processes (Rao et al., 2000).

It is acknowledge that bioprocess technology benefits from the use of new strains of microorganisms isolated from environments poorly or never studied before. It is also known that biodiversity has a huge potential as a source for new biocatalysts. In this work, we employed RSM for the planned statistical optimization of xylanase activity of a *B. circulans* strain isolated from an aquatic Amazon environment, grown in solid-state cultivation, using an industrial fibrous soybean residue as substrate.

2. Materials and methods

2.1. Microorganism

Bacillus circulans BL53 was isolated from the aquatic ambient of the Amazon environment, in Brazil. It has been selected due to its xylan-degrading properties (Heck et al., 2002). Its identification was performed by biochemical and morphological methods by two independent certified laboratories (the Microbiology Department of The Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, and The Tropical Foundation, Campinas, SP, Brazil). Isolates were maintained at 4°C on selective agar plate containing 0.1% (w/v) Tragacanth gum in basic liquid medium.

2.2. Substrate, medium and pre-inoculum

Industrial fibrous soy residue (IFSR) was chemically defined elsewhere (Heck et al., 2002) and was used as solid substrate for cultures. Basic liquid medium, used as pre-inoculum, had the following composition ($g.L^{-1}$): 0.2 $MgSO_4$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.02 $CaCl_2$, and 0.05 $FeCl_2$. Pre-inoculum for all experiments in SSC bioreactor were prepared in 250 mL Erlenmeyer flasks containing 1.2 g of IFSR and 40 mL of basic liquid medium. Flasks were inoculated from a single colony from agar plates and incubated for 18 h, 37 °C and 125 rpm.

2.3. Bioreactor and cultivation conditions

Bioreactor experiments were carried out in a 500 mL cylindrical bioreactor (60 mm diameter; 170 mm height) designed and constructed in our laboratory. Bioreactors were loaded with 18.8 g of dry IFSR soaked in 120 mL of basic liquid medium and 50 g of irregular stones with a mean diameter of 5 mm, as inert support. Cultures were run at 37 °C, 72 h. During cultivation, wet sterile air was supplied at a constant flow of 500 $mL.min^{-1}$.

2.4. Enzyme extraction

Enzymes extraction from cultivated medium was performed by the addition of 160 mL of distilled water with agitation at 250 rpm, 45 min. The enzymatic extract was then centrifuged at 10,600 g, 20 min. The supernatant was used as the source of enzymes.

2.5. Xylanase activity assay

Xylanase activity was assayed using Birchwood xylan (Sigma, USA) as substrate. Reducing sugars liberated by hydrolysis of this substrate were quantified by the dinitrossalicylic method (Chaplin, 1986). A unit of enzymatic activity was defined as the amount of enzyme producing 1 μmol of xylose per minute. Specific activity was expressed as IU/mg protein.

2.6. Protein determination

Protein concentration was determined according to the Lowry assay (Lowry et al., 1951) against a standard curve of bovine serum albumin, fraction V (Sigma, USA).

2.7. Thermal stability of the enzyme extract

Temperature stability of enzyme extracts was determined by incubation at 50, 60, 70 and 80 °C. Aliquots were withdrawn at intervals of 0, 5, 10, 20 and 30 min, and the residual enzyme activity measured.

2.8. Effects of inhibitors on enzyme activity

Xylanolytic activity inhibition was tested by incubating enzymatic extracts in the presence of 1 mM solutions of Fe^{+3}, Cu^{+2}, Co^{+2}, Mn^{+2}, Ca^{+2}, Zn^{+2}, Ba^{+2}, Mg^{+2}, EDTA and β-mercaptoethanol for 15 min before the reaction with the substrate.

2.9. Experimental design

Central composite design (CCD), with k=2, was used in order to generate 11 treatment combinations, with pH and temperature as independent variables. Five levels of each variable were chosen, the upper and lower limits of them, set to be in the range described in the literature. In the statistical model, Y denotes units of xylanase activity and the coded settings were defined as follows: X_1= (temperature – 60)/20; X_2= (pH – 7.0)/3.0. Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. This design is represented by a second order polynomial regression model, equation 1, to generate contour plots:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1^2 + b_{22} X_2^2 + b_{12} X_1 X_2 + \varepsilon \qquad (1)$$

The test factors were coded according to the following regression equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \quad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the *i*th independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. Statistic 5.0 software (Statsoft, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's t-test; the second order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination, R^2.

Table 1. Process variables used in the CCD, showing the treatment combinations and the mean experimental responses.

Treatment	Coded setting levels X_1= T; X_2= pH		Actual levels X_1= T; X_2= pH		Xylanase activity (IU/mg protein)
	X_1	X_2	X_1	X_2	
1	-1	-1	40	4	0.56
2	1	-1	80	4	0.95
3	-1	1	40	10	0.48
4	1	1	80	10	0.60
5	0	0	60	7	0.72
6	0	0	60	7	0.75
7	0	0	60	7	0.76
8	-1.41	0	32	7	0.58
9	0	-1.41	60	3	0.61
10	1.41	0	88	7	0.85
11	0	1.41	60	11	0.17

3. Results and discussion

3.1. Xylanase activity optimization

Results for experimental design for enzyme activity are shown in Table 1. Treatments 2 and 10 showed the highest levels of xylanase activity, 0.95 (80 °C, pH 4.0) and 0.85 (88 °C, pH 7.0) IU/mg protein, respectively. These results suggest that the xylanase from *B. circulans* BL53 has a higher enzyme active at high temperatures and

acidic/neutral pH. The highest activity experimentally obtained according to the CCD was 2-fold higher than the activity obtained under conditions usually described in the literature (0.50 IU/mg at 55 ºC and pH 5.0) (Saha, 2002).

The analysis of variance (ANOVA) was employed for the determination of significant parameters and to estimate the specific xylanase activity as a function of temperature and pH. Data are shown in Table 2. The computed F-value (22.27) was 4.9 fold higher than the F-value in statistic tables (Box and Wilson, 1951), indicating that the model was significant at high confidence level (95 %), with R^2=0.9369, thus 93.69 % of the total variation is explained by the model. This suggests a satisfactory representation of the process model (Francis et al., 2003) Also, there is a good correlation between the experimental and predict values.

Table 2. Analysis of variance (ANOVA) for the model regression.

Source	SS	DF	MS	F-value	F-value in Statistic table
Model	0.4017	4	0.1011	22.27	4.53
Residual	0.0272	6	0.0045		
Lack of fit	0.0262	4	0.0065	13	19.25
Pure Error	0.0010	2	0.0005		
Total	0.4312	10			

R^2 = 0.9369; Standard error of estimate = 0.0005; SS, Sum of Squares; DF, Degrees of Freedom; MS, Mean Square. Significance level = 95%.

Table 3 shows the significance of coefficients determined by Student's t-test and P-values. Significance of coefficients has been reported to be directly proportional to t-value and inversely to P-value (Akhnazaronva and Kafarov, 1982). In our work, T, pH and second-order pH were highly significant (P_T<0.005, P_{pH}<0.003 and $P_{pH..pH}$<0.003). The high significance of pH second-order model indicates that this can act as limiting factor and even small variations in its values will alter xylanase activity to a considerable extent (Adinarayana et al., 2003). The model clearly reveals significant interactions between T and pH (p<0.026). Therefore, treating them separately may not reflect their real influence on the xylanase activity (e.g., optimum T activity changes

along with pH). This interaction is essential and it would be of difficult solution using the *one-variable-at-a-time* approach (Wejse et al., 2003). Whenever possible, the model was simplified by the elimination of statistically insignificant terms. We proposed then that the quadratic model should be reduce to:

$$Y = 0.748 + 0.110\,T - 0.131\,pH - 0.156\,pH^2 - 0.068\,pH * T \quad (3)$$

where Y is the xylanase activity (UI/mg of protein), T is the temperature and pH is the pH as coded settings. To confirm the applicability of the model, xylanase activity was determined at 80 °C, pH 4.0, which are the optimum values suggested by it. In this case, the coded settings of the tested variables were T=1, pH=-1, respectively, with the model predicting enzymatic activity of 0.91 IU/mg (range from 0.87 to 0.94) in the confidence level of 95 %. Experimentally, 0.93 IU/mg of enzymatic activity was obtained, confirming the closeness of the model to the experimental results.

Table 3. Coefficient estimates by the regression model.

Independent variables (parameter)	Coefficient (β)	Standard error (β)	t-value	Significant value (p-value)
Intercept	0.748	0.012	57.62	0.000
T**	0.110	0.007	13.89	0.005
pH**	-0.131	0.007	-16.47	0.003
T.T	0.009	0.009	0.97	0.432
T. pH*	-0.068	0.011	-6.04	0.026
pH . pH**	-0.153	0.009	-16.21	0.003

* Statistically significant at 99% of confidence level.

** Statistically significant at 95% of confidence level.

Figure 1 shows contour shapes. Contour plot indicates an optimum enzyme activity in the range of pH 3.0 to 7.0 and T over 80 °C. This is extremely interesting because most xylanases known to date are optimally active at temperatures below 50 °C and few xylanases are reported to be active and stable at high temperature (Sá-Pereira et al., 2002). Souza et al. (1999) reported that the use of xylanases in many processes has been prevented by the lack of large-scale availability of enzymes that are active at around 60 °C.

The potential of enzymatic treatments of agricultural residues has been assessed and the processes have proved successful (Bajpai, 1999). Under optimized conditions (80 °C, pH 4.0), xylanases from *B. circulans* BL53 presented good ability to hydrolyze rice straw, sugar cane bagasse and soybean fiber, liberating 1.1, 0.53 and 0.45 mg of reducing sugars, respectively, from 10 mg of each substrate. Comparatively, it also converted 0.8 mg of xylan (from 10 mg) to reducing sugars. This conversion is very important since inexpensive agro-industrial by-products rich in cellulose and hemicelluloses, are the main potential substrates for producing useful biomolecules, including chemicals and organic solvents (Kang et al., 2004). The increasing interest in biotechnological processes employing lignocellulosic residues is quite justifiable because these materials are cheap, renewable and a widespread source of sugars (Roberto et al., 2003).

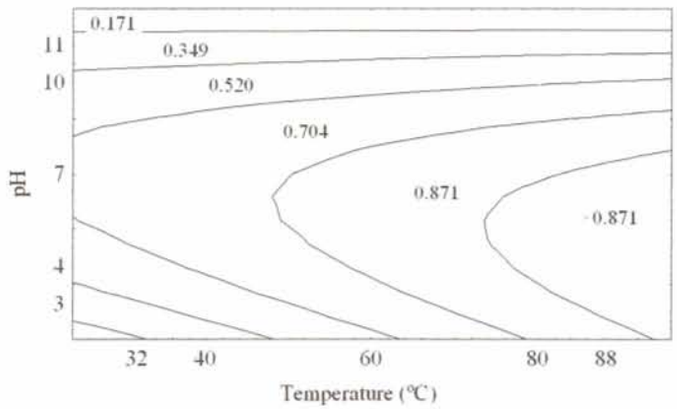

Figure 1. Contour plot for the effects of T and pH on xylanase activity of *B. circulans* BL53.

3.2. Effect of various additives on xylanase extract activities

Xylanase activity was assayed in the presence or absence of 1 mM solutions of several cations, EDTA and β-mercaptoethanol, a protein dissulphide reducing agent. As can be seen in Table 4, considerable decreases on activities were observed in the presence of Cu^{+2}, Zn^{+2} and Ba^{+2}, while Ca^{+3}, Mn^{+2} and Mg^{+2} produced discrete inhibitions. These effects might preclude the use of this enzyme in industrial processes where these chemicals are present in relevant concentrations. Stimulatory effects were, however, obtained with Fe^{+2} and Co^{+2}. The increase on xylanase activities by Co^{+2} also was

reported by Saha (2002) and Panagioutou et al. (2003). These results suggest that the xylanases from *B. coagulans* are metalloproteins, probably with Co^{+2} or Fe^{+2} at the active site. β-mercaptoethanol enhanced activity, presumably by counteracting the oxidation effects of S-S linkage of cysteine residues, thus stabilizing xylanases. This effect has been previously reported for other microbial xylanases (Sá-Pereira et al., 2002).

Table 4. Effect of the addition of chemicals upon xylanase activity. The final concentration in the reaction mixture was 1 mM. Relative activity is expressed as a percentage of control.

Compound	Relative activity (%)
Control	100.0
Fe^{+3}	112.0
Ca^{+2}	97.0
Mn^{+2}	94.7
Co^{+2}	156.5
Cu^{+2}	42.7
Zn^{+2}	43.0
Ba^{+2}	40.4
Mg^{+2}	85.2
EDTA	102.8
β-mercaptoethanol	123.9

3.3. Thermal stability of enzymes

Thermal stability is a very important aspect of industrial enzymatic bioreactors. Profiles of thermal stability of *B. coagulans* BL53 xylanase at 50, 60, 70 and 80 ºC are presented in figure 2, showing stability up to 50 ºC. Above this temperature, its activity sharply decreases, stabilizing at approximately 20 %, even when incubated at 80 ºC, irrespective of time. These results are similar to those reported by Sá-Pereira et al. (2002) for *B. subtilis*, and Nascimento et al. (2002) for *Streptomyces* sp. xylanases, although our

strain still kept some activity above 70 °C. Under storage conditions of 7 °C for 7 days and –20 °C for 30 days, no considerable loss of activities were observed (results not shown).

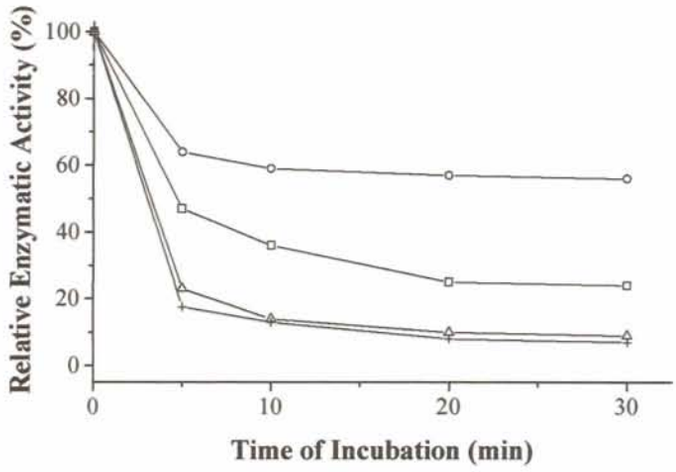

Figure 2. Thermal stability of *B. circulans* BL53 xylanases. (°) 50°C, (□) 60°C, (Δ) 70°C, (+) 80°C. Results are the mean of 3 experiments.

4. Conclusions

Although many researches regarding xylanase production have been reported, little information on the optimization of this enzyme activity is available. In this work, we demonstrated that the CCD and regression analysis methods were effective to find optimized temperature and pH conditions for xylanase extract activity of *B. circulans* BL53, a newly isolated strain, growing over a not previously used, cheap and abundant, substrate. The maximum activity was obtained at high temperatures (80 - 88 °C) and at large pH range (4.0 – 7.0). At optimal conditions, the activity predicted by the model agreed very well with experimental data, confirming the validity of it, with a 2-fold increase on xylanase activity achieved by optimization of the parameters. Our results strongly suggest that the xylanase extract from *B. circulans* BL53 may present interesting properties for industrial applications.

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5.3. RESULTADOS III. "Extraction optimization of xylanases obtained by solid-state cultivation of *Bacillus circulans* BL53" publicado – *Process Biochemistry*, 40:2891-2895, 2005.

Extraction optimization of xylanases obtained by solid-state cultivation of *Bacillus circulans* BL53

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Abstract

The present work dealt with the extraction optimization of xylanases produced in solid state cultivations of a strain of *Bacillus circulans* recently isolated from Amazon basin, with the purpose of reducing enzyme losses in order to obtain crude extracts as concentrated as possible. Substrate for growth and enzyme production was a industrial fibrous soy residue, rich in protein and hemicellulose. Several extraction solvents and temperatures were tested. A 2^3 factorial design was performed to find the best conditions of time, agitation and solid/liquid ratio. Maximum recovery was obtained by extracting in water at 7 °C, 40 minutes, 150 rpm and 1:6 solid/liquid ratio. The central composite design (CCD) used for the analysis of treatment combinations showed that a second order polynomial regression model was in good agreement with experimental results, with R^2=0.90 ($P<0.05$). Our results show that optimization of the extraction conditions is a simple way of obtaining concentrated enzyme extracts from solid state cultivation of industrially important microorganisms.

Keywords: Solid state cultivation; extraction optimization, *Bacillus circulans*; xylanases; industrial fibrous soy residue.

Introduction

Xylanase (endo-1,4-β-xylanase) and β-xylosidase (β-D-xyloside xylohydrolase) are the main constituents of microbial xylanolytic enzyme systems [1]. They are extensively used in pre-treatment of forage crops and other lignocellulosic biomass, in addition to pig and poultry cereal-based diets, to improve nutrient utilization, in flour improvement for bakery products, in saccharification of agricultural, industrial and municipal wastes

[2]. Xylanases have been produced in either solid-state (SSC) or submerged cultivations with the prevalence of the last one [3]. However, SSC has gained renewed interest in recent years and has often been employed for the production of many metabolites due to a number of economical and engineering advantages [4]. Additionally, these processes are of special economic interest for countries with abundance of biomass and agroindustrial residues, as these can be used as cheap raw materials [5].

SSC represents an interesting alternative for the production of technical enzymes used in the food industry where costs are a critical factor determining the economic feasibility of their applications. In SSC, microorganisms produce the metabolites in a concentrated way, when compared with submerged cultures [6]. If the recovery operations are done in adequate conditions, it is possible to obtain concentrated crude extracts and, consequently, reduce the downstream processing costs [7]. Ramadas et al. [8] refer to the need for studies in this area, in order to make SSC applicable for the production of high-purity enzymes. In spite of this, only a few systematic studies are available dealing with extraction of enzymes in SSC [5].

In order to improve xylanase extraction, conventional methods based on the "change-one-factor-at-a-time" in which one independent variable is studied while fixing all others can lead to unreliable results and inaccurate conclusions [9]. This experimental procedure is also expensive and time-consuming for large numbers of variables. To overcome these limitations, response surface methodology (RSM) and experimental factorial design can be employed to optimize enzyme extraction [10] performing a minimum number of experiments [11].

In this work we employed RSM for the planned statistical optimization of xylanase extraction of a strain of *B. circulans* isolated from the Amazon environment grown in SSC system using an industrial fibrous soybean residue as substrate.

2. Materials and methods

2.1. Microorganism

Bacillus circulans BL53 was isolated from the aquatic ambient of the Amazon environment, in Brazil. It has been selected due to its xylan-degrading properties [12]. Its identification was performed by biochemical and morphological methods by two independent certified laboratories (the Microbiology Department of The Federal

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University of Rio Grande do Sul, Porto Alegre, RS, Brazil, and The Tropical Foundation, Campinas, SP, Brazil). Isolates were maintained at 4 °C on selective agar plate containing 0.1% (w/v) Tragacanth gum in basic liquid medium.

2.2. Substrate, medium and pre-inoculum

Industrial fibrous soy residue (IFSR), chemically defined elsewhere [12], was used as solid substrate for cultures. Basic liquid medium, used as pre-inoculum, had the following composition ($g.L^{-1}$): 0.2 $MgSO_4$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.02 $CaCl_2$, and 0.05 $FeCl_2$. Pre-inoculum for all experiments in SSC bioreactor were prepared in 1 L Erlenmeyer flasks containing 10 g of IFSR and 400 mL of basic liquid medium. Flasks were inoculated from a single colony from agar plates and incubated for 24 h, 37 °C and 125 rpm.

2.3. Bioreactor and cultivation conditions

Bioreactor experiments were carried out in a 12 L horizontal rotated drum bioreactor (20 cm diameter; 40 cm height) designed and constructed in our laboratory. Bioreactors were loaded with 790 g of dry IFSR soaked in 4.4 L of basic liquid medium. Cultures were run at 37 °C for 96 hours. During cultivation, wet sterile air was supplied at a constant flow of 4 $L.min^{-1}$.

2.4. Influence of extraction parameters on the recovery of xylanases

Five solvents (water, 50 mM Tris-HCl buffer pH 8, 50 mM acetate buffer pH 5, 10 % solution of ethanol and 10 % solution of glycerol) pre-incubated at preset temperature (7, 25, 45 °C) were added to Erlenmeyers flasks containing the cultivated solids at solid/liquid ratio of 2 g initial substrate (dry basis)/20 mL of solvent. The mixture was mechanically stirred for 40 min at room temperature and 150 rpm. Subsequently, solids were separated from the extract by centrifugation at 10,000 *g* by 15 minutes. The extract was filtered through a Whatman No. 1 filter paper to obtain a clear extract and assayed for xylanase activity. Data were analysed by ANOVA procedure and comparisons between means were performed by Tukey's test [13].

To determine the influence of extraction parameters a central composite design (CCD), with k=3, was used in order to generate 17 treatment combinations, with time of extraction, agitation and solid/liquid ratio as independent variables. Five levels of each

variable were chosen, the upper and lower limits of them, set to be in the range described in the literature. In the statistical model, Y denotes units of xylanase activity.

Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. This design is represented by a second order polynomial regression model, equation 1, to generate contour plots:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^{\,2} + b_{22} X_2^{\,2} + b_{33} X_3^{\,2} + b_{12} X_1 X_2 + b_{13} X_{13} + b_{23} X_2 X_3 + \varepsilon \quad (1)$$

The test factors were coded according to the following regression equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \quad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the *i*th independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. Statistic 5.0 software (Statsoft, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's t-test; the second order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination, R^2.

Table 1. Process variables used in the CCD, showing the treatment combinations and the mean experimental responses. Results are the mean of three experiments.

Treatment	Coded setting levels			Actual levels			Xylanase Activity (IU mL^{-1} of extract)
	X_1	X_2	X_3	X_1	X_2	X_3	
1	-1	-1	-1	20	90	1/8	1.57
2	+1	-1	-1	60	90	1/8	3.42
3	-1	+1	-1	20	210	1/8	3.42
4	+1	+1	-1	60	210	1/8	6.45
5	-1	-1	+1	20	90	1/14	1.62
6	+1	-1	+1	60	90	1/11	3.60
7	-1	+1	+1	20	210	1/14	2.45
8	+1	+1	+1	60	210	1/14	2.65
9	0	0	0	40	150	1/11	4.39
10	0	0	0	40	150	1/11	4.33
11	0	0	0	40	150	1/11	4.31
12	-1.682	0	0	5	150	1/11	1.42
13	+1.682	0	0	75	150	1/11	4.25
14	0	-1.682	0	40	50	1/11	2.49
15	0	+1.682	0	40	250	1/11	3.65
16	0	0	-1.682	40	150	1/6	7.86
17	0	0	+1.682	40	150	1/16	4.01

X_1= time (minutes); X_2=agitation (rpm); X_3=solid/liquid ratio

2.5. Xylanase activity assay

Xylanase activity was assayed hydrolyzing birchwood xylan (Sigma, USA) as substrate. Realeased reducing sugars were quantified by the dinitrosalicylic method [14]. A unit of enzymatic activity was defined as the amount of enzyme producing 1 μmol of xylose per minute. Enzyme was expressed as volumetric activity (IU mL^{-1} extract).

Results and discussion

Initially, a comparison between agitated and static (fixed bed) extraction was made in order to determine the effect of agitation upon extraction of xylanases from the cultivated material. Although fixed bed system produced a clear enzyme extract, making unnecessary a centrifugation step, the recovery of xylanase activity was 20 % lower than in agitated system (data not shown). Ramakrishna et al. [15] verified that amyloglucosidase recovery efficiency from moldy cultivations was 8.5% higher in agitated system when compared to static bed. The higher leaching of the enzyme in agitated system is probably related to a more efficient contact of the solute with solvent, availability of a greater surface area of the solute for efficient mass transfer and enhanced diffusion of the solvent into the solid particles [16]. Based on the present results, stirred extraction was employed in the next steps of this work.

The *ideal* solvent would extract the enzyme selectively and completely at room temperature, with minimal contact time and, preferably, at the pH of the cultivated substrate [17]. Among the solvents tested (Table 2), water and acetate buffer showed the best results for extraction of xylanases (7.76 and 7.12 IU mL^{-1} at 7 and 25 °C for water; 7.19 IU mL^{-1} at 45 °C, for the buffer). Soares et al [18] also demonstrated that water was the best solvent for transglutaminase extraction on solid state cultivation. In many works on solid-state production of xylanases, the solvent used has been either acetate buffer, pH around 5.0 [19,20] or water [12,21]. Water has a higher dielectric constant compared with organic solvents. When the dielectric constant is decreased, the interaction forces between xylanase and solvent may increase, therefore, loss of enzymes would increase. Tunga et al. [22] verified an increase in protease extraction when ethanol and glycerol were used as solvents. In contrast, we obtained opposite results in our work, probably due to inhibitory effects of ethanol and glycerol on xylanase activity. Subsequent extractions with any of the solvents did not have any significant increase on enzyme activity. Hence, only one extraction step was adopted.

Table 2. Selection of extraction solvent and temperature. Means followed by different letters are statistically different ($P<0.05$). Results are the mean of four experiments.

	Xylanase Activity (IU mL^{-1})		
Solvent	7 °C	25 °C	45 °C
Water	7.76^{AB}	7.12^{AB}	3.71^{H}
Tris Buffer 50 mM pH 8.0	6.68^{BC}	5.69^{DE}	5.07^{EF}
Acetate buffer 50 mM pH 5.0	4.64^{FG}	5.41^{DE}	7.19^{AB}
10 % Ethanol	4.16^{GH}	6.02^{CD}	2.97^{H}
10 % Glycerol	6.56^{BC}	4.22^{GH}	4.69^{FG}

Treatments 4 and 16 (table 1) showed the highest levels of xylanase extraction (6.45 and 7.86 IU mL^{-1}, respectively). These results suggest that the xylanase extraction from *B. circulans* BL53 was increased at higher time of extraction, moderate/high agitation and low solid/liquid ratio. The highest extraction (7.86 IU mL^{-1}, obtained at 40 minutes, 150 rpm and 1:6 solid/liquid ratio), experimentally obtained according to the CCD, was almost 2-fold higher than the extraction obtained under conditions usually described in the literature (4.33 IU mL^{-1} at 40 minutes, 150 rpm and 1:11 solid liquid ratio). This is extremely important because concentrated crude extracts ease downstream processes, mainly the purification steps, reducing time and costs of enzymes recovery [5].

The analysis of variance (ANOVA) was employed for the determination of significant parameters and to estimate the xylanase extraction as a function of time of extraction, agitation and solid/liquid ratio. Data are shown in Table 3. The computed F-value (8.78) was 2.5-fold higher than the F-value in statistic tables, indicating that the model was significant at high confidence level (95%), with $R^2=0.90$, thus 90% of the total variation is explained by the model. This suggests a satisfactory representation of the process model [23]. Also, there was a good correlation between the experimental and predict values.

Table 3. Analysis of variance (ANOVA) for the model regression.

Source	SS	DF	MS	F-value	F-value in Statistic table
Model	40.5636	8	5.0704	8.78	3.44
Residual	4.6182	8	0.5773		
Total	45.1818	16			

R^2 = 0.90; SS. Sum of Squares; DF. Degrees of Freedom; MS. Mean Square. Significance level = 95%.

Table 4. Coefficient estimates by the regression model.

Independent variables (parameter)	Coefficient (β)	Standard error (β)	t-value	Significant value (p-value)
Intercept	4.398	0.4656	9.4448	0.0000
X_1*	1.795	0.2188	4.1028	0.0045
$X_1.X_1$**	- 1.343	0.2410	-2.7861	0.0270
X_2	0.983	0.2188	2.2464	0.0595
$X_2.X_2$**	- 1.268	0.2410	-2.6317	0.0338
X_3*	- 1.612	0.2188	-3.6844	0.0078
$X_3.X_3$	0.761	0.2410	1.5793	0.1582
$X_1.X_2$	- 0.147	0.2857	-0.2572	0.8044
$X_1.X_3$	- 0.674	0.2857	-1.1802	0.2765
$X_2.X_3$	- 1.249	0.2857	-2.1863	0.0650

* Statistically significant at 99% of confidence level.

** Statistically significant at 95% of confidence level.

Table 4 shows the significance of coefficients determined by Student´s t-test and P-values. In our work, time and solid/liquid ratio and second-order time and agitation

was highly significant (P_T<0.0045, $P_{(S/L)}$<0.0078, $P_{(T.T)}$<0.0270 and $P_{(A.A)}$<0.0338). The high significance of time and agitation on second-order model indicates that they can act as limiting factors and even small variations in their values will alter xylanase extraction to a considerable extent [10], but the interaction between them have a small effect on xylanase extraction. Whenever possible, the model was simplified by the elimination of statistically insignificant terms. However, the term agitation (P_A<0.0595), second-order solid-liquid ratio ($P_{(S/L).(S/L)}$<0.1582), time-solid/liquid ratio interaction ($P_{T.(S/L)}$<0.2765) and agitation-solid/liquid ratio ($P_{A.(S/L)}$<0.0650) were maintained in the model because of their magnitude. Some authors indeed keep all variables in the model equation [10], while others will elect some variables with P> 0.05 [11], as it is our case, in order to allow for a more flexible model since, in biological systems, we lack the accuracy of chemical reactions. We proposed then that the quadratic model should be reduce to:

$$Y = 4.4 + 1.8T - 1.34T^2 + 0.98A - 1.26A^2 - 1.6(S/L) + 0.8(S/L)^2 - 0.7T.(S/L) - 1.25A.(S/L) \quad (3)$$

where Y is the xylanase activity recovery (IU mL^{-1}), T is the time, A is the agitation and (S/L) is the solid/liquid ratio as coded settings. To confirm the applicability of the model, xylanase extraction was determined at 40 minutes, 150 rpm and 1:6 solid liquid ratio, which are the optima values suggested by it. In this case, the coded settings of the tested variables were T=0, A=0, and (S/L)=-1.68, respectively, with the model predicting enzymatic activity recovery of 9.24 IU mL^{-1}. Experimentally, 8.25 IU mL^{-1} of enzymatic activity recovery was obtained, confirming the closeness of the model to the experimental results.

Figure 1a shows the shapes contour of time against agitation. A time of extraction lower than 30 min seems not to be enough for total solubilization of enzymes present in the cultivated solids. Contour plot indicates an optimum enzyme extraction in the range of time 30 to 75 minutes and agitation between 90 to 250 rpm. According to Ghildyal et al. [16] prolonged periods of time could cause losses of enzyme activity due to the prolonged mechanical agitation or to a greater extraction of denaturant agents, but this was not observed in our work.

Figure 1b shows contour plot of time against solid/liquid ratio and figure 1c shows contour of agitation against solid/liquid ratio. The solid/liquid ratio investigated was 1:6 to 1:16. Maximal enzyme extraction was obtained at lower solid/liquid ratio. Extract volume naturally increased with extractant volume. As both enzyme activity and protein content (data not show) varied at nearly similar rates, specific and volumetric activity showed similar variations along the experiments. The use of small volumes of solvent for extraction of a required metabolite greatly reduces the energy requirements, equipment sizes and pollution problems [17]. This characteristic of the SSC system over submerged cultivations needs to be maintained during the extraction of the cultivated solids for the recovery of products, since a diluted extract would eliminate this economically important advantage [16]. However, inadequate lower ratios could lead to unsatisfactory recoveries, since a significant fraction of the solution could be retained in the cultivated mass [17]. In our study, ideal low solid/liquid ratios presented the best xylanases extraction.

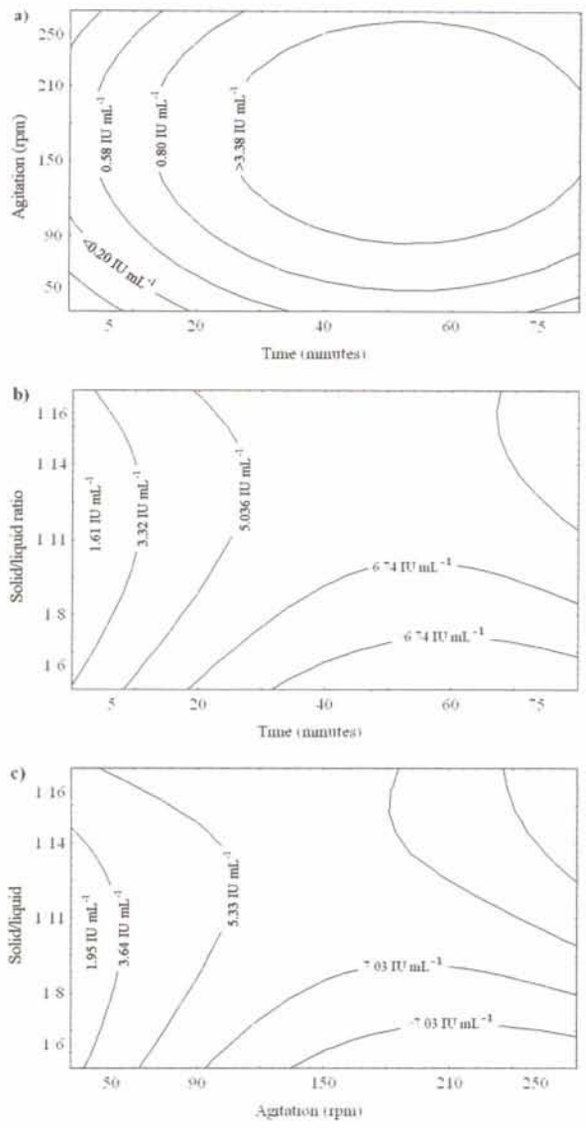

Figure 1. Contour plot for the effect of (a) Time x Agitation, (b) Time x Solid/Liquid ratio, and (c) Agitation x Solid/Liquid ratio on xylanolytic activity recovery. Not plotted variable is fixed at zero level in all of the three graphs.

Conclusions

In the present work, several extraction parameters were studied, aiming at an improvement of the extraction of xylanases produced in solid state cultivations of a newly isolated strain of *Bacillus circulans* from the Amazon environment. The extraction step was optimized by studying the combined influence of solvent nature, temperature, time of contact, agitation and solid/liquid ratio. The most concentrated

crude extracts were obtained employing water at 7 ºC by 40 minutes, 150 rpm and 1:6 solid/liquid ratio, indicating the optimization of the extraction parameters. The results show that optimization of enzyme extraction from SSC is an important tool aiming to reduce costs of downstream processing.

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5.4. RESULTADOS IV. "Optimization of xylanase and mannanase production by *Bacillus circulans* strain BL53 on solid-state cultivation" - *Enzyme and Microbial Technology* – in press.

Optimization of xylanase and mannanase production by *Bacillus circulans* strain BL53 on solid-state cultivation

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Abstract

A 2^3 central composite design (CCD) was applied to determine the optimal conditions of cultivation time, aeration and temperature to xylanase and mannanase production by *Bacillus circulans* BL53, a strain isolated from the Amazonian environment. The results suggest that xylanase production by *B. coagulans* BL53 is higher at a moderate cultivation aeration and at low temperatures and following CCD modeled conditions, we were able to increase 2.5 fold enzyme activities previously obtained and published by our group. For mannanase, the results showed that production was increased at higher aeration rate, and lower temperature. The highest enzymatic activity experimentally obtained according to the CCD was 3.7-fold higher than previous conditions employed. The predicted optimal parameters were tested in the laboratory and the final xylanase and mannanase activities obtained were very close to the predicted value (0.928 U mg^{-1}, predicted; 0.953 U mg^{-1}, tested, and 0.544 U mg^{-1}, predicted; 0.540 U mg^{-1}, tested, respectively). Our results show that optimization of the enzymes production is an useful way of obtaining concentrated enzyme extracts from solid-state cultivation of industrially important microorganisms.

Keywords: Xylanase; Mannanase; Bioprocess optimization; Solid-state cultivation; *Bacillus circulans*, Amazonian biodiversity.

Running headline: Xylanase and mannanase optimization in solid-state cultivation.

1. Introduction

Hemicelluloses form a complex group of branched and linear polysaccharides, strongly bound to lignin and cellulose in higher plant cell walls [1]. Endo-β-1,4-xylanases (EC 3.2.1.8) are enzymes that hydrolyze xylan, the major structural component of hardwood cell walls. The growing interest in xylanase production for industrial applications is due to its importance in the bioconversion of agro-industrial residues, as well as food and beverage improvers, in bakery products or for the clarification of wines and fruit juices [2–4]. Endo-β-1,4-mannanases (EC 3.2.1.78) randomly hydrolyze the main chain of hetero-mannans, the major softwood hemicellulose. Mannanases have been useful tested in several industrial processes, such as the extraction of vegetable oils from leguminous seeds and viscosity reduction of the extracts during the manufacture of instant coffee [5]. In the paper industry, both enzymes have a synergistic action in the biobleaching of the wood pulp, significantly reducing the amount of chemicals used [6-7].

Xylanase and mannanase have been produced in either solid-state (SSC) or submerged cultivations with the prevalence of the later [8–10]. However, SSC has gained renewed interest in recent years and has often been employed, chiefly with fungi species, for the production of many enzymes [11–12]. This cultivation technique offers distinct advantages over submerged liquid cultivations, including equipment and installations economy, greater product yields and provision of easer growth conditions [13].

Several different strategies in order to enhance enzyme production by various microorganisms have been successfully described [3,8-9,14–15]. In general, culture conditions optimization by the traditional 'one-factor-at-a-time' technique are used. This method is not only time consuming but also often leads to an incomplete understanding of the system behavior, resulting in a bafflement and failure of predictive response. To overcome these limitations, response surface methodology (RSM) and experimental factorial design can be employed to optimize enzyme activity, performing a minimum number of experiments [16].

This work integrated statistical experimental designs to optimize the growth conditions for enzyme production, with the use of a cheap byproduct from the soy protein industry as the main substrate, aiming at the production of xylanase and

mannanase by *Bacillus circulans* BL53, a newly isolated strain from the Amazonian environment.

2. Materials and methods

2.1. Microorganism

Bacillus circulans BL53 was isolated from the aquatic ambient of the Amazonian environment, in Brazil. It has been selected due to its xylan-degrading and mannan-degrading properties. The bacterium was characterized and identified in a previous work of our group [14]. Isolates were maintained at 4 °C on selective agar plate containing 0.1% (w/v) tragacanth gum and 0.1% (w/v) locust bean gum in basic liquid medium.

2.2. Substrate, media and inoculum

Industrial fibrous soy residue (IFSR), chemically defined elsewhere [14] was used as solid substrate for cultures. Basic liquid medium, used as inoculum, had the following composition (g l^{-1}): 0.2 $MgSO_4$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.02 $CaCl_2$, and 0.05 $FeCl_2$. The inocula for all experiments in SSC bioreactor were prepared in 500 ml Erlenmeyer flasks containing 200 ml of Luria-Bertani (LB) medium. Flasks were inoculated from a single colony from agar plates and incubated for 18 h, 37 °C and 125 rpm.

2.3. Bioreactor cultivation conditions

Bioreactor experiments were carried out in a 500 ml cylindrical bioreactor (60 mm diameter; 170 mm height) designed and constructed in our laboratory. Bioreactors were loaded with 20 g of dry IFSR soaked in 100 mL of basic liquid medium and inoculated with 20 ml of inocula which had its OD adjusted to 1.0.

2.4. Experimental design

A 2^3 full factorial central composite design for three independent variables, each one at five levels with six star points and three replicates at the central point, was employed to fit a second order polynomial model in which 17 experiments were required for this procedure. The test variables chosen in this study were cultivation time, aeration and temperature. The upper and lower limits of them were set to be in the range described in the literature. In the statistical model, Y denotes either units of xylanase or mannanase activity and the coded settings were defined as follows:

X_1= (cultivation time – 5)/2; X_2= (aeration – 500)/300; X_3= (temperature – 37)/7.

Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. This design is represented by a second order polynomial regression model, equation 1, that generates the contour plots:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_{13} + b_{23} X_2 X_3 + \varepsilon \quad (1)$$

The test factors were coded according to the following regression equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \quad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the *i*th independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. Statistica 5.0 software (Statsoft, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's t-test; the second order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination, R^2.

Table 1. Process variables used in the CCD, showing the treatment combinations and the mean experimental responses. Results are the mean of three experiments.

Treatment	Coded setting levels			Actual levels			Xylanase Activity (U mg^{-1} of protein)	Mannanase Activity (U mg^{-1} of protein)
	X_1	X_2	X_3	X_1	X_2	X_3		
1	-1	-1	-1	3	200	30	0.602	0.340
2	+1	-1	-1	7	200	30	0.748	0.410
3	-1	+1	-1	3	800	30	0.645	0.400
4	+1	+1	-1	7	800	30	0.668	0.560
5	-1	-1	+1	3	200	44	0.302	0.490
6	+1	-1	+1	7	200	44	0.292	0.230
7	-1	+1	+1	3	800	44	0.384	0.410
8	+1	+1	+1	7	800	44	0.266	0.450
9	0	0	0	5	500	37	0.483	0.180
10	0	0	0	5	500	37	0.407	0.130
11	0	0	0	5	500	37	0.429	0.120
12	-1.68	0	0	2	500	37	0.639	0.440
13	+1.68	0	0	8	500	37	0.422	0.260
14	0	-1.68	0	5	0	37	0.452	0.190
15	0	+1.68	0	5	1000	37	0.484	0.500
16	0	0	-1.68	5	500	25	1.037	0.350
17	0	0	+1.68	5	500	49	0.193	0.430

X_1= time (days); X_2=aeration (ml min^{-1}); X_3= temperature (°C)

2.5. Enzymes extraction

Enzymes recovery from cultivated medium was performed by the addition of 120 ml of distilled water with agitation at 250 rpm and 40 min. The enzymatic extract was then centrifuged at 10,000 *g* for 15 min. The supernatant was used as the source of both enzymes.

2.6. Enzymes assays

Xylanase activity was assayed hydrolyzing a suspension of 1 % (w/v) of birchwood xylan in 20 mM and pH 5.0 acetate buffer as substrate [17]. Mannanase activity was measured by using a suspension of locust bean gum 0.5% (w/v) in 50 mM citrate buffer pH 5.3 [18]. The released reducing sugars were quantified by the dinitrossalicylic acid method [19]. One unit of enzymatic activity was defined as the amount of enzyme liberating 1 μmol of xylose or mannose respectively per minute. Specific activities were expressed as U mg^{-1} of protein.

2.7. Protein determination

Soluble protein concentration was determined according to the Lowry assay [20], against a standard curve of bovine serum albumin, fraction V (Sigma, USA).

3. Results and discussion

Based on previous studies, cultivation time, aeration and temperature were identified as the major factors affecting xylanase and mannanase production of *Bacillus* sp. [21]. In a previous work we were able to demonstrate that initial culture pH had no important effect on enzymes production by *B. circulans* BL53. It was also noted a strong buffering characteristic of IFSR as substrate, with final cultivation pH always close to 7.0 (results not shown), which has been described as the best pH for xylanase and mannanase production by Kansoh et al. [22]. Therefore, Central Composite Design (CCD) was used to obtain the best conditions of time rum of cultivation, temperature and aeration for xylanase and mannanase production by *Bacillus circulans* BL53 on SSC. Experimental design matrix and results obtained from it for enzymes activities are shown in Table 1.

3.1 Xylanase production

Treatment 16 showed the highest xylanase activity (1.037 U mg^{-1} of protein). This productivity was obtained at a moderate aeration rates (500 ml min^{-1}, which corresponds to 25 vmm) and at low temperature (25 °C). This strikingly low temperature contrasts strongly with results obtained for *B. subtilis* growing in submerged cultures, where highest enzyme activity was achieved at 60 °C [23], and for *Streptomyces* sp. that showed maximum xylanase production at 50 °C, both temperatures predicted by central composite design [8]. The time for highest xylanase activity (5 days) corresponds to mid-stationary growth phase (results not shown), in this case in agreement with findings

by Bocchini et al. [21], who reported optimum cultivation time for xylanase activity of *B. circulans* D1 in submerged cultures of 48 h. The highest activity experimentally obtained according to the CCD was 2.5-fold higher than the activity obtained under conditions previously used in our laboratory (0.437 U mg^{-1} of protein at 5 days, 500 ml min^{-1} and 37 °C). An interesting result is that temperature was the only significant variable to protease production and at 25 °C protease activity was completely inhibited (data not shown), therefore contributing to avoid xylanase degradation in culture.

The results of the second order response surface model fitting in the form of analysis of variance (ANOVA) are given in Table 2. The analysis of variance (ANOVA) of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's *F*-test. The computed *F*-value (10.56) is much higher than the tabular *F*-value Tabular (3.68 at the 5% level), indicating that the differences in treatment are highly significant, with R^2=0.93, thus 93% of the total variation is explained by the model. This suggests a satisfactory representation of the process model [24]. Also, there was a good correlation between the experimental and predict values.

Table 2. Analysis of variance (ANOVA) for the model regression for optimization of xylanase production.

Source	SS	DF	MS	F-value	F-value in Statistic table
Model	0.647	9	0.072	10.56	3.68
Residual	0.048	7	0.007		
Lack of fit	0.045	5	0.009	5.75	19.30
Pure Error	0.003	2	0.002		
Total	0.694	16			

R^2 = 0.93; SS, Sum of Squares; DF, Degrees of Freedom; MS, Mean Square. Significance level = 95%.

The Student's *t*-test and *P*-values were used as a tool to check the significance of each coefficient (Table 3), which, in turn, are necessary to understand the patterns of the mutual interactions between the test variables [25]. The larger magnitude of the *t*-value and smaller the *P*-value, the more significant is the corresponding coefficient [26]. This implies that the quadratic main effects of all factors are insignificant, whereas the first order main effect of temperature is highly significant as is evident from its *P*-value (P<

0.002). By applying multiple regression analysis on the experimental data, the experimental results of the CCD design were fitted with a second-order polynomial equation. The model was simplified by the elimination of statistically insignificant terms. However, the t term (P_t<0.152), t^2 (P_{t2}<0.231), T^2 (P_{T2}<0.051), t.A ($P_{t.A}$<0.170) and t.T (Pt.T<0.119) were maintained in the model because of its magnitude. We proposed then that the quadratic model should be reduce to:

$$Y = 0{,}443 - 0{,}023t + 0{,}019t^2 - 0{,}207T + 0{,}049T^2 - 0{,}029t.A - 0{,}036t.T$$

where Y is the specific xylanase activity produced (U mg^{-1}), t is the cultivation time, T is the temperature and A is the aeration as coded settings. A validation of the model is given in Figure 1, which shows the cultivation of *B. circulans* BL53 for the xylanase production under optimal conditions of 500 ml min^{-1} and 25 ℃. The maximal xylanase activity obtained was 0.953 U mg^{-1} in five days. In this case, the coded settings of the tested variables were t = 0, A = 0, T = –1.68, respectively, with the model predicting xylanase activity of 0.928 U mg^{-1} in five days. The experimental value was found to be 2.7 % higher than the predicted value, confirming the closeness of the model to the experimental result.

Table 3. Coefficient estimates by the regression model for optimization of xylanase production.

Independent variables (parameter)	Coefficient (β)	Standard error (β)	t-value	Significant value (p-value)
Intercept	0.4434	0.0226	19.561	0.002
X_1	-0.0235	0.0106	-2.206	0.158
$X_1.X_1$	0.0199	0.0117	1.696	0.231
X_2	0.0050	0.0106	0.475	0.681
$X_2.X_2$	-0.0022	0.0117	-0.195	0.863
X_3*	-0.2075	0.0106	-19.484	0.002
$X_3.X_3$	0.0497	0.0117	4.242	0.051
$X_1.X_2$	-0.0292	0.0139	-2.103	0.170
$X_1.X_3$	-0.0365	0.0139	-2.627	0.119
$X_2.X_3$	0.0111	0.0139	0.801	0.507

* Statistically significant at 99% of confidence level. ** Statistically significant at 95% of confidence level. X_1= time (days); X_2=aeration (mL.min^{-1}); X_3= temperature (°C).

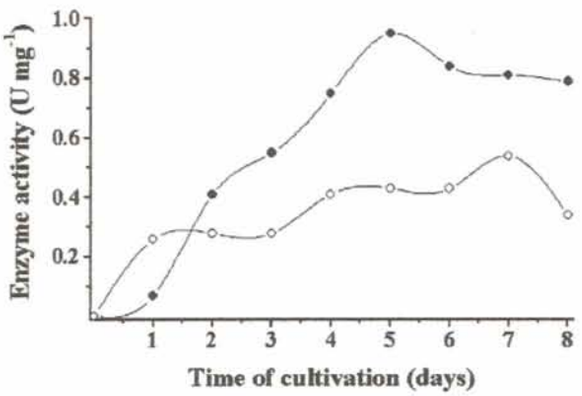

Figure 1. Xylanase and mannanase production profile under the optimal conditions suggested by the model. Xylanase (•), A = 500 ml min^{-1} and T = 25 °C; mannanase (○), A = 800 ml min^{-1} and T = 30 °C.

Figure 2 shows contour shapes. Each contour curve represents an infinite number if combinations of two test variables with the other maintained at their respective zero level. These plots demonstrated that the production of xylanase can be increased either by extending time of cultivation under moderate aeration or, at increased aeration rate, reducing the cultivation time (Figure 2a). This phenomena could be explained due to the fact that aeration tends to remove media moisture that is essential for cell growth as we demonstrated elsewhere [27]. Temperature affected more directly the amount of xylanase produced (Figure 2b and 2c). The non-elliptical nature of these contour plots depicts that there is no mutual interaction between temperature and each of the other two variables [26].

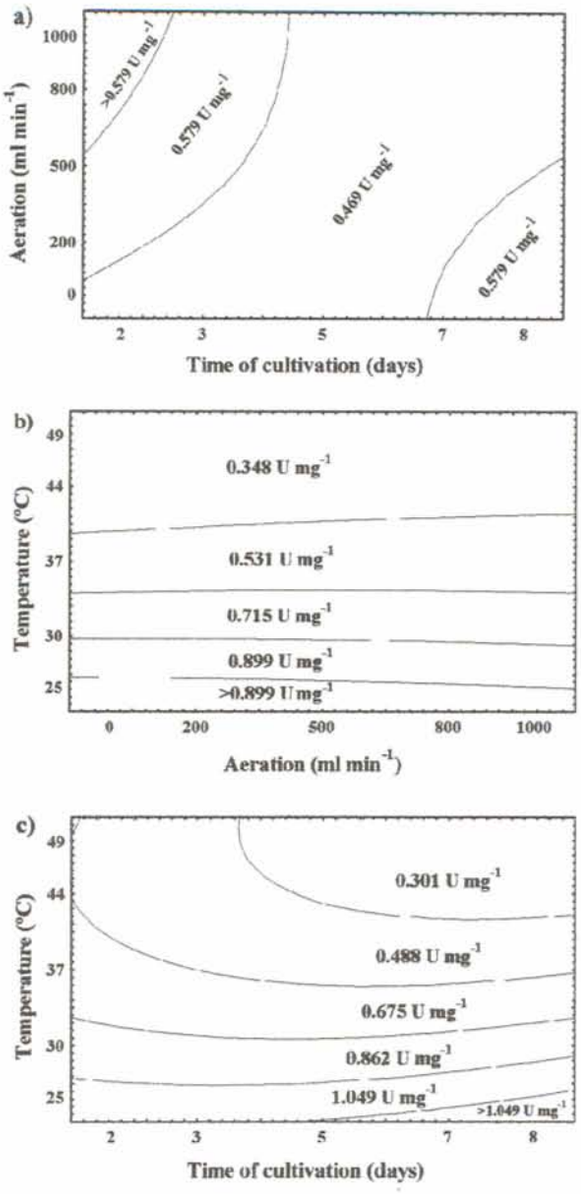

Figure 2. Contour plot for the effect of (a) Agitation x Time, (b) Temperature x Aeration and (c) Temperature x Time on xylanolytic activity production. Not plotted variable is fixed at zero level in all of the three graphs.

3.2 Mannanase production

Treatment 4 shows the highest level of mannanase activity production (0.560 U mg^{-1} of protein). These results suggest that the mannanase production by *B. circulans* BL53 was increased at higher cultivation time and aeration rate and lower temperature. The highest enzymatic activity experimentally obtained according to the CCD was 3.7-fold higher than that obtained under conditions previously employed in our laboratory (0.150 U mg^{-1} of protein at 5 days, 500 ml min^{-1} of air flow and 37 °C).

The ANOVA was employed for the determination of significant parameters and to estimate the mannanase production as a function of cultivation time, aeration and temperature. Data are shown in Table 4. The computed F-value (5.99) was 1.7-fold higher than the F-value in statistic tables (3.58), indicating that the model was significant at high confidence level (95%), with R^2=0.88.

Table 4. Analysis of variance (ANOVA) for the model regression for optimization of mannanase production.

Source	SS	DF	MS	F-value	F-value in Statistic table
Model	0.264	9	0.0293	5.99	3.68
Residual	0.034	7	0.0048		
Lack of fit	0.029	4	0.0072	4.13	9.12
Pure Error	0.005	3	0.0017		
Total	0.298	16			

R^2 = 0.88; SS, Sum of Squares; DF, Degrees of Freedom; MS, Mean Square. Significance level = 95%.

Table 5 shows the significance of coefficients determined by Student's t-test and P-values. In our work, second-order cultivation time, second-order aeration and second-order temperature were highly significant ($P_{t.t}$<0.007, $P_{A.A}$<0.008 and $P_{T.T}$<0.007). The high significance of variables on second-order model indicates that they can act as limiting factors and even small variations in their values will alter mannanase production to a considerable extent [28]. The model clearly reveals significant interactions between t and A (P<0.046) and t and T (P<0.032). Therefore, treating them separately may not reflect their real influence on the mannanase production (e.g.,

optimum cultivation time production changes along with aeration). This interaction is essential and it would be of difficult solution using the *one-variable-at-a-time* approach [15]. Whenever possible, the model was simplified by the elimination of statistically insignificant terms. We proposed then that the quadratic model should be reduce to:

$$Y = 0{,}141 - 0{,}02t + 0{,}082t^2 + 0{,}064A + 0{,}080A^2 + 0{,}092T^2\ 0 + 0{,}049t.A - 0{,}056t.T$$

where Y is the specific mannanase activity produced (U mg^{-1}), t is the cultivation time, T is the temperature and A is the aeration as coded settings. To confirm the applicability of the model mannanase production was determined at different times, 800 ml min^{-1} of aeration and 30 °C that are the optimum values suggested by it. The maximal mannanase activity obtained was 0.540 U mg^{-1} in seven days (Figure 1). In this case, the coded settings of the tested variables are t = 1, A = 1, T = -1, respectively, with the model predicting mannanase activity of 0.544 U mg^{-1} in five days. The experimental value was found to be 0.7 % lower than the predicted value, confirming the closeness of the model to the experimental results.

Table 5. Coefficient estimates by the regression model for optimization of mannanase production.

Independent variables (parameter)	Coefficient (β)	Standard error (β)	t-value	Significant value (p-value)
Intercept	0.1407	0.0241	5.826	0.010
X_1	-0.0214	0.0113	-1.889	0.155

optimum cultivation time production changes along with aeration). This interaction is essential and it would be of difficult solution using the *one-variable-at-a-time* approach [15]. Whenever possible, the model was simplified by the elimination of statistically insignificant terms. We proposed then that the quadratic model should be reduce to:

$$Y= 0{,}141 - 0{,}02t + 0{,}082t^2 + 0{,}064A + 0{,}080A^2 + 0{,}092T^2\ 0+ 0{,}049t.A - 0{,}056t.T$$

where Y is the specific mannanase activity produced (U mg^{-1}), t is the cultivation time, T is the temperature and A is the aeration as coded settings. To confirm the applicability of the model mannanase production was determined at different times, 800 ml min^{-1} of aeration and 30 ºC that are the optimum values suggested by it. The maximal mannanase activity obtained was 0.540 U mg^{-1} in seven days (Figure 1). In this case, the coded settings of the tested variables are t = 1, A = 1, T = -1, respectively, with the model predicting mannanase activity of 0.544 U mg^{-1} in five days. The experimental value was found to be 0.7 % lower than the predicted value, confirming the closeness of the model to the experimental results.

xylan fraction and higher xylanolytic activity in five days, whereas mannanase peak was only observed after seven days of cultivation, during stationary growth phase.

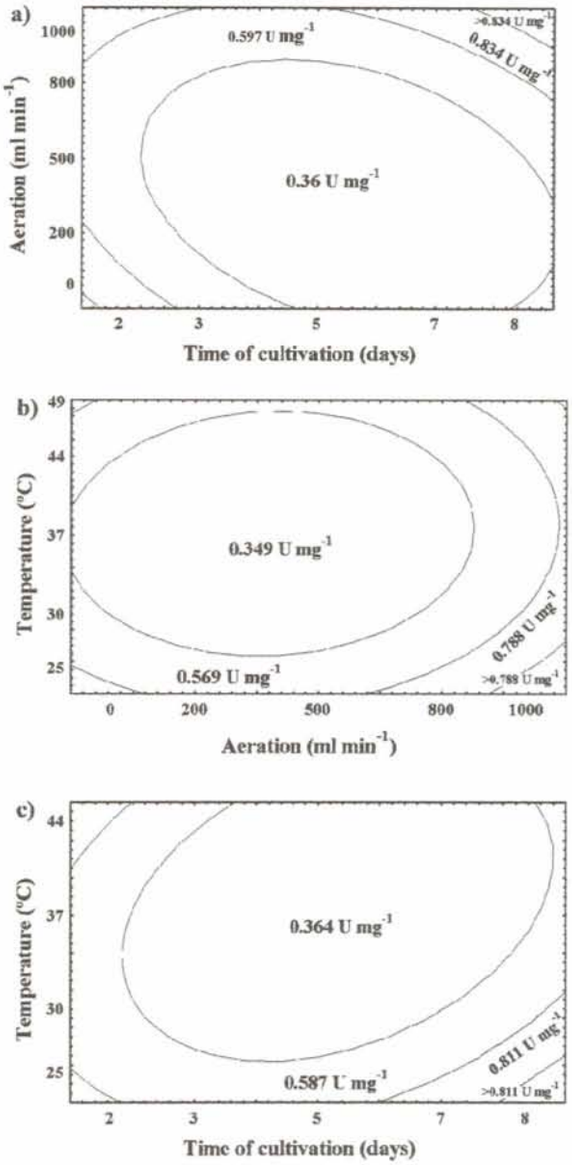

Figure 3. Contour plot for the effect of (a) Agitation x Time, (b) Temperature x Aeration and (c) Temperature x Time on mannanase activity production. Not plotted variable is fixed at zero level in all of the three graphs.

Conclusions

In this work it was possible to determine the optimal growth conditions for the production of xylanase and mannanase by *B. circulans* BL53 in SSC using the method of experimental factorial design and response surface methodology. The maximal amount of enzyme produced was 0.953 U mg^{-1} (2-fold increase) and 0.540 U mg^{-1} (3.6-fold) for xylanase and mannanase, respectively, when the optimized conditions were employed. The enzyme activity predicted by the model at optimal conditions agreed fittingly with experimental data, thus confirming the model validity. The results obtained in this study are satisfactory, as the gain in enzymes production achieved will greatly facilitate further work on the purification of the xylanase and mannanase produced by *B. circulans* BL53.

Acknowledgments

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5.5. RESULTADOS V. "Purification and properties of a xylanase produced by *Bacillus circulans* BL53 on solid-state cultivation" - Enzyme and Microbial Technology, submetido em maio de 2005.

Purification and properties of a xylanase produced by *Bacillus circulans* BL53 on solid-state cultivation

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Abstract

Xylanase from amazon isolate *B. circulans* BL53 grown on solid-state cultivation was purified to apparent homogeneity by ammonium sulfate fractioning, cation-exchange and gel filtration chromatography. A purification factor of 428-fold was achieved, with the purified enzyme presenting a specific activity of about 37 U mg^{-1} protein. The xylanase molecular weight was calculated as 38 kDa by SDS-PAGE. Determination of pH and temperature for the maximum activity was obtained using a 2^2 factorial design over an extensive range of pH (5.0 – 8.0) and temperatures (40 – 80 °C). The enzyme follows Michaelis-Menten kinetics with K_M and V_{max} values of 9.9 mg xylan mL^{-1} and 25.25 μmoles min^{-1}, respectively. The purified enzyme hydrolyzes soybean hull, soybean fiber, rice straw, grape skin and sugar cane bagasse. Its activity was stimulated by Co^{+2}, Mn^{+2} and protein disulphide reducing reagents, but strongly inhibited by Hg^{+2} ions and SDS.

Keywords: Xylanase; Hemicellulose; Solid-state cultivation; *Bacillus circulans*.

Running headline: Purification of a *Bacillus circulans* xylanase.

Introduction

Xylans are a complex group of hemicelluloses composed of a xylose-based backbone with a variable degree of side substitutions. Xylans are environmentally important in the recycling cicles of vegetal biomass since they constitute up to 30 % of the plant cell

wall components in hardwoods and annual crops. For the majority of bioconversion processes, the xylans must be first be converted to xylose units or xylo-oligosaccharides. The core chain hydrolysis is accomplished by the action of xylanases (1,4-β-xylan xylanohydrolases, EC 3.2.1.8), which release xylooligosaccharides of different sizes [1,2]. Xylanases have recently found original applications in facilitating the bleaching of kraft pulps, as additives in poultry feeding, in wheat flour for dough handling and quality improvement of baked products, in combination with pectinases and cellulases for fruit juices clarification, and in the recovery of fermentable sugars from hemicelluloses [3–5].

Xylanases have been produced in either solid-state (SSC) or submerged cultivations with the prevalence of the last one [5]. However, SSC has gained renewed interest in recent years for the production of many enzymes due to lower operation costs and energy requirements, and simpler plant and equipment projects when compared to submerged microbial cultures. [6].

The aim of this study was to purify and biochemically characterize an extracellular xylanase produced by the amazon isolate *Bacillus circulans* BL53 grown on solid-state cultivation using an industrial fibrous soybean residue as the sole substrate for carbon and nitrogen sources.

Materials and methods

2.1. Microorganism

Bacillus circulans BL53 was isolated from the Amazon environment, in Brazil, as part of a program for the study of biodiversity potentials for new products. This isolate has been extensively studied elsewhere and has been selected due to its xylan-degrading properties [7]. Isolates were maintained at 4 °C on selective agar plate containing 0.1% (w/v) of gum tragacanth in basic liquid medium.

2.2. Substrate, medium and inoculum

Industrial fibrous soy residue (IFSR) was chemically defined elsewhere [7] and used as the sole solid substrate for cultivations. Basic liquid medium, used as inoculum, had the following composition (g l^{-1}): 0.2 $MgSO_4$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.02 $CaCl_2$, and 0.05 $FeCl_2$. Inocula for all experiments in SSC bioreactor were prepared in 250 mL Erlenmeyer flasks containing 1.2 g of IFSR and 40 mL of basic liquid medium.

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Flasks were inoculated with a single colony from agar plates and incubated for 18 h at 37 °C and 125 rpm.

2.3. Bioreactor and cultivation conditions

Bioreactor experiments were carried out in a 500 mL cylindrical bioreactor (60 mm diameter; 170 mm height) designed and constructed in our laboratory. Bioreactors were loaded with 18.8 g of dry IFSR soaked in 120 mL of basic liquid medium. Ideal process conditions were established in our previous work [6]. and set as 25 °C. 120 hours and 500 mL min^{-1} of sterile aeration.

2.4. Enzyme extraction

Enzyme extraction from cultivated medium was performed by addition of 100 mL of distilled water at 7 °C, with agitation at 150 rpm for 40 min, which were the best conditions established in a previous work [8]. The enzymatic extract was then centrifuged at 10,000 *g* for 20 min. The supernatant was used for the subsequent purification steps.

2.5. Xylanase activity assay

Xylanase activity was assayed using Birchwood xylan in 100 mM acetate buffer pH 5.0 as the substrate. Reducing sugars freed by enzymatic hydrolysis were quantified by the dinitrosalicylic acid method [9]. One unit of enzymatic activity was defined as the amount of enzyme producing 1 μmol of xylose per minute. Specific activity was expressed as U mg^{-1} protein.

2.6. Protein determination

Protein concentration was determined according to the Lowry assay [10] against a standard curve of bovine serum albumin, fraction V (Sigma, USA).

2.7. Purification procedures

The purification steps were all performed at room temperature since previous experiments showed no difference in enzyme stability at 4 or 22 °C. Firstly, proteins were precipitated between 20 and 80% of ammonium sulphate saturation and centrifuged at 15,000 *g* for 30 min. The pellet was resuspended in 50 mM sodium acetate buffer pH 5.5 and dialyzed for 18 h against this buffer. The solution was applied

to a CM–Sepharose FF column (1.0 x 20.0 cm) previously equilibrated with the above mentioned buffer at a flow rate of 0.8 mL min^{-1}. The proteins were eluted with a NaCl gradient (0 – 0.5 M) in the same buffer. The active xylanases fractions were collected and concentrated by liofilization. The concentrated enzyme solution was further purified by gel filtration on a Sephacryl S–200 HR column (1.5 x 55 cm) equilibrated with 50 mM acetate buffer pH 5.5. The flow rate was 0.4 mL min^{-1}. The highly active xylanase fractions were pooled and used as the purified enzyme for subsequent studies.

2.8. Optimal temperature and pH for enzyme activity, and thermal stability

A central composite design (CCD), with $k = 2$, was used in order to generate 11 treatment combinations for enzyme activity, with temperature and pH as independent variables. In the statistical model, Y denotes units of xylanase activity and the coded settings were defined as follows: $X1 = (\text{temperature } 60)/20$; $X2 = (\text{pH } 7.0)/3.0$. Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. This design is represented by a second-order polynomial regression model, Eq. (1), to generate the contour plots:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1^2 + b_{22} X_2^2 + b_{12} X_1 X_2 + \varepsilon \qquad (1)$$

The tested factors were coded according to the following regression equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \qquad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the *i*th independent variable, X_0 is the actual value at the central point, and ΔX_i is the step change value. Statistica 5.0 software (Statsoft, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's *t*-test; the second order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination, R^2. The thermal stability of purified xylanase was determined by incubation at 50, 60, 70 and 80 °C. Aliquots were withdrawn after 0, 10, 20, 30 and 60 min of incubation, and the residual enzyme activity measured.

Table 1. Process variables used in the CCD, showing the treatment combinations and the mean experimental responses.

Treatment	Coded setting levels		Actual levels		Xylanase activity (U mg^{-1} protein)
	X_1	X_2	X_1	X_2	
1	-1	-1	40	5	25.2
2	1	-1	80	5	22.4
3	-1	1	40	9	20.9
4	1	1	80	9	13.6
5	0	0	60	7	68.9
6	0	0	60	7	70.9
7	0	0	60	7	72.1
8	-1.41	0	32	7	17.6
9	0	-1.41	60	3.6	57.2
10	1.41	0	88	7	12.8
11	0	1.41	60	10.4	8.3

X_1= temperature (°C); X_2= pH

2.9. Effect of inhibitors and activators on purified xylanase activity

The effect of various compounds on xylanase activity was determined by incubating the purified enzyme in the presence of 2 mM solutions of each compound for 15 min before the reaction with the substrate. Residual activity was expressed as the percentage of the activity observed by the standard assay described above.

2.10. Kinetic parameters

The effect of substrate concentration, ranging from 2.0 to 20 mg mL^{-1}, on xylanase activity was evaluated under optimal assay conditions (60 °C and pH 7.0). Xylan presents low solubility, so higher concentrations could not be used. The kinetic parameters of Michaelis–Menten constant, K_M, and maximal reaction velocity, V_{max}, were estimated by linear regression according to the Lineweaver and Burk double-reciprocal plot.

2.11. Hydrolysis of agro-industrial residues

In order to assess the enzyme ability to hydrolyze several different agro-industrial residues, reaction mixtures containing 1 mL (40 μg) of purified enzyme solution and 50 mg dry weight of selected agro-industrial residues (soybean hull, soybean fiber, rice straw, grape skins, sugarcane bagasse and kraft pulp cellulose) suspended in 100 mM acetate buffer pH 5.0 were incubated for 1h at 60 °C and 100 rpm. The reaction was stopped by placing the mixture in boiling water for 5 min, and then centrifuged at 5,000 *g* for 10 min. Reducing sugars liberated by hydrolysis of these substrates were quantified by the dinitrosalicylic acid method.

2.12. Cellulose Binding Domain (CBD) assay

Binding assays were carried out in 1 mL acetate buffer (50 mM, pH 5.0) containing 2 mg of insoluble microcrystaline cellulose and 40 μg of enzyme. The mixture was stirring incubated at 4 °C for 30 min. CMC was sedimented by centrifugation at 12,000 rpm for 5 min. The amount of enzyme bound to the insoluble polysaccharides was determined by measuring the enzyme concentration in the supernatant. These assays were carried out in triplicates.

2.13. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10 % acrylamide gel [11], and protein bands were revealed by silver staining. Broad range molecular weight standards were used as size markers.

Results and discussion

3.1. Purification

The supernatant obtained by extraction of a five day solid-state cultivation was used as crude extract, and the enzyme purification was performed by ammonium sulfate precipitation (20–80 % saturation), cation-exchange chromatography and gel filtration (Table 2). Although the salting-out procedure yielded only 16 % of enzyme recovery, it was considered a critical step in our work because *B. circulans* BL53 produces an exo-polysaccharide that modifies the medium viscosity and makes chromatographic processing more difficult. The elution profile of xylanase activity in CM-sepharose at pH 5.5 is shown in Figure 1. Xylanase activity was detected in fractions 32, 33 and 34 and the specific activity of the pooled fractions reached 13.15 U mg^{-1} with no cellulase and protease activities observed. Figure 2 shows the results of gel filtration, which

allowed xylanase purification to protein homogeneity. Although rendering low recovery yields of 5.7%, this was an efficient strategy to purify the enzyme, resulting in high specific activity, which increased from 0.087 U mg^{-1} in the extract to approximately 37.22 U mg^{-1}, equivalent to a purification factor of 428-fold.

Table 2. A summary of xylanase purification from *B. circulans* BL53.

Steps	Total Activity (U)	Total protein (mg)	Specific Activity (U mg^{-1} protein)	Purification (fold)	Recovery (%)
Crude extract	286.17	3296.51	0.087	1.00	100.0
Ammonium sulphate	46.20	110.91	0.417	4.79	16.1
CM-sepharose	28.45	2.16	13.152	151.18	9.9
Gel filtration	16.38	0.44	37.222	427.83	5.7

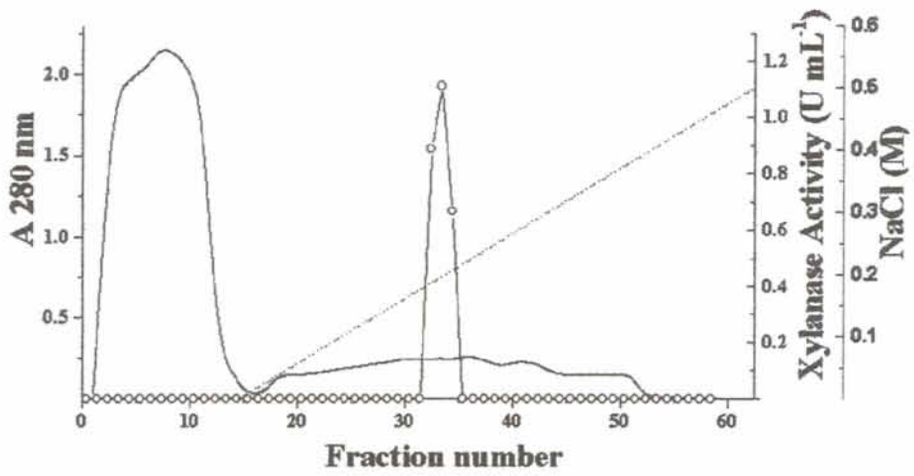

Figure 1. Elution profile of xylanase on CM-Sepharose. (–) xylanase activity. (–o–) Absorbance at 280 nm, (---) NaCl. Fraction volume of 3 mL.

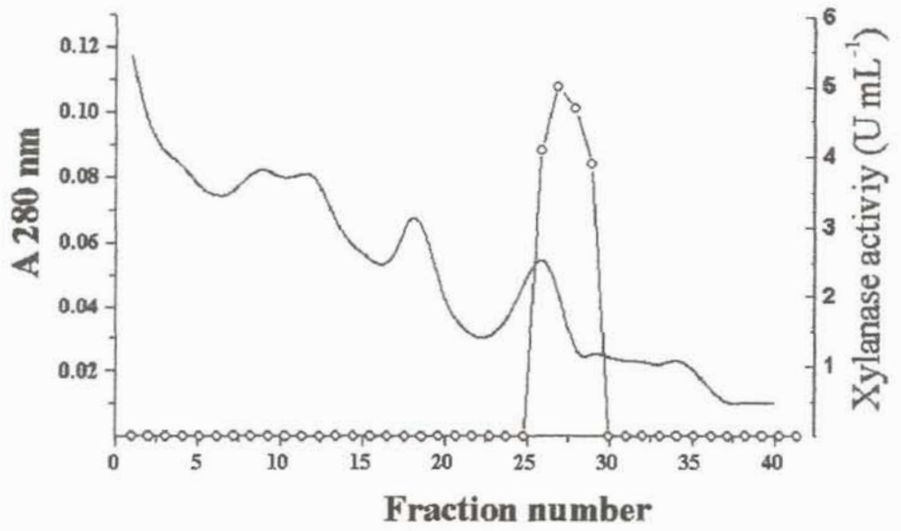

Figure 2. Elution profile of xylanase activity (–) and absorbance at 280 nm (–o–), in a Sephacryl HR S-200 column. Fraction volume of 2 mL.

3.2. Biochemical properties of B. circulans xylanase

SDS-PAGE of the purified protein revealed a single band, suggesting that xylanase from *B. circulans* BL53 is a monomeric polypeptide with an estimated molecular mass of 38 kDa (Figure 3). Many xylanases produced by the *Bacillus* genus are in the range of 22 to 45 kDa [12–15].

Kinetic parameters that reflect the effect of substrate concentration on the reaction velocity are depicted in the double reciprocal plot in Figure 4. The values for K_M and V_{max} are of 9.9 mg mL^{-1} and 25.25 μmoles min^{-1}, respectively. These values are consistent with the reported range of K_M values for microbial xylanases (0.27–14 mg mL^{-1}) [16].

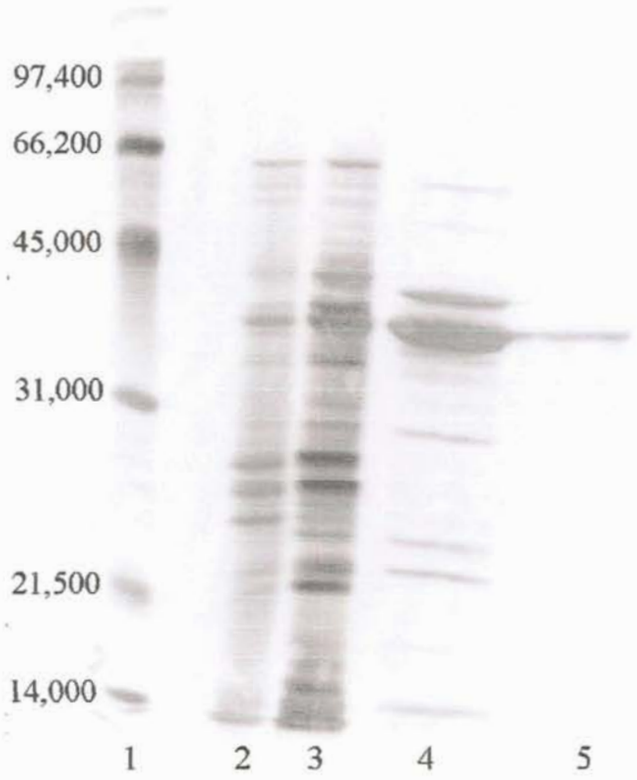

Figure 3. SDS-PAGE of xylanase purification steps. Lane 1: molecular weight standards; lane 2: crude enzyme extract; lane 3: after ammonium sulfate precipitation; lane 4: after cation-exchange chromatography on CM-Sepharose; lane 5: after gel filtration on Sephacryl S-200 HR. Protein bands were stained with silver.

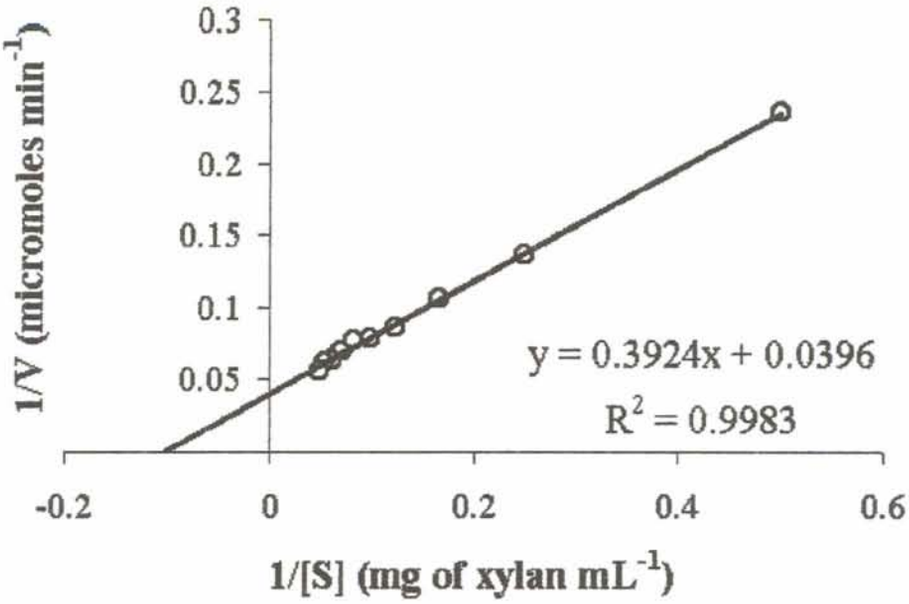

Figure 4. Lineweaver-Burk plot for the purified *B. circulans* BL53 xylanase. Enzyme activity was measured at 60 °C and pH 7.0.

3.3. Optimal temperature and pH, and thermal stability

Treatments 5, 6 and 7, which correspond to the central points in the experimental design, showed the highest xylanase activities of 68.9, 70.9 and 72.1 U mg^{-1} protein, respectively (Table 1). These results suggest that the xylanase from *B. circulans* BL53 has an optimal enzyme activity at broad temperature (40 – 80 °C) and pH (5 – 8) ranges (Figure 5). The optimal temperature and pH values of purified xylanase were within the range reported for many xylanases from *Bacillus* [15,17]. However, the wide temperature and pH range of maximal activity might be an advantage of this enzyme for different applications. The highest activity experimentally obtained according to the CCD was 1.4-fold higher than the activity obtained under conditions usually described in the literature (54.4 U mg^{-1} at 55 °C and pH 5.0) [18]. The analysis of variance (ANOVA) was used for the determination of significant parameters and to estimate the specific xylanase activity as a function of temperature and pH. The computed F-value (10.63) was 2.0-fold higher than the tabled F-value, indicating that the model was significant at high confidence level (95 %), with R^2=0.92, thus 92 % of the total variation is explained by the model. This suggests a satisfactory representation of the process model [19]. Also, there is a good correlation between the experimental and predicted values, and a significance of coefficients determined by Student´s t-test and P-

values (not shown). In this work, T, pH and second-order T and pH were significant at confidence level of 95% (P_T<0.001, $P_{pH..}$<0.0004, $P_{T.T}$<0.002 and $P_{pH..pH}$<0.05), but the interaction between them have no significant effect on xylanase activity. We proposed then that the quadratic model for pH and temperature interactions on enzyme activity should be reduce to:

$$Y = 70.64 + 5.72\,T - 17.53T^2 + 2.44pH - 31.02pH^2 \qquad (3)$$

where Y is the specific xylanase activity (U mg^{-1}), T is the temperature, and pH is the pH as coded settings.

Thermal stability of xylanase was determined by heating the purified enzyme at different temperatures up to 60 minutes. Data presented in Figure 6 show that the enzyme was stable at 60 °C following a rapid loss of activity above 70 °C. No activity loss was observed after one month at 4°C or −18°C.

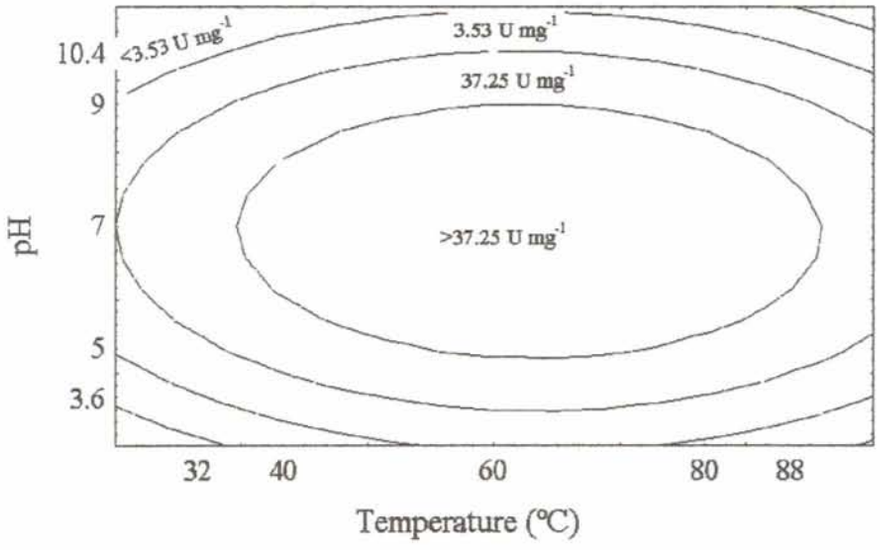

Figure 5. Contour plot for the effects of temperature and pH on purified xylanase activity of *B. circulans* BL53.

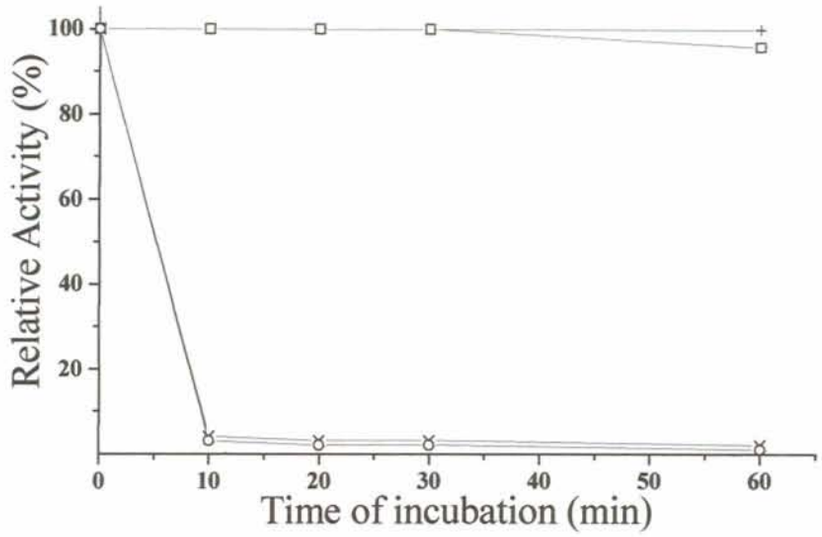

Figure 6. Thermal stability of purified *B. circulans* BL53 xylanase. (+) 50 °C, (□) 60 °C, (×) 70 °C, (o) 80 °C. Results are the mean of 3 experiments.

3.4. Effect of potential inhibitors and activators on purified xylanase activity

The effect of potential inhibitors or activators on purified xylanase is shown in Table 3. Results show that Hg^{2}+ and SDS were strong inhibitors of enzyme activity, while Pb^{+2}, Na^{+2}, Cu^{+2}, Fe^{+2}, Zn^{+2}, Mg^{+2} and Ca^{+2} had a slight inhibition effect on activity. The inhibition of the enzyme was generally obtained with ions that reacted with sulphydryl groups such as Hg^{+2} ions, suggesting that there was an important cysteine residue in or close to the active site of the enzyme [2]. Inactivation of xylanases by Hg^{+2} had also been reported by other authors [20]. PHMB produced low inhibition, suggesting the presence of a relative hydrophobic pocket at the catalytic site [14]. Stimulatory effects were, however, obtained with Co^{+2} and Mn^{+2}. The high increase on xylanase activities by these cations were also reported elsewhere [18,21]. The purified xylanase activity was also stimulated in the presence of the protein disulphide reducing reagents, β-mercaptoethanol and DTT, confirming the presence of a reduced thiol group of cysteine in the enzyme [22]. The addition of EDTA did not affect activity, suggesting that this enzyme has no metallic cation on its active site.

Table 3. Effect of the addition of chemicals on xylanase activity. The final concentration in the reaction mixture was 2 mM. Relative activity is expressed as a percentage of control.

Reagent	Relative Activity (% of control)
Control	100.00
Na^{+2}	92.10
Co^{+2}	151.67
Hg^{+2}	0.00
NH_4^{+1}	109.21
Mn^{+2}	161.88
Cu^{+2}	91.44
Fe^{+2}	81.90
Pb^{+2}	78.93
$Zn+^{2}$	91.11
Mg^{+2}	82.55
Ca^{+2}	95.39
PHMB	77.29
DMSO	106.58
SDS	37.46
β-mercaptoethanol	152.66
EDTA	114.48
DTT	111.60

3.5. Hydrolytic abilities

Hydrolysis experiments using several agro-industrial residues were carried out at optimal conditions (60 °C and pH 7.0) using the purified enzyme. In this experiment, purified xylanase released 78.0 mg of reducing sugars per g of xylan (which works as a control), 29.5 mg g^{-1} of soybean hull, 21.5 mg g^{-1} of soybean fiber, 14.0 mg g^{-1} of rice straw, 13 mg g^{-1} of grape skins, 4.8 mg g^{-1} of sugar cane bagasse. However, no sugar was released from Kraft pulp. These conversion abilities are very important since inexpensive agro-industrial by-products, rich in cellulose and hemicelluloses, are the

main potential substrates for the production of useful biomolecules, including chemicals and organic solvents. The increasing interest in biotechnological processes employing lignocellulosic residues is quite justifiable because these materials are cheap, renewable and a widespread source of sugars [24, 23].

3.6. Binding experiments

Xylanase from *B. circulans* BL53 presented no CBDs, which are noncatalytic domains that to increase the affinity of cellulases and hemicellulases by recalcitrant insoluble substrates, such as the plant cell wall, and are present in some of the bacterial and fungal xylanases previously described [25].

Conclusions

In this work we were able to purify to homogeneity a xylanase produced by *B. circulans* BL53 isolated from the Amazon region, which was successfully grown on solid-state cultivation, using a cheap and abundant industrial fibrous residue as substrate. This enzyme exhibits some properties that suggest its potential for future application. Reducing sugars were released from all the five lignocellulosic materials, soybean hull, soybean fiber, rice straw, grapes skin and sugar cane bagasse, with application in the recovery of fermentable sugars from hemicelluloses. The maximal enzyme activity was obtained using a 2^2 factorial design over a large range of temperature (40 - 80°C) and pH (5.0 – 8.0). At optimal conditions, the activity predicted by the model agreed very well with experimental data, confirming the validity of it, with a 1.4-fold increase on xylanase activity achieved by optimization of the parameters.

Acknowledgments

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5.5.1. Figura não incluída no manuscrito original

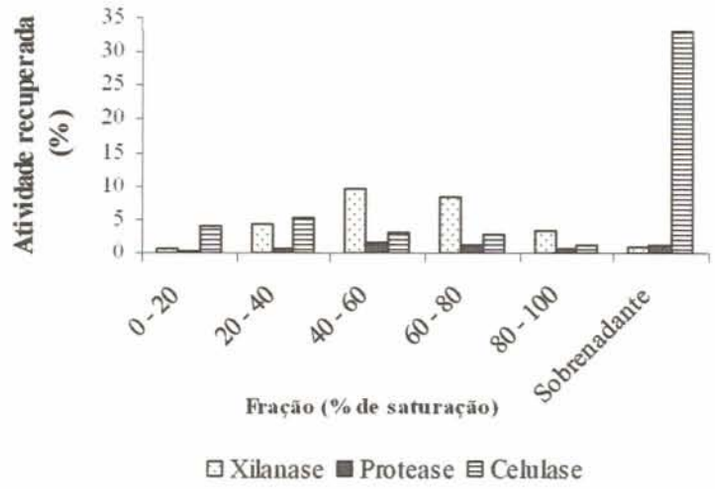

Figura 1. Precipitação fracionada com sulfato de amônio do extrato bruto produzido pelo isolado *B. circulans* BL53 em CSS.

5.6. RESULTADOS NÃO APRESENTADOS NOS MANUSCRITOS

5.6.1. Comparativo da produção de xilanase e atividade metabólica em Biorreatores Cilíndricos Verticais Estáticos e Biorreatores Cilíndricos Horizontais Agitados

É sabido que um leito sólido é extremamente mais difícil de ser homogeneizado eficientemente do que um meio líquido e, como conseqüência disso, o suprimento de oxigênio e a remoção do calor metabólico são operações extremamente restritivas em Cultivo Semi-sólido (MARSH *et al*, 1998). Os estudos referentes à comparação entre sistemas de CSS em leito fixo e em leito agitado são escassos na literatura, principalmente quando bactérias são os microrganismos estudados. Neste projeto, foram realizados alguns experimentos prospectivos, comparando-se a produção de xilanases em biorretores de leito fixo (aqui denominados de Biorreatores Cilíndricos Verticais Estáticos – BCVE) e biorreatores de leito agitado (aqui denominados de Biorreatores Cilíndricos Horizontais Agitados – BCHA). As metodologias de cultivo, para ambos os sistemas, foram detalhadas nos Materiais e Métodos.

Os resultados obtidos sugerem que a atividade metabólica do *B. circulans* BL53 foi maior durante os cultivos nos BCHA. Isto pode ser envidenciado pela maior atividade enzimática (Figura 1) e maiores concentrações de proteína e açúcares redutores no meio (Figuras 2 e 3), os quais são indicadores de atividade enzimática (glicosídicas e proteolíticas) e conseqüentemente de crescimento celular (MITCHELL e LONSANE, 1992).

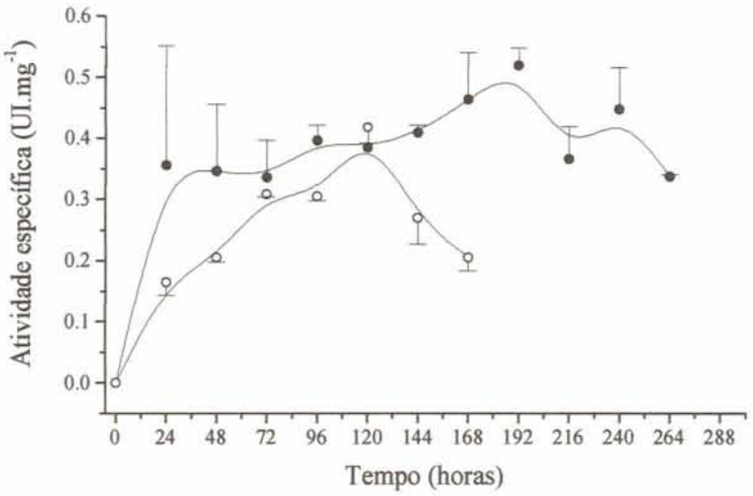

Figura 1. Atividade Específica (xilanase) ao longo de cultivos semi-sólidos em (•) BCHA e em (°) BCVE (os resultados expressam a média de 3 experimentos, com os respectivos desvios padrões).

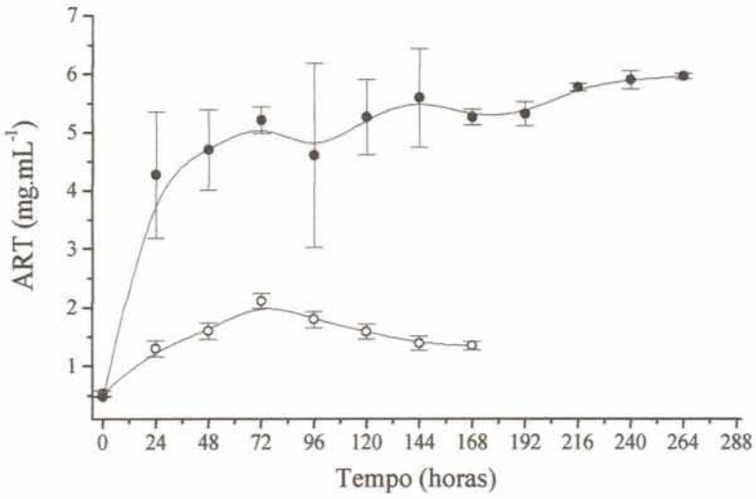

Figura 2. Açúcares Redutores Totais (ART) ao longo de cultivos semi-sólidos em (•) BCHE e em (°) BCVA (os resultados expressam a média de 3 experimentos, com os respectivos desvios padrões).

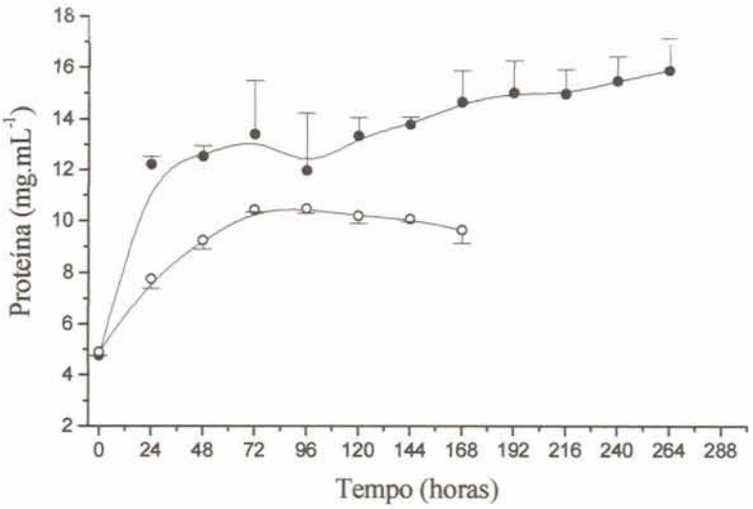

Figura 3. Proteína total ao longo de cultivos semi-sólidos em (•) BCHA e em (°) BCVE (os resultados expressam a média de 3 experimentos, com os respectivos desvios padrões).

Uma causa possível para a menor atividade metabólica nos BCVE pode ser o acúmulo de gás carbônico em algumas regiões destes biorreatores (STUART *et al.*, 1999). Ademais, a homogeneização constante do substrato nos BCHA faz com que este seja colocado constantemente em contato com a parede do biorreator, o que melhora a dissipação do calor metabólico e permite que os microrganismos sejam expostos constantemente a substratos menos exauridos (MITCHELL e LONSANE, 1992). Em CSS com biorreatores de leito fixo o superaquecimento é o principal problema. Ao passar através da coluna e remover calor do substrato, o ar tende a ser aquecido conforme atravessa o leito. Com isso se forma um gradiente de temperatura, com a região mais quente encontrando-se próximo da saída do biorreator. Portanto, a aeração requerida nesses biorreatores deve não apenas suportar o microrganismo com oxigênio, mas também impedir o superaquecimento das regiões da parte mais alta da coluna (MARSH *et al*, 1998). Por este motivo, as aerações neste tipo de biorreator, tendem a ser maiores do que nos biorreatores de leito agitado (MITCHELL e LONSANE, 1992).

O efeito da de rotação na *performance* dos cultivos semi-sólidos tem recebido especial atenção, em especial a extensão do efeito da agitação em biorreatores do tipo tambor é controversa. Em alguns estudos (KARGI e CURME, 1985) a rotação reduz a

produtividade ou a esporulação, presumivelmente devido ao efeito deletério da agitação, principalmente com fungos filamentosos (STUART *et al.*, 1999). No entanto, outros estudos mostram que a produtividade pode ser aumentada quando se utiliza agitação no biorreator. AIDOO *et al.* (1984) sugerem que o fato de a remoção do calor ser mais eficiente nos cultivos agitados é a chave para o sucesso dos cultivos em biorreatores do tipo tambor. STUART *et al.* (1999) e AIDOO *et al* (1984) afirmam que a dissipação do calor metabólico acaba sendo o fator mais limitante em CSS, principalmente com biorreatores estáticos e relataram que pode haver diferenças de até 10°C entre a temperatura desejada e o substrato, neste tipo de biorreator. Nos nossos experimentos a temperatura do substrato nos BCVE foi, em média, 1°C maior que a temperatura da estufa e nos BCHA a temperatura do substrato foi idêntica a do banho-maria que controlava a temperatura (dados não mostrados), demonstrando a eficaz transferência de calor no nosso sistema.

A manutenção das condições de umidade do substrato durante o cultivo semi-sólido é outro aspecto fundamental para o desenvolvimento microbiano (PANDEY *et al.*, 2000), pois o teor de umidade afeta diretamente a transferência de calor no meio e quando acontecem perdas consideráveis de umidade a transferência do calor é prejudicada (MARSH *et al*, 1998). Os resultados apresentados na figura 4 sugerem que não houve perdas consideráveis de umidade em decorrência da evaporação da água do meio em nenhum dos dois sistemas. Além disso, os resultados sugerem também que a adição de ar úmido permitiu que a água formada pelo metabolismo microbiano fosse retirada praticamente na mesma razão em que o ar úmido foi adicionado ao biorreator, ao contrário do que foi verificado por STUART *et al.* (1999), que constaram um aumento de umidade durante os períodos de maior metabolismo microbiano em um biorreator do tipo tambor horizontal agitado.

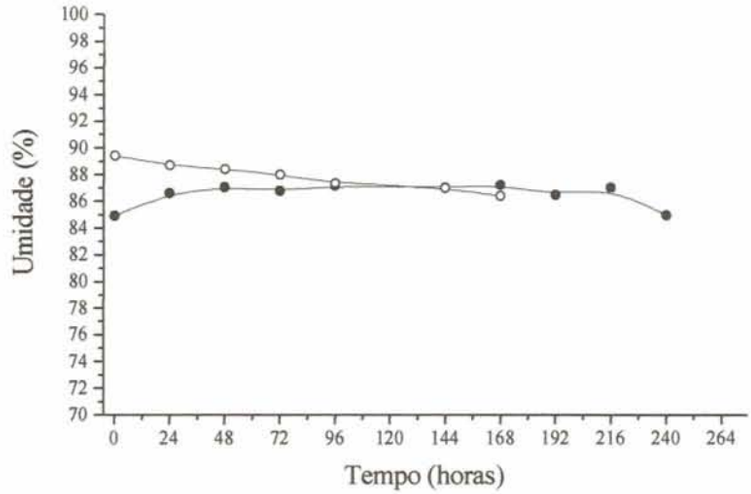

Figura 4. Perfil de umidade ao longo de cultivos semi-sólidos em (•) BCHA e em (°) BCVE (os resultados expressam a média de 2 experimentos).

Por fim, fica evidente o quanto o desenho e a operação do biorreator podem afetar as características ambientais do cultivo (temperatura, oxigênio, disponibilidade de nutrientes), tendo conseqüências diretas no desenvolvimento microbiano e afetando a produtividade da enzima. Portanto, estudos posteriores sobre os fenômenos de transporte de massa e calor envolvidos nesses biorreatores são fundamentais, além de avaliar-se as possíveis alterações na a_w do substrato durante o cultivo.

6. CONCLUSÕES GERAIS

Neste trabalho foram aprofundados os conhecimentos sobre uma série de aspectos referentes a xilanases bacterianas, no que se refere, principalmente, à produção, purificação e aplicações dessa enzima; e também sobre as diversas operações envolvidas nos processos de cultivo semi-sólido de bactérias e ao aproveitamento de resíduos agroindustriais para aplicações biotecnológicas.

Em um primeiro momento estudou-se a produção, em Biorreatores Cilíndricos Verticais Estáticos, e aplicação de uma xilanase produzida pelo *Bacillus coagulans* BL69 e que apresentou propriedades importantes, sendo a principal o fato de o extrato bruto ser completamente livre de qualquer atividade celulolítica. Microrganismos capazes de produzir xilanases sem produzir, paralelamente, celulases são relativamente raros. Por isso o isolado *B. coagulans* BL69 é extremamente interessante e potencialmente apto a produzir xilanases para serem aplicadas em operações de branqueamento da polpa de papel (polpa Kraft). Além disso, a xilanase produzida por esse isolado demonstrou faixas ampliadas de temperatura (45-75°C) e pH (4,5 a 10,0) para sua atividade ótima, o que é fundamental para aplicações industriais. No entanto, neste projeto, os trabalhos com o isolado *B. coagulans* BL69 foram suspensos prematuramente em virtude das razões expostas no item 5.1.1.

Em segundo momento direcionaram-se todas as atenções à xilanase produzida pelo isolado *B. circulans* BL53 em CSS. Uma série de estudos sobre a otimização da produção e da recuperação dessa enzima foram extensivamente realizados.

Ainda que muitos pesquisadores tenham realizados estudos sobre a produção dessa enzima, pouca informação sobre a otimização dos processos de produção e recuperação estão disponíveis na literatura. E acreditamos que parte dessa lacuna possa

ser preenchida com os nossos resultados. Foram determinadas as condições ótimas de produção das xilanases em CSS empregando-se metodologias de planejamento experimental e de superfície de resposta. Os resultados obtidos foram extremamente satisfatórios e possibilitaram um aumento na produção da enzima de 0,437 $U.mg^{-1}$ de proteína, nas condições antigas, para 1,037 $U.mg^{-1}$ de proteína, nas condições otimizadas. Também foi eficientemente otimizada a extração da xilanase produzida, novamente empregando-se metodologias estatísticas, para determinar as melhores condições de temperatura, agitação, tempo de extração, relação sólido-liquido e o solvente empregado. A otimização dessas duas operações é extremamente importante em processos biotecnológicos industriais, pois tende a reduzir os custos e facilitar as operações de purificação da enzima.

Por fim, a xilanase produzida pelo isolado BL53 foi purificada a homogeneidade e bioquimicamente caracterizada. A enzima exibiu algumas propriedades que sugerem seu potencial para futuras aplicações, uma vez que açúcares redutores foram liberados quando enzima foi incubada com diversos resíduos agroindustriais, indicando que a xilanase possa ser empregada na hidrólise de resíduos hemicelulósicos para obtenção de açúcares fermentescíveis, além de ter apresentado amplos intervalos de temperatura e pH para sua atividade ótima.

Em conclusão, é importante ressaltar que os estudos aqui realizados apontam firmemente para a viabilização do emprego de processos de Cultivo Semi-sólido e do aproveitamento de resíduos agroindustriais baratos e abundantes na Biotecnologia moderna, principalmente em países carentes de recursos financeiros, como é caso do Brasil. Ademais, também ficou evidente que a exploração da diversidade microbiana de ambientes ainda inexplorados (ou pouco explorados) pode revelar microrganismos e enzimas com aplicações práticas importantes, como foi o caso dos *B. coagulans* BL69, do *B. circulans* BL53 e das xilanases produzidas por eles.

7. PERSPECTIVAS

Este trabalho demonstrou a possibilidade de utilizar formas alternativas de produção de metabólitos de interesse, empregando um substrato de cultivo barato e abundante em um sistema de cultivo de microrganismo extremamente simples e que ainda é pouco empregado. Isso abriu a possibilidade de que outros projetos fossem elaborados utilizando-se dessas alternativas, inclusive duas dissertações de Mestrado já concluídas (uma sobre a produção de proteases e a outra sobre a produção de goma xantana em CSS).

Outra perspectiva aberta por este trabalho e também já colocada em prática em um projeto de Pós-doutoramento é a busca por novas enzimas lignocelulolíticas, como mananases, produzidas pelo isolados *B. circulans* BL53.

Além disso, evidenciou-se a aplicabilidade do emprego de ferramentas estatísticas e de planejamento experimental em processos biotecnológicos, permitindo que se alcance o máximo rendimento de cada etapa envolvida (produção, extração, atividade enzimática e hidrólise) e resultando em uma melhora global do processo. Isso contribui para consolidar essas metodologias como ferramentas úteis e importantes para a Biotecnologia, abrindo a possibilidade de que o seu emprego possa se tornar corrente entre os biotecnologistas.

O escalonamento dos processos biotecnológicos utilizados é um campo de pesquisa que precisa e merece ser considerado em trabalhos futuros, principalmente quando se objetiva a transposição da produção e do emprego das enzimas da escala de laboratório para uma escala industrial. Para tanto, não se pode deixar de considerar também a necessidade de estudos de modelagem matemática, pois esses podem

contribuir efetivamente para um maior conhecimento dos fenômenos físicos, químicos e biológicos envolvidos nos processos em questão.

Outra perspectiva, que não deixa de ser uma necessidade neste caso, é a busca por formas eficientes e reprodutíveis de determinação de biomassa bacteriana nos cultivos semi-sólidos. Este é, sem dúvida, o grande desafio que se impõe aos pesquisadores desta área, principalmente aos que utilizam-se de bactérias em seus experimentos. Para tanto, acreditamos que o emprego de técnicas de cromatografia gasosa, que permite a mensuração da atividade metabólica da bactéria, possa ser uma solução concreta para esse problema e, nesse sentido, esforços já estão sendo conduzidos.

E por último, não se pode deixar de aventar a perspectiva de estudos de manipulação genética com vistas à clonagem e caracterização dos genes das xilanases e sua expressão em sistemas mais eficientes.

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9. APÊNDICES

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CELLULASE AND XYLANASE PRODUCTION BY ISOLATED AMAZON *BACILLUS* STRAINS USING SOYBEAN INDUSTRIAL RESIDUE BASED SOLID-STATE CULTIVATION

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ABSTRACT

In Brazil, a large amount of a fibrous residue is generated as result of soybean (*Glycine max*) protein production. This material, which is rich in hemicellulose and cellulose, can be used in solid state cultivations for the production of valuable metabolites and enzymes. In this work, we studied the bioconversion of this residue by bacteria strains isolated from water and soil collected in the Amazon region. Five strains among 87 isolated bacteria selected for their ability to produce either celullases or xylanases were cultivated on the aforementioned residue. From strain BL62, identified as *Bacillus subtilis*, it was obtained a preparation showing the highest specific cellulase activity, 1.08 UI/mg protein within 24 hours of growth. Concerning xylanase, the isolate BL53, also identified as *Bacillus subtilis*, showed the highest specific activity for this enzyme, 5.19 UI/mg protein within 72 hours of cultivation. It has also been observed the production of proteases that were associated with the loss of cellulase and xylanase activities. These results indicated that the selected microorganisms, and the cultivation process, have great biotechnological potential.

Key words: Solid-State cultivation, cellulase, xylanase, soybean residue, Amazon biodiversity

INTRODUCTION

A great deal of research on the enzymatic degradation of cellulose and hemicellulose has been developed in the last 30 years (18). Organic wastes from renewable forest and agricultural residues are rich sources of cellulose, hemicellulose and lignin, in an average ratio of 4:3:3 (3) although the exact percentage of these components varies from source to source (24).

Cellulose, the largest renewable carbon source available (approximately 150 billion tons of organic material is photosynthesized annually), is frequently found in close association with other compounds, such as hemicellulose, lignin and other polysaccharides, which makes its bioconversion more difficult (17). Cellulose is an unbranched glucose polymer, composed of anhydro-D-glucose units linked by 1,4-β-D-glucoside bonds, which can be hydrolyzed by cellulolytic enzymes (18) produced by both bacteria and fungi. Cellulolytic bacteria include aerobic species such as *Pseudomonas* and Actynomicetes, facultative anaerobes such as *Bacillus* and *Cellulomonas*, and strict anaerobes such as *Clostridium*. The commercial possibility of using cellulase preparations to produce glucose, alcohol and protein from cellulose is under intensive study (11).

Hemicellulose is the second most abundant plant fraction available in nature. It is a storage polymer in seeds being also a structural component of cell walls in woody plants. Waste residues contain up to 40% hemicelluloses formed by pentose sugars (12). The monomers of various hemicelluloses are useful in the production of different antibiotics, alcohols, animal feed, chemical specialities and fuels (26). Xylan is the most abundant of the hemicelluloses. It has a linear backbone comprised of β-1,4-linked D-xylopyranose residues, which, depending on the origin, may present ramifications containing mainly acetyl, arabinosyl and gluconosyl residues (9). There is a great interest in the enzymatic hydrolysis of xylan due to possible applications in feedstock, fuel and chemical production, and paper manufacturing (6).

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Solid state cultivation (SSC) can be defined as the microbial cultivation process in the absence or near absence of free water in the substrate (15). However, there must be enough moisture present to support cell growth (16). As bacteria, yeasts and filamentous fungi can grow on solid substrates SSC has been used in bioprocesses (19). Besides, SSC has some advantages over submerged cultivation, including conditions that are similar to those of the natural habitat of the microorganisms, lower costs and improved enzyme stability (9). SSC has been used for the production of cellulolytic and xylanolytic enzymes. At the end of the cultivation, enzymes can be extracted from the substrate by simply percolating the bioreactor with appropriated buffers which is an easy, economical operation (17).

Brazil is the second largest soybean (*Glycine max*) world producer. The industrial processing of this seed, to obtain isolated protein, generates a large amount of fibrous residues. This material is mainly composed of 23% hemicellulose, 16% cellulose, and 28% insoluble protein (dry basis). It has been estimated by our studies that approximately 10,000 tn/year of this rich by-product are currently being produced, which is partially used for animal feeding complement or is simply disposed off in the environment (10).

The aims of this research are to study the bioconversion of soybean fibrous residue into value-added industrial products, such as enzymes, using SSC, and the selection of new bacteria strains from the Amazon region biodiversity for bioprocess applications.

MATERIALS AND METHODS

Industrial fibrous soybean residue

Industrial fibrous soybean residue (IFSR), a by-product from soybean processing for protein isolation, was used as the cultivation substrate. IFSR composition was determined according to the method of Van Soest and Robertson for cellulose and hemicellulose (27). Sucrose and protein contents were determined by Fehling and Kjeldahl methods respectively (1).

Microorganisms

The five strains of *Bacillus* used in this study were isolated from samples of soil and water of the Amazon region, and kindly provided by Prof. Spartaco Astolfi-Filho. They were selected according to their cellulolytic and xylanolitic activities. Strains were maintained on agar medium containing 0.1% tragacanth gum and 0.1% carboxymethyl cellulose. Bacteria were identified according to standard biochemical methods (22).

Medium

A basic liquid mineral medium showing the following composition (g l^{-1}): 0.2 $MgSO_4$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.02 $CaCl_2$, and 0.05 $FeCl_2$ was used for both inoculum preparation and SSC. SSC inoculum was prepared in 200 mL Erlenmeyer flasks containing 1.2 g of IFSR and 40 mL of basic liquid mineral medium. Flasks were inoculated with a single colony from an agar plate culture and incubated for 18 – 20 h at 37°C under agitation (125 rpm).

Bioreactor and Cultivation Conditions

Bioreactor experiments were carried out using a 500 mL cylindrical bioreactor (60 mm diameter, 170 mm height) designed and built in our laboratory. SSC medium was prepared with 20 g of dry IFSR and 50 g of inert support (irregular stones with approx. 5 mm of diameter) soaked in 120 mL of the liquid medium. Bioreactors filled with the substrate and the inert support, were sterilized in autoclave at 121°C for 15 min. After inoculation with the entire content of an Erlenmeyer flask, cultivation proceeded at 37°C for 72 hours. During cultivation, humidified sterile air was supplied at a constant rate of 150 mL min^{-1}.

Enzyme Extraction

Enzymes were extracted from the entire bioreactor content using eight volumes of water (160 mL) and by shaking this mixture at 150 rpm for 30 min. Separation of solid-liquid phase was accomplished by centrifugation at 10,600 g for 20 min.

Sugar content

Total reducing sugar was measured by the dinitrosalicylic method (DNS) (4).

Soluble Protein

Soluble protein was measured by the Bradford (2) procedure, using bovine albumin as the standard.

Enzymes Assays

Cellulase activity was determined by filter paper activity (FPA), according to Mandels *et al.* (13). Xylanase activity was measured according to Tavares *et al.* (25). Cellulase and xylanase activities were expressed as micromoles of glucose equivalent liberated per minute per mL of enzyme solution (IU/mL). Specific activities were expressed as IU/mg protein.

Proteolytic Activity Assay

Proteolytic activity was measured by the azocasein method, according to Sarath *et al.* (21). One enzymatic activity unit (U) was defined as the amount of enzyme needed to produce, using a 1cm cuvette, the change in one unit of absorbance under the conditions of the method. Specific activity was expressed as U/mg protein.

Statistical Analysis

Data were analysed by ANOVA procedure (20) and comparisons between means were performed by Tukey's test.

RESULTS AND DISCUSSION

The five bacteria strains used in this research presented the ability to produce either cellulases or xylanases, or both enzymes: BL 15 and BL 62 produced both enzymes; BL 53 and BL 69 produced xylanase, and strain BL 16 produced cellulases. BL 53 and BL 62 were identified as *Bacillus subtilis,* whereas BL 15, BL 16 and BL 69 were identified as *Bacillus* sp.

The time course of bioreactor SSC cultivations is shown in Figure 1. Strains BL 16, BL 62, and BL69 presented similar behavior towards reducing sugar liberation and consumption (Fig. 1a), as a peak of sugar concentration was observed in 24 hours of cultivation, followed by sharp decline, which indicates carbon source consumption. BL 53 strain showed an anticipated sugar liberation peak within 12 hours followed by a second peak of sugar liberation at the end of the run, probably due to *de novo* enzyme synthesis necessary to the hydrolysis of the substrate. BL 15 strain liberated the lowest amount of reducing sugar whose concentration remained constant up to the end of the process. Fig. 1b shows the results for protein concentration. With the exception of strain BL 69, which showed very little protein variation during cultivation, all others presented an initial decrease of soluble protein, indicating consumption of available nitrogen sources. During a later stage of growth, these cultures showed increased protein liberation into the medium, indicating proteolytic activity. To confirm this hypothesis, we have tested the proteolytic activity of cultures (Fig. 2). We obtained a very high enzymatic activity for all isolates, except for BL 69, confirming their ability to hydrolyze the residual soy protein.

Cell lyses, causing the liberation of basic amino acids and peptides into the medium, were probably responsible for a strong pH variation of cultures. The high values of pH obtained at the end of cultivations for the degradation of IFSR, shown in Figure 1c, contrasted with results obtained for the biodegradation of other cellulosic material, as described by Cen and Xia (5), who reported a highly buffering property, with pH staying in between 5.5 and 7.0. Our results point to the need of pH control during cultivation of *Bacillus* over IFSR when enzymatic activities may be halted by high pH values.

Cellulase and xylanase activities

With the exception of strain BL69 and BL53 that showed very low cellulolytic activity, the other isolates presented considerable cellulase activity, being its maximum at 24 hours of cultivation as it is shown in Fig. 3a. Studies with cellulolytic bacteria suggest that prokaryotic cellulases may present very different action from those of fungi, with best temperature and pH varying among them. Therefore, comparison in terms of enzyme activity is difficult to establish (8). To overcome this limitation, and aiming at to compare the enzymes produced by the strains investigated in this work to commercial enzyme preparations (Table 1), we measured enzyme specific activity

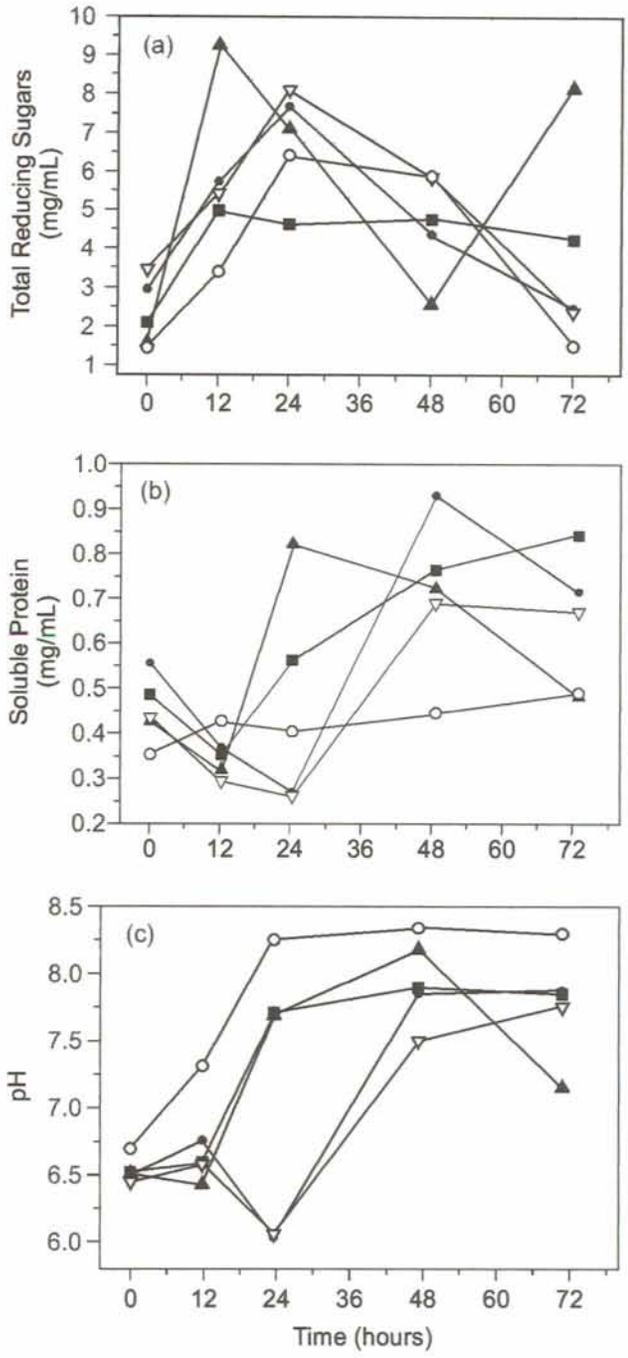

Figure 1. Cultivation time course for bioreactor SSC at 37°C and 150 mL air/min. (a) total reducing sugar; (b) soluble protein; (c) pH. Showing data for -■- *Bacillus* sp. BL 15, -●- *Bacillus* sp BL16, -▲- *Bacillus subtilis* BL53, -▽- *Bacillus subtilis* BL62, -○- *Bacillus* sp BL69. Results represent the average of two experiments.

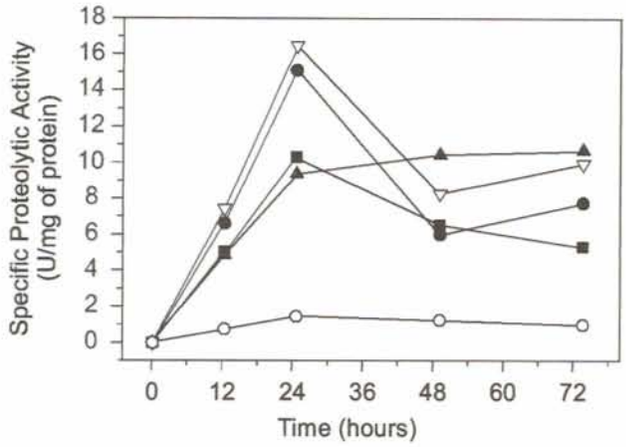

Figure 2. Specific proteolytic activity throughout SSC of IFSR at 37°C and 150 mL air/min. Showing data for -■- *Bacillus* sp. BL 15, -●- *Bacillus* sp BL16, -▲- *Bacillus subtilis* BL53, -▽- *Bacillus subtilis* BL62, -○- *Bacillus* sp BL69. Results represent the average of two experiments.

Table 1. Specific enzyme activities of commercial preparations and enzymatic extracts obtained in this work. Activity represents the higher value obtained during cultivation. Enzyme concentration of commercial preparations was equivalent to 1% (w/v) of crude powder. Means followed by different letters on column are statistically different (P<0.05).

Ezyme	Cellulase Specific Activity (UI/mg ptotein)	Xylanase Specific Activity (UI/mg ptotein)
Prozyn*	0.87^{A}	ND
ACX6000-P**	ND	1.37^{A}
HBC-5**	ND	1.03^{A,C}
Bacillus sp. BL15	0.72^{A}	1.75^{B}
Bacillus sp. BL16	0.89^{A}	0.88^{C}
Bacillus subtilis BL53	0.23^{B}	5.19^{D}
Bacillus subtilis BL62	1.08^{C}	4.06^{E}
Bacillus sp. BL69	0.06^{B}	1.28^{A}

* Commercial Cellulase - Novo Nordisk; ** Commercial hemicellulase - Trigotec Ltda.; ND - Not determinated.

for both cases. Results shown that isolates BL15, BL16, and BL62 have similar cellulase specific activity as shown by Prozyn™, a widely used industrial enzyme. Concerning the production of xylanase, all strains presented some activity but profiles varied among them as shown in Figure 3b. The isolate BL53 presented the highest xylanase activity within 72 hours of cultivation (5.19 UI/mg protein). BL62, which presented the

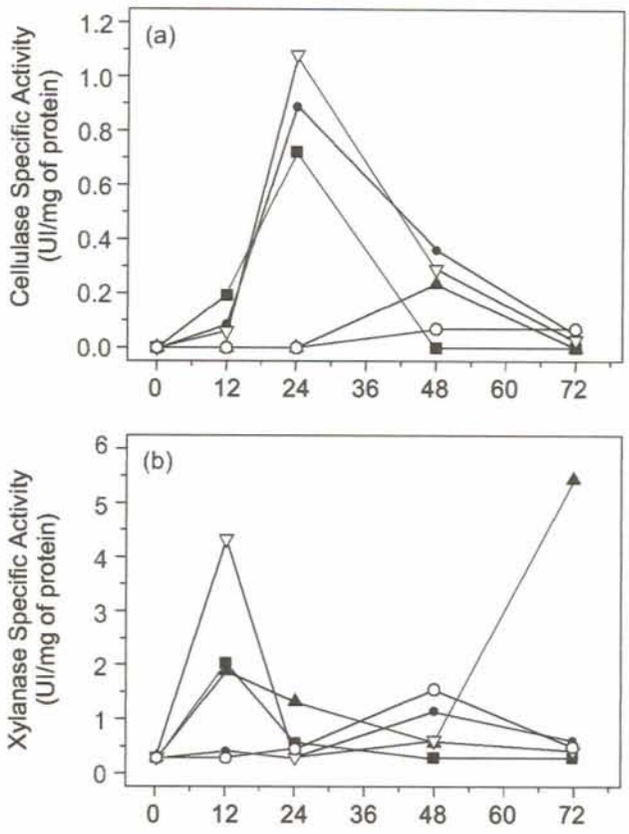

Figure 3. Specific enzyme activities of (a) Cellulase, and (b) Xylanase throughout SSC of IFSR at 37°C and 150 mL air/min. Showing data for -■- *Bacillus* sp. BL 15, -●- *Bacillus* sp. BL16, -▲- *Bacillus subtilis* BL53, -▽- *Bacillus subtilis* BL62, -○- *Bacillus* sp BL69. Results represent the average of two experiments.

highest cellulase activity, also showed a reasonable xylanase production (4.07 UI/mg protein) but, in contrast with BL53, its peak was at the initial phase of cultivation, 12 hours. This difference might be related to the ability of strains in initially using carbon sources other than cellulose and hemicellulose that are present in IFSR. The complete analysis of the SSC substrate residue showed 17% (dw) content on sugars such as raffinose, stachyose, and arabinose, which may be used or not, depending on the metabolic capabilities of strains. Protein preparations obtained from strains BL53 and BL62 compared favorably to commercial xylanases, as shown in Table 1, with activities being four to five times higher. Silva *et al.* (23) described SSC production of xylanase by *Aspergillus fumigatus* Fresenius growing for seven days. The maximum productivity obtained in their work was of 6.27 UI/mg protein, similar to that for BL53 within 72 hours of cultivation.

According to Moo-Young (14), the actions of cellulases and xylanases are synergetic over substrate, especially for microorganisms isolated from environments where wood and agro-residues are biodegraded. In our work, most of the strains were capable of producing both enzymes, confirming these observations. Another important aspect for industrial applications of enzymes is the need for reduced costs of production (17). Gessesse and Mamo (7) postulate that the great advantages of using SSC over submerged cultivation for enzyme production are the lower water content of enzymatic extract, easy of control, lower probability of contamination, among others. The selection of better solvents for enzyme extraction from the medium is another important point of research when selecting SSC as a biotechnological system.

CONCLUSIONS

In this work we demonstrated that some bacteria are as good producers of cellulase and xylanase as fungi are. Although is not common to use bacteria as microorganisms in SSC, in our work, the isolates from Amazonian environment proved to be excellent biological systems, showing some advantage over filamentous fungi, such as faster growth and lower probability of contaminations. Yet, recovery of extracellular enzymes is eased by the simplicity of the process. The use of agro-industrial residues as substrate for microbial growth also proved to be possible, with good perspectives for scaling up. Moreover, results obtained in this work point to the strong necessity of better understanding of the microbial diversity of Amazon region, one of the largest ecosystems on earth.

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RESUMO

Produção de celulase e xilanase por cepas de *Bacillus* isoladas da Amazônia em culivo semi-sólido utilizando resíduo da indústria da soja

No Brasil, uma grande quantidade de resíduos fibrosos de soja (*Glycine max*) são gerados no processo de produção de proteína de soja. Estes materiais, ricos em celulose e hemicelulose, podem ser usados como substratos para cultivos microbianos visando a produção de valiosos metabólitos e enzimas. Neste trabalho, estudou-se a produção de enzimas, utilizando estes resíduos, por bactérias isoladas da água e do solo da região amazônica. Cinco cepas, dentre 87 iniciais, foram selecionadas e crescidas em cultivo semi-sólido (CSS). Preparações obtidas do isolado BL 62, identificado como *Bacillus subtilis*, apresentaram a maior atividade específica para celulase, 1,08 UI/ mg de proteína, em 24 horas de cultivo. No que se refere às xilanases, preparações obtidas do isolado BL 53, também identificado como *Bacillus subtilis*, apresentaram a maior atividade específica para esta enzima, com um valor de 5,19 UI/ mg de proteína, em 72 horas de cultivo. Também foi demonstrada a produção simultânea de proteases, o que pode ser associado à perda das atividades de celulase e xilanase durante o cultivo. Os resultados indicam que os microrganismos selecionados e o processo de cultivo empregado utilizando resíduo da soja apresentam grande potencial biotecnológico.

Palavras-chave: Cultivo semi-sólido, celulases, xilanases, resíduo de soja, biodiversidade amazônica.

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9.2. Comprovantes de publicação dos artigos referentes aos resultados apresentados em Resultados I e III e IV, respectivamente.

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Optimization of cellulase-free xylanase activity produced by *Bacillus coagulans* BL69 in solid-state cultivation

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Optimization of xylanase and mannanase production by *Bacillus circulans* strain BL53 on solid-state cultivation

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CURRICULUM VITÆ RESUMIDO

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2. FORMAÇÃO: Químico Industrial de Alimentos (UNIJUÍ, 1998); Mestre em Microbiologia Agrícola e do Ambiente (PPGMAA, UFRGS, 2001)

3. ESTÁGIOS: Estágio curricular obrigatório no Núcleo de Alimentos do Polo de Modernização Tecnológica – Fronteira Noroeste (Santa Rosa, fevereiro a julho de 1996); Estágio curricular obrigatório na empresa VEGA Industrial e Mercantil de Produtos Alimentícios (Pelotas, julho de 1997); Estágio curricular obrigatório na Usina-Escola de Laticínios da Universidade Federal de Santa Maria (Santa Maria, janeiro e fevereiro de 1998); Estágio curricular obrigatório no Frigorífico e Matadouro Prenda SA (Santa Rosa, maio a setembro de 1998); Bolsista PIBIC/UNIJUÍ no Laboratório de Análises de Águas do Núcleo de Alimentos do Polo de Modernização Tecnológica – Fronteira Noroeste (de janeiro de 1995 a agosto de 1996, sob a orientação da professora MSc. Vera Maria Klajn).

4. EXPERIÊNCIA PROFISSIONAL

- Professor substituto do Departamento de Química Inorgânica da UFRGS (40 horas), atuando nas disciplinas de Química Geral Experimental e Química Analítica Qualitativa. De dezembro de 2001 a maio de 2002.

- Professor do curso de Especialização em Ciência e Tecnologia de Alimentos do ICTA/UFRGS, atuando na disciplina da Química e Bioquímica de Alimentos.

- Professor substituto do Departamento de Biotecnologia da Escola Técnica da UFRGS (20 horas), atuando nas disciplinas de Microbiologia, Processos Fermentativos e Introdução à Biotecnologia para os cursos de Técnico em Biotecnologia e Técnico Analista de Processos – Química. Desde março de 2005.

5. ARTIGOS COMPLETOS PUBLICADOS EM PERIÓDICOS

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