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Produção de enzimas celulolíticas de *Trichoderma reesei* por fermentação em estado sólido e sua aplicação na sacarificação de resíduos agroindustriais lignocelulósicos

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil

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Produção de enzimas celulolíticas de *Trichoderma reesei* por fermentação em estado sólido e sua aplicação na sacarificação de resíduos agroindustriais lignocelulósicos

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Engenharia de Processos, Área de Concentração em Desenvolvimento de Processos Agroindustriais e Ambientais, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Engenharia de Processos**.

Orientador: Prof. Dr. Sergio Luiz Jahn
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Produção de enzimas celulolíticas de Trichoderma reesei por fermentação em estado sólido e sua aplicação na sacarificação de resíduos agroindustriais lignocelulósicos

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como requisito parcial para obtenção do grau de
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RESUMO

Dissertação de Mestrado
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PRODUÇÃO DE ENZIMAS CELULOLÍTICAS DE *Trichoderma reesei* POR FERMENTAÇÃO EM ESTADO SÓLIDO E SUA APLICAÇÃO NA SACARIFICAÇÃO DE RESÍDUOS AGROINDUSTRIAIS LIGNOCELULÓSICOS

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Data e Local da Defesa: Santa Maria, 18 de fevereiro de 2014.

O bagaço de cana-de-açúcar (BC) é um resíduo lignocelulósico abundante em regiões sucroalcooleiras no Brasil e é um potencial substrato para produção de etanol de segunda geração, além de possuir características estruturais que o classificam como bom indutor para produção de celulases por microrganismos. O alto custo da produção industrial de celulases, no entanto, é um grande empecilho na hidrólise desse tipo de material para posterior fermentação, o que inviabiliza a utilização desse processo na produção de etanol em larga escala. Nesse contexto, o desenvolvimento de processos de fermentação mais eficientes e de menor custo para a produção de celulases em escala industrial, bem como alternativas mais eficazes de hidrólise enzimática desse material são necessários a fim de viabilizar economicamente o processo. Para essa finalidade, esse trabalho tem como proposta o desenvolvimento de um processo para produção de celulases utilizando uma cepa do fungo filamentoso *Trichoderma reesei*, bem como a utilização do extrato enzimático produzido na hidrólise enzimática de bagaço a fim de avaliar os efeitos do ultrassom no processo. O processo otimizado de produção das celulases consistiu em cinco dias de crescimento do pré-inóculo em placas de Petri, seguido de dois dias de crescimento em meio líquido otimizado, e quatro dias de FES de BC suplementado com 1% de farelo de soja (FS) e 15% de água de maceração de milho (AMM), 65% de umidade, 28±1°C e densidade de 0,5 mililitros de inóculo por grama de substrato. Essa condição experimental em escala de bancada (5 g) resultou em uma produção de 1,4 FPU/g, valor esse que aumentou aproximadamente três vezes com o aumento de escala de produção em um biorreator de leito fixo com aeração forçada com capacidade para 70 g de substrato. Para hidrólise enzimática assistida por banho de ultrassom, a condição que atingiu melhores eficiências foi de 43,4±2°C e 18,5% (v/v) de concentração de enzima, atingindo um máximo de 229 gramas de açúcares redutores por quilograma de substrato utilizado, e foi observado um aumento médio de 12% na eficiência de hidrólise naqueles experimentos em que a hidrólise foi assistida por ultrassom. Já nas sacarificações utilizando a sonda ultrassônica, os resultados utilizando sonicação indireta durante a sacarificação foram, em média, 158% maiores que aqueles utilizando sonicação direta. Dessa forma, conclui-se que a utilização de sonicação indireta é mais indicada como auxiliar nas hidrólises, uma vez que a sonicação direta pode causar desnaturação da enzima e diminuir a eficiência do processo.

Palavras-chave: Fermentação em estado sólido. Resíduos agroindustriais lignocelulósicos. Produção de enzimas. Celulases. Biorreator de leito fixo. Hidrólise enzimática. Ultrassom.

ABSTRACT

Master Dissertation
Graduate Program in Process Engineering
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PRODUCTION OF CELLULOLYTIC ENZYMES FROM *Trichoderma reesei* BY SOLID STATE FERMENTATION AND ITS USE IN THE SACHARIFICATION OF LIGNOCELLULOSIC RESIDUES

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Defense Place and Date: Santa Maria, February 18th, 2014.

Sugarcane bagasse is an abundant lignocellulosic residue in traditional regions of sugar and ethanol production in Brazil. It is not only a potential substrate for second generation ethanol production but also have structural features to be classified as good inducer for cellulases production by microorganisms. However, the high cost of cellulases industrial production is the major bottleneck in the hydrolysis of this raw material for subsequent fermentation, which makes unfeasible in large scale the ethanol production using this process. In this context, the development of more efficient and less expensive fermentation processes for industrial cellulases production, as well as better alternatives of enzymatic hydrolysis of lignocellulosic material is crucial to achieve economic feasibility in this process. For this purpose, this work aims to develop a cellulase production process using *Trichoderma reesei*, as well as assessing the use of produced enzymatic extract in sugarcane bagasse hydrolysis, in order to evaluate the ultrasound effects in the hydrolysis process. The optimized process of cellulases production consisted in five days of grow in pre-inoculum Petri dishes, followed by two days of grow in optimized liquid medium and four days of solid state fermentation, using sugarcane bagasse supplemented with 1% of soybean bran and 15% (v/w) of corn steep liquor as substrate, moisture of 65%, 28±1°C and 0.5 mL of inoculum per gram of substrate. This experimental condition in bench scale (5 g) resulted in a production of 1.4 FPU/g of cellulases, and the production was approximately three-fold high in a fixed-bed bioreactor with forced aeration for 70 g of substrate capacity. For ultrasound assisted enzymatic hydrolysis using an ultrasound bath, the condition that achieved higher efficiencies were 43.4±2°C and 18.5% (v/v) of enzyme concentration, resulting in a maximal hydrolysis efficiency of 229 grams of reducing sugar per kilogram of used substrate, achieving an average increase of 12% in efficiency in those experiments where the hydrolysis was assisted by ultrasound compared with those without sonication. Regarding the saccharification using the ultrasonic probe, results using the indirect sonication during process were, on average, 158% higher than those using the direct sonication. Thus, it can be concluded that indirect sonication is more suitable to be used as an auxiliary in the hydrolysis, since the direct sonication can cause denaturation of the enzyme, reducing the process efficiency.

Key words: Solid state fermentation. Lignocellulosic agroindustrial residues. Enzyme production. Celullases. Fixed bed bioreactor. Enzymatic hydrolysis. Ultrasound.

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LISTA DE ABREVIATURAS E SIGLAS

AMM: água de maceração de milho

BC: Bagaço de cana

BG: β-glucosidase

BM: Banho Maria sem ultrassom

DCCR 11E: Delineamento composto central rotacional com 11 experimentos

DCCR 17E: Delineamento composto central rotacional com 17 experimentos

EE: concentração de extrato enzimático (%, v/v)

EL: Concentração de extrato de levedura

EnG: Endoglucanases

ExG: Exoglucanases

FES: Fermentação em estado sólido

FPA: Atividade em Papel Filtro

FPU: Unidade de atividade em papel filtro

FS: farelo de soja

FSub: Fermentação Submersa

PDA: meio ágar batata dextrose

r: razão sólido líquido

U: Unidade de atividade enzimática

UI: Atividade enzimática em Unidades Internacionais.

US: Banho Maria com ultrassom

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1 INTRODUÇÃO

A importância da sustentabilidade nas atividades produtivas tem sido amplamente reconhecida, havendo a necessidade da substituição de processos químicos baseados em insumos não renováveis por processos químicos ou bioquímicos que utilizem insumos renováveis. Reconhece-se também a necessidade da substituição das múltiplas etapas de processos químicos por processos biotecnológicos mais eficientes. Este contexto favorece a utilização de matérias primas renováveis através de biotransformação e biocatálise (KUMAR et al., 2008; LYND et al., 2005; HOFFERT et al., 2002).

Entre as alternativas de matérias primas renováveis para utilização em bioprocessos destacam-se os resíduos lignocelulósicos. Estes são os resíduos mais abundantes no mundo e apresentam um enorme potencial para a obtenção de produtos de interesse industrial como bioetanol, glicose e biomassa proteica. Tal biomassa representa uma abundante fonte de carboidratos e sua bioconversão tem recebido grande atenção nos últimos anos. Processos utilizando esse tipo de resíduo como matéria-prima podem minimizar a falta de alimentos, resolver problemas de desperdício e diminuir a dependência do homem por combustíveis fósseis através do fornecimento de uma conveniente e renovável fonte de energia na forma de glicose (OJUMU et al., 2003). Além disso, a utilização desses resíduos impede a sua acumulação, o que representa um ganho ambiental devido ao potencial de contaminação de rios e águas subterrâneas que esse material possui (LEITÃO et al., 2010).

A hidrólise de materiais lignocelulósicos pode ser feita de duas maneiras distintas: via química ou via enzimática. Embora a hidrólise ácida de biomassa seja eficiente e relativamente barata, gera resíduos poluentes e, muitas vezes, produtos que acabam por inibir uma fermentação posterior (AGUIAR, 2010). Em razão disso, a sacarificação enzimática tem sido objeto da maior parte dos estudos, já que processos enzimáticos oferecem potencial de redução de custos em longo prazo. Isso porque a utilização de enzimas em processos industriais muitas vezes pode eliminar o uso de altas temperaturas, solventes orgânicos e condições extremas de pH, enquanto que, ao mesmo tempo, oferece um aumento da especificidade da reação e da pureza do produto, além de reduzir o impacto ambiental (CHERRY e FIDANTSEF, 2003). Nesse contexto, o uso e produção de enzimas provenientes de fontes renováveis têm se tornado uma das áreas de maior interesse da indústria biotecnológica.

Por outro, a hidrólise dos resíduos lignocelulósicos se processa inicialmente em um sistema heterogêneo (substrato insolúvel e enzimas solúveis) e vários fatores têm sido relacionados como limitantes à hidrólise e podem ser divididos em dois grandes grupos: os associados às mudanças na estrutura do substrato e os relativos ao comportamento das enzimas durante o processo hidrolítico (RAMOS, 2000). Consequentemente é necessário otimizar as condições de hidrólise para conseguir o funcionamento satisfatório dos processos de sacarificação (RABELO, 2007). Nesse sentido, algumas tecnologias emergentes, como o pré-tratamento ou hidrólise assistida por ultrassom, vêm sendo utilizadas com intuito de melhorar a eficiência do processo e contornar essas limitações (BENAZZI et. al, 2013; SFALCIN et. al, 2012; LEÃES et. al, 2013).

No âmbito de produção de etanol a partir de materiais lignocelulósicos, as celulases são insumos que impactam significativamente o processo, e, segundo Zhuang et al. (2007), podem representar até 18% do custo operacional de uma planta de hidrólise enzimática. As tecnologias de produção de celulases e sua aplicação na hidrólise de materiais lignocelulósicos são processos em contínuo desenvolvimento, e chave para o aumento da produtividade e economicidade da rota enzimática (ZÚÑIGA, 2010). Nesse contexto, é de extrema importância o desenvolvimento de novas rotas tecnológicas para a produção dessa enzima com custos mais baixos, para que a produção de etanol de segunda geração torne-se viável economicamente, com preços de custo competitivos em relação aos demais biocombustíveis, e, em uma perspectiva mais otimista, até mesmo aos combustíveis fósseis.

As celulases podem ser produzidas por duas metodologias de cultivo: fermentação em estado sólido (FES) ou semissólido e em cultivo submerso ou fermentação submersa (FSub). A produção das enzimas comerciais utiliza, tradicionalmente, a tecnologia de fermentação submersa, já que sua tecnologia já foi bastante estudada, e permite o controle dos níveis de aeração, pH e temperatura do meio, para o crescimento ótimo do microrganismo. Contudo, a fermentação em estado sólido tem sido bastante estudada nos últimos anos. Isto se explica pelo grande número de vantagens econômicas e de processo, nos quais se destacam a natureza dos substratos utilizados como suporte e/ou fonte indutora na produção da enzima, que podem ser resíduos lignocelulósicos, por exemplo, bem como o menor volume de reação necessário, o que leva à necessidade de utilização de biorreatores menores e, consequentemente, de menor custo (SCHMIDELL e FACCIOTTI, 2001).

1.1 Estrutura da dissertação

Assim, segue a resumida apresentação dessa dissertação, no que tange ao conteúdo de cada capítulo contido nela:

O capítulo dois apresenta a ***Revisão Bibliográfica*** dos aspectos relevantes desse trabalho, que sequencia as informações técnicas necessárias à compreensão e ao embasamento do estudo.

O capítulo três, intitulado ***Metodologia Experimental***, apresenta resumidamente os fluxogramas do processo desenvolvido.

No capítulo quatro, ***Artigo 1***, encontra-se o artigo, submetido para a revista *Industrial Crops and Products*, referente aos resultados da primeira parte experimental do trabalho, que abrange a otimização das condições de processo para produção de celulases, o aumento de escala, a hidrólise *in situ* e a avaliação dos efeitos da sonicação de baixa intensidade utilizando um banho de ultrassom.

O capítulo cinco, ***Artigo 2,*** apresenta o segundo artigo, submetido para a revista *Biomass and Bioenergy*, com os resultados da segunda parte experimental do trabalho, onde são avaliados e comparados os efeitos da hidrólise assistida por sonicação direta e indireta, por meio de uma sonda ultrassônica.

O capítulo seis apresenta as ***Considerações Finais***, relacionando os principais resultados dos dois artigos apresentados anteriormente.

Já o sétimo capítulo apresenta as C***onclusões e Sugestões,*** onde são explicitadas as principais conclusões obtidas no decorrer do trabalho, e apresenta sugestões para o desenvolvimento de trabalhos futuros.

No oitavo e último capítulo, estão identificadas as ***Referências Bibliográficas***.

1.2 Objetivos

1.2.1 Objetivo geral

O principal objetivo do presente trabalho é a produção de enzimas celulolíticas de *Trichoderma reesei* por fermentação em estado sólido e sua aplicação na sacarificação de resíduos lignocelulósicos.

1.2.2 Objetivos específicos

- Definição uma metodologia padrão para crescimento do microorganismo em placas de Petri.
- Otimização do meio de cultura para crescimento do pré-inóculo em meio líquido.

- Definição e otimização das condições de processo em escala de bancada para posterior aumento de escala.
- Aumento de escala do processo, utilizando um biorreator de leito fixo com aeração forçada para produção de celulases.
- Avaliação das condições de hidrólise enzimática de bagaço de cana *in natura* em banho de água convencional e em banho de água com ultrassom, utilizando o extrato enzimático produzido e comparação das eficiências de hidrólise nos dois processos.
- Utilização do extrato enzimático produzido para avaliação das condições de sacarificação de bagaço de cana *in natura* assistida por ultrassom, e comparação das eficiências nos processos com sonicação direta e indireta, utilizando uma sonda ultrassônica.

2 REVISÃO BIBLIOGRÁFICA

Neste capítulo, será apresentada uma revisão teórica sobre os resíduos lignocelulósicos disponíveis e sua potencial utilização como substrato e a produção de etanol de segunda geração, assim como para produção de enzimas celulolíticas, destacando os principais microorganismos produtores de enzimas e o processo de fermentação em estado sólido. Além disso, será abordada uma revisão sobre a hidrólise enzimática de biomassa lignocelulósica e o uso do ultrassom nesses processos.

2.1 Resíduos lignocelulósicos e a produção de etanol de segunda geração

As tendências mundiais para o avanço científico e tecnológico na área de novos combustíveis destacam a importância da utilização de resíduos agroindustriais lignocelulósicos como matéria-prima nos processos de produção. A reutilização e reciclagem destes resíduos podem minimizar os problemas ambientais ligados ao seu acúmulo e diminuir o uso de combustíveis fósseis, além de resultar em uma melhora no aproveitamento da matéria-prima, que é de grande interesse na atualidade (RABELO, 2007). Uma das potenciais aplicações desses materiais é a produção de etanol de segunda geração.

O bioetanol de segunda geração vem sendo produzido pela hidrólise e fermentação de materiais lignocelulósicos desde o fim do século XIX, mas somente nos últimos 20 anos essa tecnologia tem sido proposta para atender o mercado de combustíveis. Os principais programas de pesquisa e desenvolvimento são conduzidos nos Estados Unidos e na Europa, basicamente em escalas experimentais de produção, mas seu sucesso poderia transformar o bioetanol em um biocombustível passível de ser produzido em quase todas as regiões do mundo, aproveitando a alta disponibilidade de resíduos orgânicos de diversas fontes. Praticamente todos os resíduos de biomassa, produzidos nas atividades agrícolas e industriais, e mesmo o lixo urbano, apresentam elevados teores de materiais lignocelulósicos (MACEDO et al., 2008).

A composição de resíduos lignocelulósicos é bastante variável, pois os constituintes possuem características químicas semelhantes às da madeira e são identificados em diferentes quantidades percentuais, dependendo da espécie e condições de crescimento. A biomassa lignocelulósica é composta de celulose (~35-50%), hemicelulose (~20-35%), lignina (~10-25%), além de pequenas quantidades de outros componentes (extrativos) (~5-20%), e sua estrutura está representada na Figura 1 (TAMANINI, 2004; RABELO, 2007).

A celulose de fórmula $(C_6H_{10}O_5)_n$ é um polissacarídeo C6 formado por uma longa cadeia de moléculas de glicose. A hemicelulose de fórmula $(C_5H_8O_4)_n$ e $(C_6H_{10}O_5)$ é um componente relativamente amorfo, que é mais facilmente quebrada quimicamente por calor do que a celulose e é constituída por uma mistura de polissacarídeos C_6 (galactose e manose) e polissacarídeos C_5 (xilose e arabinose). A lignina é essencialmente o cimento que propicia a rigidez estrutural das plantas e árvores, formada por uma rede polimérica tridimensional de unidades metoxilas, arilpropanos e hidroxifenóis. A fórmula empírica deste polímero complexo é $C_9H_{10}O_2(OCH_3)_n$ no qual n é a razão de CH_3O para grupos C_9: n = 1,4; 0,94 e 1,18 para as madeiras duras, moles e gramíneas, respectivamente. O que dá a rigidez a esta rede polimérica são as ligações cruzadas (RODRIGUES, 2011). A Tabela 1 apresenta a composição química parcial de alguns principais resíduos lignocelulósicos.

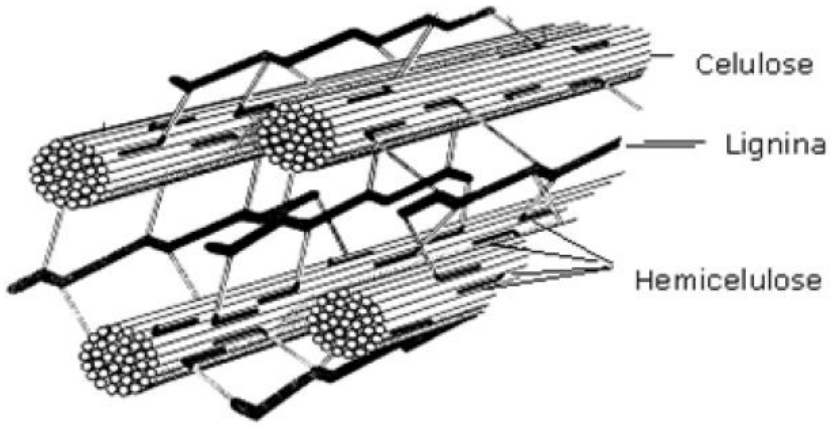

Figura 1: Esquema estrutural simplificado das fibras do material lignocelulósico (LEE, 1997; SILVA, 2010).

Tabela 1: Composição química parcial dos principais resíduos lignocelulósicos

Resíduos lignocelulósicos	**Celulose (%)**	**Hemicelulose (%)**	**Lignina (%)**
Farelo de cevada	23,0	32,7	24,4
Sabugo de milho	31,7	34,7	20,3
Folhas de milho	37,6	34,5	12,6
Bagaço de cana	40,2	26,4	25,2
Palha de arroz	43,5	22,0	17,2
Palha de trigo	33,8	31,8	20,1
Palha de sorgo	34,0	44,0	20,0
Casca de aveia	30,5	28,6	23,1
Farelo de cevada	23,0	32,7	24,4

Fonte: SILVA, 2010.

No Brasil, a quantidade de resíduos lignocelulósicos gerada anualmente é de aproximadamente 350 milhões de toneladas. Com tal quantidade de resíduo estima-se a capacidade de produção de 147 milhões de toneladas de açúcares, gerando um potencial de produção de etanol de 92 bilhões de litros. Essa produção corresponde a 2,6 vezes a produção do Brasil e dos Estados Unidos, juntas. Se considerarmos as eficiências de hidrólise e fermentação, a capacidade de produção de etanol é de aproximadamente 60 bilhões de litros (AGUIAR, 2010). Já mundialmente, a quantidade de resíduos lignocelulósicos produzidos é estimada em 1,55 bilhões de toneladas/ano incluindo sabugo de milho, palha de cevada, de trigo, de arroz e de sorgo e bagaço de cana (EPOBIO, 2007).

O bagaço de cana é, sem dúvida, o resíduo agroindustrial obtido em maior quantidade no Brasil, correspondendo a aproximadamente 290 quilogramas por tonelada moída (SILVA et al., 2007). A Tabela 2 mostra a composição média característica do bagaço de cana em que a fibra é a matéria insolúvel em água contida na cana-de-açúcar e o Brix o teor de sólidos solúveis em água.

Tabela 2: Composição média do bagaço de cana-de-açúcar - base seca

Composição Química	
Carbono	39,7 – 49%
Oxigênio	40 – 46%
Hidrogênio	5,5 – 7,4%
Nitrogênio e cinzas	0 – 0,3%
Propriedades Físico-químicas	
Umidade	50%
Fibra	46%
Brix	2%
Impurezas minerais	2%
Composição média da fibra do bagaço	
Celulose	26,6 – 54,3%
Hemicelulose	14,3 – 24,4%
Lignina	22,7 – 29,7%

Fonte: (Rosa e Garcia, 2009).

As tecnologias para a obtenção de bioetanol de segunda geração, produzido a partir de materiais lignocelulósicos, envolvem a hidrólise dos polissacarídeos da biomassa em açúcares fermentáveis e sua posterior fermentação. Para executar essa tarefa, o processo de hidrólise

utiliza tecnologias complexas e multifásicas, com base no uso de rotas ácidas e/ou enzimáticas para a separação dos açúcares e remoção da lignina (PEREIRA Jr. et al., 2008).

A Figura 2 ilustra o fluxograma simplificado do processo de produção de etanol de segunda geração. A primeira etapa no processamento da biomassa para produção de etanol é o pré-tratamento mecânico ou químico, que visa destruir a estrutura celular das plantas, de modo a permitir as operações posteriores. A Tabela 3 relaciona as principais técnicas que estão sendo desenvolvidas (ROSA E GARCIA, 2009).

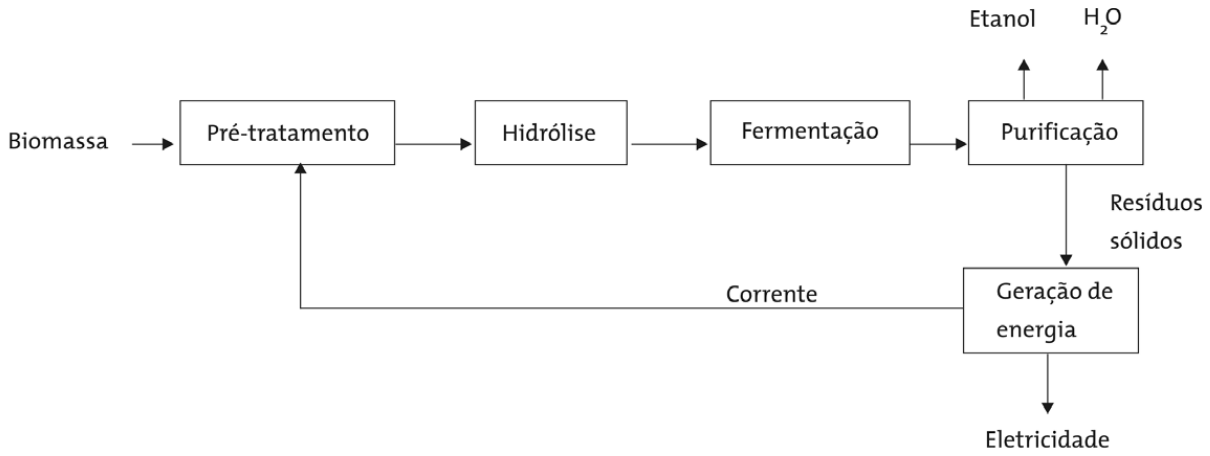

Figura 2: Fluxograma do processo convencional de produção de etanol de matérias primas lignocelulósicas (HAMELINCK et al., 2005).

O pré-tratamento desorganiza a estrutura da biomassa celulósica, beneficiando o trabalho das enzimas e ácidos que atuam na conversão de carboidratos em açúcares. A Figura 3 representa a ação do pré-tratamento sobre o material lignocelulósico.

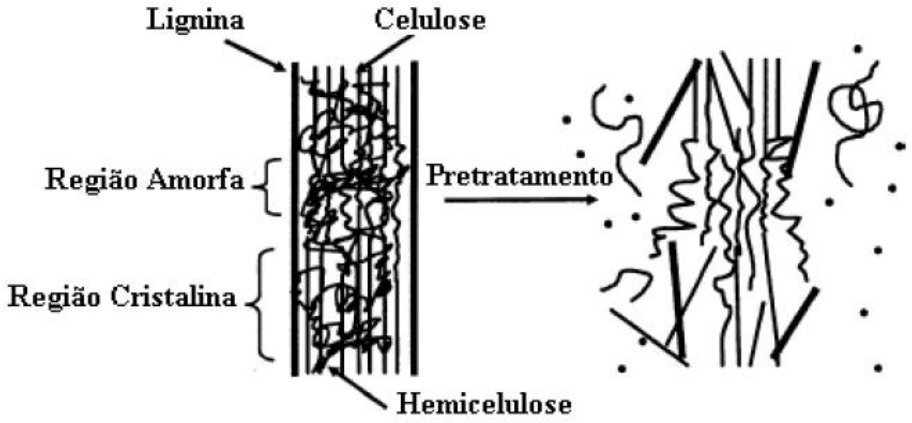

Figura 3: Representação esquemática da ação do pré-tratamento sobre o material lignocelulósico (MOSIER et al., 2005).

Tabela 3: Principais processos de pré-tratamento da biomassa utilizados na produção de etanol de segunda geração

PROCESSO	*DESCRIÇÃO*	*TEMPO DE REAÇÃO*	*RENDIMENTO EM XILOSE*
	FÍSICOS		
Explosão a vapor	A biomassa triturada é tratada com vapor (saturado, 160°-260° C) seguido de uma rápida descompressão.	1-10 min	45 – 65%
Termo-hidrólise	Utiliza água quente a alta pressão (pressões acima do ponto de saturação) para hidrolisar a hemicelulose.	30 min	88-98%
	QUÍMICOS		
Hidrólise ácida	Por meio do uso de ácidos sulfúrico, clorídrico ou nítrico, concentrados ou diluídos.	2 – 10 min	75 – 90%
Organosolv	Uma mistura de um solvente orgânico (metanol, bioetanol e acetona, por exemplo) com um catalisador ácido (H_2SO_4, HCl) é usada para quebrar as ligações internas da lignina e da hemicelulose.	40 – 60 min	70 – 80%
	COMBINADOS		
Afex (ammonia fiber explosion)	Exposição à amônia líquida a alta temperatura e pressão por certo período de tempo, seguida de uma rápida descompressão.	-	50%-90%
Explosão de CO_2	Similar à explosão de vapor.	-	75%
	TECNOLOGIAS AVANÇADAS		
CO_2 Supercrítico	Utilização de CO_2 (60°C, 200 bar) sob condições supercríticas.	30 – 120 min	-
Ultrassom	Aplicação de ultrassom em diferentes frequências.	1 – 6 horas	75%

(ROSA e GARCIA, 2009; BENAZZI et al., 2013)

Após o pré-tratamento, a biomassa é submetida à separação da lignina e da hemicelulose. A lignina é muito difícil de ser tratada quimicamente, e seu papel nos processos em desenvolvimento estará limitado, normalmente a fornecer energia térmica. Quanto à hemicelulose, pode ser hidrolisada sem maiores problemas com as técnicas hoje disponíveis. A operação central dos processos de produção de etanol de segunda geração é a hidrólise propriamente dita, que possibilita, pela atuação de determinados catalisadores, a quebra das cadeias de polissacarídeos, tendo como resultado moléculas de açúcar. Uma vez obtidos os

açúcares, seguem-se as etapas de fermentação e destilação, exatamente como no processo convencional (SILVA, 2010; PEREIRA Jr et al., 2008).

As rotas tecnológicas distinguem-se entre si pela natureza do catalisador, que pode ser químico (ácido ou alcalino) ou biológico (enzimas). Na situação atual, de acordo com as perspectivas das tecnologias de segunda geração, a quase totalidade dos esforços concentra-se no desenvolvimento da hidrólise enzimática (ROSA e GARCIA, 2009). Para isso, é necessário, também, que se desenvolvam rotas tecnológicas mais eficientes e baratas para a produção de enzimas a serem utilizadas na hidrólise dos materiais lignocelulósicos.

2.2 Enzimas celulolíticas

2.2.1 Caracterização, classificação e mecanismos de ação.

As enzimas celulolíticas, também denominadas como complexo celulase, são caracterizadas por realizar a hidrólise das ligações O-glicosídicas da celulose, e estão divididas em três grandes grupos, com base no modo de catálise sobre a fibra de celulose: endoglucanases, exoglucanases e β-glucosidases (CASTRO et al., 2010, FERREIRA, 2010). A Tabela 4 apresenta as classificações das enzimas celulásicas segundo a *Enzyme Commission* e a IUBMB - I*nternational Union of Biochemistry and Molecular Biology*.

Tabela 4: Classificações das enzimas celulolíticas segundo *Enzyme Commission* e a IUBMB - I*nternational Union of Biochemistry and Molecular Biology*.

Celulase	Classificação	IUBMB
Endoglucanases	EC 3.2.1.4	1,4-ß-D-glucana-4-glucanoidrolases
Exoglucanases	EC 3.2.1.91	1,4-ß-D-glucana-4-glucanoidrolases
β-glucosidase	EC 3.2.1.21	ß-glicosídeo glucohidrolases

Individualmente, as enzimas do complexo celulásico não hidrolisam a celulose de maneira eficiente, sendo necessária uma ação complementar e sinérgica, ou seja, uma ação em conjunto para que o rendimento das celulases quando atuam simultaneamente seja melhor do que a soma dos rendimentos individuais (SOCCOL et al., 2010). A Figura 4 é uma representação simplificada da ação enzimática de cada classe de enzimas, tendo como resultado a ação sinérgica da degradação da celulose à glicose. As endo-1,4-β-glucanases ou 1,4-β-D-glucana-4-glucano-hidrolases (EC 3.2.1.4) atuam randomicamente nas regiões

amorfas da celulose e de seus derivados, hidrolisando ligações glicosídicas β-(1,4). Sua atividade catalítica pode ser medida através da diminuição da viscosidade do meio decorrente da diminuição de massa molar média de celulose ou derivados de celulose. As celobio-hidrolases (exo-1,4-β-D-glucanases, EC 3.2.1.91) atuam nos terminais redutores das cadeias de celulose, liberando D-celobiose, que pode ser detectada pelas técnicas de Cromatografia Líquida de Alta Eficiência (CLAE) ou Cromatografia Gasosa (CG). As "β-D-glucosidases" ou β-D-glucoside gluco-hidrolases (EC 3.2.1.21) catalisam a liberação de unidades monoméricas de D-glicose a partir da celobiose e celodextrinas solúveis (OGEDA e PETRI, 2010; ARANTES & SADDLER, 2010).

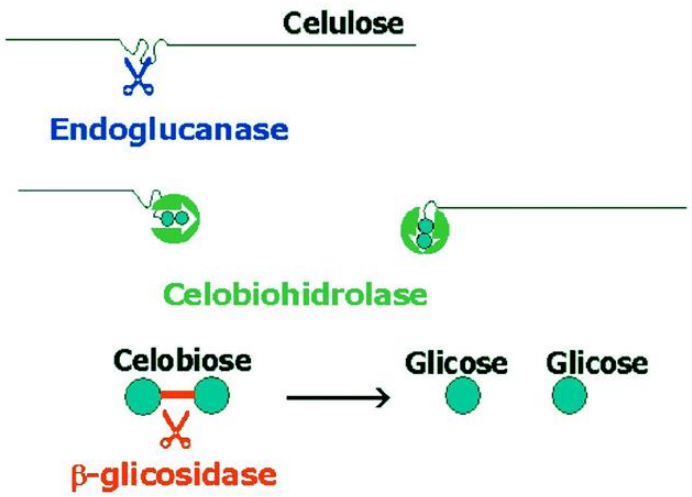

Figura 4: Representação esquemática da ação catalítica do complexo enzimático (celulase) sobre celulose com geração de glicose (OGEDA e PETRI, 2010).

2.2.2 Micro-organismos produtores

A celulose, principal componente polimérica do material vegetal, é o polissacarídeo mais abundante na Terra. Na natureza, há uma variedade de micro-organismos conhecidos para a produção de um conjunto de enzimas capazes de degradar este polímero insolúvel a açúcares solúveis, principalmente celobiose e glicose (ANDERSEN, 2007). Além de microorganismos, essas enzimas podem ser obtidas de plantas e animais, porém em quantidades muito menores, sendo os microorganismos a fonte mais utilizada (PAULO, 1995).

A capacidade de fungos filamentosos para secretar grandes quantidades de proteínas tem motivado a sua ampla utilização para a produção de enzimas industriais. No entanto, a morfologia, mecanismos de crescimento celular e formação de produto não são bem conhecidos. A fermentação fúngica é amplamente reconhecida como um processo complexo

com vários problemas, alguns dos quais estão relacionados com a diversidade de morfologias fúngicas cultivadas (DOMINGUES et al., 2000). As espécies de fungos filamentosos mais estudadas são: *Trichoderma reesei, Penicillium pinophilum, Humicola insolens, Trichoderma koningii, Penicillium funiculosum, Fusarium solani, Myrothecium verrucaria, Sporotrichum pulverulentum e Aspergillus niger* (RABELO, 2007).

Tais fungos são capazes de excretar celulases de alta atividade no meio de cultura, uma vez que seu modo de crescimento hifal (pelo alongamento das pontas e ramificações) permite a penetração parcial no material sólido e a produção de uma ampla variedade de enzimas extracelulares para degradar as macromoléculas, características que fazem estes organismos especialmente apropriados para FES. No curso de crescimento, microcolônias espalham-se radialmente sobre a superfície do substrato formando um "tapete" micelial. As hifas aeriais podem se prolongar na fase gasosa (com ramificações, na forma de árvore) e as hifas penetrativas podem entrar na matriz sólida, até certo ponto, dependendo do fornecimento de oxigênio e da disponibilidade de nutrientes, bem como da estrutura e das propriedades físicas do material sólido (HÖLKER e LENZ, 2005; LIMA, 2009).

2.2.3 Determinação da atividade enzimática

A produção de enzimas é quantificada em termos de atividade enzimática, que consiste na medida da velocidade de reação. Segundo a *Enzyme Commission*, "uma unidade (U) de atividade é a quantidade de enzima que catalisa a transformação de 1 µmol de substrato ou a formação de 1 µmol de produto por minuto", nas condições do ensaio (temperatura, pH, concentração de substrato). A atividade específica é expressa em termos de atividade por miligrama de proteína (U/mg), ou atividade por grama (U/g), como expresso na Tabela 4.

A velocidade das reações enzimáticas varia com fatores diversos como concentração de enzima ou de substrato, temperatura e pH. A caracterização da atividade das celulases apresenta algumas particularidades que não se encontram noutros tipos de enzimas. A atividade sobre celulose é medida em termos de açúcares redutores solúveis e é entendida como resultado da ação total de todo o complexo celulolítico (SHUANGQI et al., 2011).

De acordo com Paulo (1995), não existem métodos padrão para medir as atividades das celulases. Existem, no entanto, recomendações específicas da IUPAC para a medição das atividades celulolíticas sobre os seguintes substratos:

- Papel de filtro (Whatmann n° 1): possui em sua estrutura celulases amorfas e cristalinas e, portanto, são utilizadas para determinação de celulases totais;
- Carboximetilcelulose (CMC): possui estrutura amorfa, utilizada para determinação de endocelulases.
- Avicelose ou Celulose microcristalina: em decorrência da estrutura cristalina destas celuloses, são utilizadas na determinação de exocelulases.
- Celobiose: utilizada para determinação de celobiases.

Apesar da existência das recomendações da IUPAC, a maioria dos investigadores não as segue, preferindo desenvolver seus próprios métodos de determinação de atividade enzimática de celulases (PAULO, 1995).

2.2.4 Fermentação em estado sólido

As principais rotas biotecnológicas possíveis para a produção das celulases utilizando microorganismos se referem ao emprego de fermentação em estado sólido ou fermentação submersa. A fermentação em estado sólido é um processo cujo crescimento microbiano ocorre na ausência de água livre, sendo mais indicado para o cultivo de fungos e leveduras que bactérias. O material sólido pode atuar como suporte inerte ao crescimento do microrganismo ou como suporte e fonte de carbono, sendo o último mais utilizado na produção de enzimas. Os substratos usados em fermentação em estado sólido são, na grande maioria das vezes, compostos por resíduos agroindustriais, como bagaço de cana, casca de arroz, farelo de trigo, farelo de soja, entre outros (PARIS, 2008; SCHMIDELL e FACCIOTTI, 2001; RAHARDJO et al., 2006).

A fermentação em estado sólido (FES) tem se mostrado como uma alternativa na produção de enzimas microbianas, pois apresenta uma série de vantagens sobre a fermentação submersa, tais como: possibilidade de adição de nutrientes suplementares ao substrato sólido; o volume de meio reacional é reduzido, implicando em um menor investimento capital em biorreatores; os esporos dos microorganismos podem ser utilizados diretamente na inoculação, evitando etapas prévias como pré-cultivos que envolvem grandes volumes de meio e tanques para seu desenvolvimento; o crescimento dos microrganismos ocorre em condições similares às do seu habitat natural; o fato do meio de cultivo apresentar baixa atividade de água reduz o problema de contaminações, especialmente por bactérias; a aeração do meio é facilitada devido ao maior espaço entre as partículas e pela difusão do oxigênio, o

qual é saturado em água para ajudar na manutenção da umidade do meio; altos rendimentos quanto à formação de metabólitos, e simplicidade nas etapas de purificação, pois os produtos estarão concentrados no líquido da extração (FERNÁNDEZ-FERNÁNDEZ & PÉREZ-CORREA, 2007; PANDEY, 2003; CUNHA, 2009).

Apesar das vantagens apresentadas, a fermentação no estado sólido ainda não é amplamente aplicada na indústria, principalmente devido à dificuldade de manutenção das condições ideais de cultivo, ocasionada pela falta de ferramentas para desenvolver e otimizar a operação de biorreatores industriais (MITCHELL et al., 2006). Em contrapartida, a fermentação submersa vem sendo amplamente estudada e sua tecnologia encontra-se bastante desenvolvida, já que é aplicada desde o final da década de 40 devido à necessidade de produção de antibióticos (HÖLKER e LENZ, 2005).

Segundo Lima (2009), embora existam dificuldades na aplicação industrial, existem alguns processos comerciais de sucesso para a fermentação no estado sólido. Entre eles estão: a produção de *koji* para molho de soja, alimentos fermentados tradicionais do oriente, ácido cítrico, ácido glucônico e enzimas (celulase, amilase, pectinase e lipase) (MITCHELL et al., 1999). Destes processos comerciais, a tradicional indústria de *koji* para produção de molho de soja parece ter desenvolvido grande conhecimento para a utilização de biorreatores em escala industrial, porém são poucos os dados disponíveis na literatura (MITCHELL et al., 2006). Algumas poucas informações podem ser encontradas em patentes (WILLIAMS, 1980 e IZUMI, 1977).

Em um trabalho prévio realizado pelo grupo (GASPAROTTO et al., 2012), fez-se uma busca de patentes na base brasileira (INPI), relacionadas à FES, e constatou-se que das dezesseis patentes encontradas, apenas duas eram sobre configurações de biorreatores para FES. Dessa forma, percebe-se que o Brasil ainda é emergente no desenvolvimento dos processos em fermentação em estado sólido e, embora existam algumas patentes nesse sentido, ainda não há processo industrial utilizando FES no país.

Apesar de a produção de enzimas por FES não estar bem difundida industrialmente, as pesquisas para produção de celulases a partir de substratos residuais lignocelulósicos, em escala de bancada, são inúmeras, utilizando diferentes tipos de microorganismos. Alguns exemplos de trabalhos, bem como os resultados obtidos em termos de atividade enzimática, estão dispostos na Tabela 5.

Tabela 5: Estudos reportando a produção de celulases por fermentação em estado sólido a partir de resíduos lignocelulósicos nos últimos anos

Matéria prima	Microorganismo	Tempo de ferm. (h)	Atividade máx. (U/g)*	Tipo de biorreator	Ref.
Palha de arroz e farelo de trigo (3:2)	Culturas mistas de *Aspergillus niger* e *Trichoderma reesei*	96 (FPase, beta-glucosidase), 120 (CMCase, xylanase); 30	35,8[1]; 33,71[3]; 131,34[2]; 3106,34[4]	Fermentação em bandeja	Dhillon et al., 2011
Palha de milho	*Aspergillus fumigatus* Z5	96; 30	526,3[2]; 144,6[1]	Erlenmeyer	Liu et al., 2011
Sabugo de milho, bagaço de cana	*Aspergillus niger* MS82	NA ; 35	50,32[2]; 25,16[3]	Erlenmeyer	Sohail et al., 2009.
Farelo de trigo e casca de arroz (pré-tratados em micro-ondas (450W)	*Trichoderma* sp. 3.2942	48 e 36; 110	7,69[1]; 13,91[2]	Béquer de 500 mL	Zhao et al., 2010
Farelo de trigo, bagaço de cana, farelo de soja e casca de laranja	*Aspergillus fumigatus*	120; 65	160,1[2]; 5,0[1]	Tubos	Delabona et al., 2012
Fibras da fruta de Palma	*Penicillium verruculosum* COKE4E	144; 30	6,5[2]	Erlenmeyer	Kim e Kim, 2012
Bagaço de cana	*Aspergillus niger*	24; 32	1,052 U/mL[2]	Erlenmeyer	Cunha et al., 2012
Farelo de trigo	*Aspergillus niger* NS-2	240	310[2]; 17[1]; 33[3]	Erlenmeyer	Bansal et al., 2012
Resíduo da produção de óleo de palma	*Aspergillus fumigatus* SK1	288	54,27[2]; 3,36[1]; 4,54[3]; 418,70[4]	Erlenmeyer	Ang et al., 2013

* 1: FPase; 2: CMCase; 3: β-glucosidase; 4: xilanase.

2.3 Hidrólise enzimática de material lignocelulósicos

Uma das mais emergentes aplicações das enzimas do complexo celulolítico atualmente é a hidrólise de biomassas. As matérias-primas de origem lignocelulósica, conforme apresentado na Tabela 1, contêm de 20 a 60% de celulose, que pode ser totalmente convertida

em glicose, por ação enzimática (CASTRO & PEREIRA Jr., 2010; CASTRO & CASTRO, 2012). Devido à natureza polissacarídica, os materiais lignocelulósicos não são diretamente utilizados pelos micro-organismos produtores de substâncias de interesse industrial, tendo a necessidade de proceder-se a hidrólise dos seus componentes (BORTOLAZZO, 2011).

Apesar de a sacarificação da celulose representar uma alternativa interessante para a destinação de biomassas residuais como o bagaço de cana e resíduos de atividades florestais, as características do substrato que atribuem maior eficiência à taxa de hidrólise da celulose ainda não estão completamente compreendidas. Algumas das características mais influentes incluem a acessibilidade, o grau de cristalinidade, o grau de polimerização e a distribuição da lignina (PALONEN et al., 2004). Além disso, bioprocessos baseados na hidrólise enzimática requererão substratos produzidos com a qualidade adequada, a partir de biomassa residual.

A sacarificação da celulose pode ser conduzida de duas formas principais: por tratamento químico (utilizando-se ácido diluído ou concentrado) ou por rota enzimática, utilizando-se as celulases. A utilização de celulases na hidrólise da celulose ocorre em condições mais brandas de pressão, temperatura e pH do que os processos químicos, e exibe elevada especificidade, eliminando a chance de ocorrência de substâncias tóxicas (furfurais e derivados de lignina) às células microbianas que serão utilizadas para fermentação do meio hidrolisado. Na rota enzimática, embora o custo de produção dos biocatalisadores ainda seja alto, são detectados pontos de economia no processo, tanto do ponto de vista energético, como metalúrgico, visto que os equipamentos podem ser confeccionados com materiais menos nobres (CASTRO e PEREIRA Jr, 2010).

É notória a imprescindibilidade de pré-tratamento aos materiais utilizados para os processos de hidrólise. A adsorção das celulases em substratos celulósicos insolúveis é facilitada quando estes são submetidos previamente a tratamentos, sejam eles químicos ou termopressurizados, que promovem, pela remoção de frações indesejáveis nesse processo (lignina e hemicelulose), um aumento na área superficial exposta à ação enzimática e a redução da cristalinidade deste glicopolissacarídeo. Um recurso para o aumento da eficiência de hidrólise que vem sendo reportado é a adição de surfactantes ao meio reacional, cujo intuito é promover maior adsorção das celulases ao polímero celulósico (MOSIER et al., 2005; CASTRO e PEREIRA Jr, 2010). Paralelamente, estudos têm sido realizados na busca de enzimas capazes de hidrolisar a celulose de maneira cada vez mais efetiva, seja pela otimização de processos fermentativos, pela combinação de enzimas para a obtenção de complexos celulolíticos mais eficientes ou pelo melhoramento de espécies através de métodos de engenharia genética (JORGENSEN et al., 2004; KANG et al., 2004).

A hidrólise enzimática da celulose é realizada pelas enzimas celulases e este processo tem como produtos açúcares redutores, incluindo a glicose. Durante a reação enzimática, podem-se ressaltar os seguintes fatores em se tratando do comportamento das enzimas (RAMOS, 2000; PITARELO, 2007; RABELO, 2007):

a. Inibição retroativa das celulases, devida ao acúmulo do produto final de hidrólise no meio de reação (glicose e celobiose);
b. Inativação das enzimas pelo efeito prolongado da temperatura e agitação;
c. Adsorção inespecífica e/ou não produtiva de um ou mais componentes enzimáticos sobre complexos lignina-carboidrato (hemicelulose e lignina);
d. Concentração de enzima;
e. Tempo de duração da hidrólise;
f. pH do meio.

Em relação à estrutura da celulose, sua parte cristalina é altamente resistente ao ataque enzimático, pois a maioria das ligações glicosídicas na microfibrila é inacessível às enzimas, e todas as ligações clivadas pela ação das endoglucanases podem prontamente ser reformadas devido à orientação estável das ligações glicosídicas. Consequentemente, a degradação da celulose cristalina requer a ação sinérgica das endoglucanases e exoglucanases. O máximo sinergismo é obtido com uma elevada quantidade de exoglucanases na mistura e depende do tipo de substrato usado. Já as exoglucanases removem rapidamente as unidades de celobiose das extremidades recentemente criadas pela ação das endoglucanases, impedindo assim a reformação das ligações glicosídicas. As duas enzimas podem agir consecutivamente ou em harmonia. As exo e endoglucanases são inibidas pela celobiose, e a ação da ß-glucosidase é, portanto, considerada como a etapa limitante na degradação da celulose (RABELO, 2007).

O efeito do complexo da enzima celulase é expresso pela ação sinérgica destas três enzimas diferentes na celulose e este sistema complexo de enzimas necessita ser mantido estável para que a atividade celulolítica se mantenha elevada. Em consequência da ação dos primeiros dois grupos de enzimas (endoglucanases e exoglucanases) na celulose, a celobiose e a glicose são obtidas, e enquanto sua concentração no meio reacional aumenta gradualmente, as atividades das celulases respectivas são inibidas por estes produtos, tendo por resultado uma diminuição final na taxa e no rendimento do processo de sacarificação. A celobiose apresenta um poder de inibição maior no complexo celulolítico sendo mais expressivo que a inibição por glicose (AGUIAR, 2010).

A atividade catalítica das enzimas é altamente dependente da temperatura, como no caso dos catalisadores convencionais, porém, à medida que se eleva a temperatura dois efeitos

ocorrem simultaneamente: (a) a taxa de reação aumenta, como se observa na maioria das reações químicas; e (b) a estabilidade da proteína decresce devido à desativação térmica. O aumento da atividade é chamado de ativação pela temperatura e a redução é chamada de inativação pela temperatura ou desnaturação térmica. Toda enzima tem uma temperatura ótima para que atinja sua atividade máxima, ou seja, é a temperatura máxima na qual a enzima possui uma atividade constante por um período de tempo (FURIGO Jr., 2001; SHULER, 1992).

Outro fator que afeta positivamente a hidrólise enzimática da celulose, por via de regra, é a concentração enzimática, que aumenta a taxa de hidrólise até uma concentração limite, a partir da qual não é compensatório continuar adicionando enzimas ao processo, pois os sítios da matriz do substrato já se encontram saturados pelos biocatalisadores (CASTRO e PEREIRA Jr, 2010).

Tabela 6: Condições de aplicação de celulases na hidrólise de materiais lignocelulósicos

Fonte de celulases	**Temperatura (°C); pH**	**Carga enzimática**	**Fonte celulásica**	**Teor de produto (g/L); Tempo de processo (h)**
C-1184®,8 de *A. niger*	60; 4,8	100 µg/g $_{biomassa}$	Casca de arroz	1,2[2]; 5
Celuclast®,4 Novozymes 188®,4	50; 4,8	40 FPU/g $_{celulose}$	Madeira Conífera	70[1]; 30
Celuclast®,4 Novozymes 188®,4	45; 4,8	80 FPU/g $_{celulose}$	Madeira Douglas fir	1,5[1]; 72
Rapidase Pomaliq®,10	50; 5,0	26 U ExG/g $_{biomassa}$	Sabugo de milho	110[1]; 48
Preparados de *T. viride* e *A. niger*	50; 4,8	28 U ExG/g $_{biomassa}$	Bagaço de cana	20[2]; 72
Preparado da Novozymes	50; 5,3	322 FPU/g $_{biomassa}$	Palha de trigo	17[2]; 7
Celuclast 1.5L®,4 Novozymes 188®,4	40; 4,8	15 FPU/g $_{biomassa}$ 20 U BG/g $_{biomassa}$	Bagaço de cana	17[1]; 7
Preparados de *T. viride* e *A. niger*	50; 4,8	41600 U/g $_{celulose}$	CMC	0,36[1]; 3

BG: β-glucosidase; ExG: exoglucanases; FPU: unidades de atividade em papel filtro; CMC: carboximetilcelulose

[1]Concentração de produto expressa como glicose; [2]Concentração de produto expressa como açúcares redutores totais;

Fonte: Adaptado de CASTRO e PEREIRA Jr., 2010.

A Tabela 6 apresenta algumas condições de aplicação de celulases, comerciais ou não, na hidrólise enzimática de diferentes matérias primas de origem lignocelulósica, bem como o teor de produto obtido após a reação de sacarificação. É notória a preferência pela utilização de enzimas comerciais nos experimentos de hidrólise mostrados na tabela acima, e isso ocorre principalmente pela dificuldade de produção dos complexos celulósicos e caracterização dos mesmos, sendo assim mais fácil comparar resultados utilizando um mesmo complexo enzimático comercial. É também possível verificar que a carga enzimática utilizada é bastante variada, sendo utilizada em termos de atividade de diferentes enzimas em relação à quantidade de biomassa ou de celulose total, tornando extremamente difícil a comparação entre os resultados obtidos, sendo somente possível uma comparação direta entre aqueles trabalhos cuja unidade de carga enzimática, bem como a metodologia de determinação de atividade enzimática utilizada é exatamente a mesma. Além disso, o teor de produto é apresentado nessa tabela em duas unidades distintas, concentração de glicose e concentração de açúcares redutores totais, sendo possível a comparação dos valores para aqueles experimentos com a mesma unidade de medida. Por exemplo, para os dois trabalhos utilizando as enzimas Celuclast®,4 Novozymes 188®,4, observa-se que a formação de produto foi muito maior utilizando Madeira conífera como fonte celulásica e, mesmo utilizando a metade da carga enzimática (40 FPU/g $_{celulose}$) utilizada para madeira Dougras fir, a produção de glicose foi mais de 45 vezes maior (70 g/L), nas condições de processo utilizadas.

2.4 Utilização da tecnologia de ultrassom na hidrólise enzimática

A sonoquímica é um campo da ciência que estuda a aplicação de ondas sônicas e ultrassônicas em processos químicos. Essas ondas provocam o aumento da velocidade das reações químicas e da transferência de massa, reduzindo, assim, a quantidade de reagentes e tornando as condições reacionais menos drásticas (ADEWUYI, 2001). Na busca de novas tecnologias para a produção de biocombustíveis renováveis, muitos autores têm avaliado o efeito da sonoquímica em etapas de pré-tratamento de biomassa (HROMADKOVA et al., 1999; LIU et al., 2006; MA et al., 2009; SCHUCHARDT e GONÇALVES, 2002; SUN e TOMKINSON, 2002) e também na etapa de hidrólise enzimática (LI et al., 2004; LI et al., 2005; IMAI et al., 2004; LEÃES et al., 2013; SFALCIN et al., 2012).

O princípio da sonoquímica se baseia no fenômeno da cavitação, isto é, a energia transmitida pela onda sonora é absorvida pelo líquido formando microcavidades que, num pequeno intervalo de tempo, colapsam-se liberando enormes quantidades de energia ao meio

reacional. A temperatura e pressão dessas microcavidades podem alcançar 5000 K e 1200 bar, respectivamente. Sob essas condições extremas, as moléculas mais voláteis se vaporizam e sofrem degradação pela temperatura, gerando radicais livres (KARDOS e LUCHE, 2001). A água, sob radiação ultrassônica, se desassocia a radicais hidroxilas e átomos de hidrogênio. Além do efeito de lise das moléculas, o colapso das microcavidades formadas durante a radiação gera também forças mecânicas, as quais são capazes de romper, homoliticamente ou heteroliticamente, macromoléculas de forma não aleatória. A cavitação sofre grande influência pelas propriedades físico-químicas do solvente, soluto ou gases (ADEWUYI, 2001). As cavidades são mais rapidamente formadas em solventes com alta pressão de vapor e baixa viscosidade e pressão superficial. Entretanto, a intensidade da cavitação é favorecida em solvente com características opostas (baixa pressão de vapor, alta viscosidade e tensão superficial) (AVVARU et al., 2006), já que as forças intermoleculares do líquido devem ser superadas para que as bolhas sejam formadas. Então, em solventes com alta densidade, tensão superficial e viscosidade geralmente as bolhas necessitam de grande quantidade de energia para serem formadas, provocando, assim, condições severas de cavitação (YOUNG, 1989).

Como pré-tratamento, o ultrassom normalmente é usado na indústria para limpeza de materiais, solda de plásticos, processos químicos, preparação de emulsão e suspensão, desgaseificação de solventes, entre outros. O ultrassom é um método físico que reduz o grau de cristalinidade, diminui o grau de polimerização, aumenta a fração solúvel da fibrila de celulose e também ajuda a reduzir o tamanho das partículas, podendo ser usado diferentes potências (MIGUEL, 2009).

São poucos os trabalhos que utilizam a tecnologia do ultrassom para auxiliar nas reações de hidrólise enzimática. Vargas et al. (2004) demonstraram que é possível potencializar o processo de inversão de carboidratos por ultrassom e Sun et al. (2004) conseguiram a solubilização de até 91% da lignina e de 92% da hemicelulose de bagaço de cana usando solução alcalina e peróxido de hidrogênio sob impacto de ondas ultrassônicas. Nos últimos anos, porém, a aplicação de ultrassom na potencialização de reações enzimáticas passou a ser mais estudada, conforme pode ser observado na Tabela 7.

O estudo desenvolvido por Leães e colaboradores (2013), avaliou os efeitos da irradiação de ultrassom na atividade de amilases. Constatou-se que a irradiação de ultrassom alterou o comportamento das enzimas, uma vez que as mesmas responderam de maneira diferente às alterações na temperatura e no pH, quando expostas ou não à sonicação. Para temperaturas maiores que 50°C, as atividades enzimáticas foram sempre mais altas, e a energia de ativação sofreu considerável declínio, nas hidrólises assistidas por ultrassom. Já o

estudo desenvolvido por Silva e colaboradores (2013) obteve um aumento da atividade enzimática com complexo celulásico em até 381% utilizando gás liquefeito de petróleo comprimido combinado com ultrassom durante a hidrólise.

Tabela 7: Estudos avaliando o efeito do ultrassom na atividade enzimática de diferentes enzimas em diferentes matérias primas

Matéria prima	**Enzimas**	**Efeito observado**	**Referência**
Amido de batata solúvel	Amilase comercial (STARGEN™ 002)	Atividades até 3 vezes maior em presença de ultrassom para T>40°C e redução da energia de ativação.	SOUZA et al., 2013.
Amido de batata solúvel	Alfa-amilase (Liquozymes SC DS) e amiloglucosidase (Spirizymes Fuel)	Para T>50 °C, a atividade enzimática foi sempre maior na presença de ultrassom, e a energia de ativação sempre menor.	LEÃES et al., 2013.
Material lignocelulósico	Complexo celulolítico de *Trichoderma reesei* (NS50013)	Aumento da atividade enzimática em até 381% utilizando GLP* comprimido combinado com ultrassom.	SILVA et al. 2013.
Carboximetil celulose (CMC)	Celulases comerciais (Novozymes)	Aumento de 18,17% na atividade de cellulases livres com tratamento em ultrassom de baixa intensidade: 15 W, 24 kHz por 10 min	WANG et al. 2012.
Carboximetil celulose (CMC); Papel Filtro Whatmann No. 1	Celulases comerciais	Atividade aumentada em 18,7% quando a solução de CMC em concentração de 1.68 mg/mL foi exposta a sonicação por 80 segundos com 6 W/mL de intensidade, e de 39.4% para papel filtro.	NGUYEN e LE, 2013.
Dextrana	Dextranase produzida por *Chaetomium erraticum*	Aumento da atividade em 13,43% quando tradada com ultrassom a 25 kHz, 40 W por 15 min.	BASHARIA et al. 2013.
Alho	alinases	Nas condições de 0,5 W/cm² de intensidade e 40 kHz de frequência, a atividade da alinase aumentou em 47.1%.	WANG et al., 2011.

*GLP = gás liquefeito de petróleo

Dessa forma, observa-se que o uso do ultrassom em reações no laboratório tem se tornado comum e, a extensão da tecnologia para reações em escala industrial, virá atrelada aos

recentes avanços no entendimento da natureza da cavitação e os efeitos químicos do ultrassom (MIGUEL, 2009).

2.5 Conclusão acerca do estado da arte

A produção de enzimas celulolíticas, como se pode perceber na revisão da literatura, vem sendo muito estudada, não só na exploração de novos microorganismos produtores, mas também na variedade de substratos que podem ser utilizados em fermentação em estado sólido, principalmente em escala laboratorial. Quando industrializadas, estas costumam passar por um processo de purificação, onde ocorrerá a separação dos diversos tipos de celulases produzidos, para que estas sejam aplicadas nos mais diferentes processos, incluindo a hidrólise enzimática de materiais lignocelulósicos.

Para que a hidrólise da biomassa seja mais eficiente, porém, é necessário que se tenha a ação sinérgica das endocelulases, exocelulases e celobiases do complexo celulásico, bem como enzimas acessórias, como as xilanases. Dessa forma, o desenvolvimento de um processo que produza estas enzimas nas devidas proporções para serem aplicadas diretamente na hidrólise enzimática, sem necessidade da etapa de purificação parece bastante promissor. Nesse sentido, um dos diferenciais desse trabalho é a realização da produção de celulases e hidrólise *in situ*, ou seja, a produção da enzima seguida de extração do extrato enzimático e imediata hidrólise, utilizando o mesmo substrato já fermentado.

O uso do ultrassom para inativação de enzimas já é bastante comum no processamento de alimentos, assim como vem sendo bastante estudada a sua utilização em etapas de pré-tratamento de matéria prima lignocelulósica. Porém, seu uso na melhoria da atividade enzimática em reações ainda é pouco difundido. Existem alguns trabalhos, publicados principalmente nos últimos dois anos, avaliando o efeito da sonicação sobre a atividade enzimática de enzimas como amilases, dextranase, xilanases, alinases e até mesmo celulases (SOUZA et al., 2013; LEÃES, et al., 2013; BASHARIA et al., 2013; SILVA et al. 2014; WANG et al., 2012; WANG et al., 2013; NGUYEN e LE, 2013; SILVA et al., 2013; SZABÓ e CSISZÁR, 2013). A maioria deles, todavia, utiliza em seus testes de hidrólise matérias primas puras ou pré-tratadas. Com isso, esse trabalho apresenta como segundo diferencial a avaliação dos efeitos do ultrassom de baixa intensidade na hidrólise enzimática do bagaço de cana sem pré-tratamento, utilizando o extrato enzimático bruto, produzido em condições de processo de FES otimizadas, utilizando o microorganismo *Trichoderma reesei*.

Além disso, compararam-se os efeitos da sonicação direta e indireta na hidrólise enzimática do bagaço de cana, utilizando uma sonda ultrassônica acoplada direta ou

indiretamente ao meio reacional, sendo este um estudo inédito em se tratando de hidrólise enzimática de material lignocelulósico.

3 METODOLOGIA EXPERIMENTAL

A parte experimental desse trabalho é extensa e os resultados serão apresentados nos próximos dois capítulos na forma de artigos submetidos para publicação em revistas internacionais. Para facilitar o entendimento do trabalho como um todo, esse capítulo apresenta os fluxogramas dos processos que serão detalhados no decorrer dos dois artigos a seguir. A Figura 5 apresenta de maneira simplificada, as etapas do desenvolvimento todo o processo experimental desenvolvido durante esse trabalho.

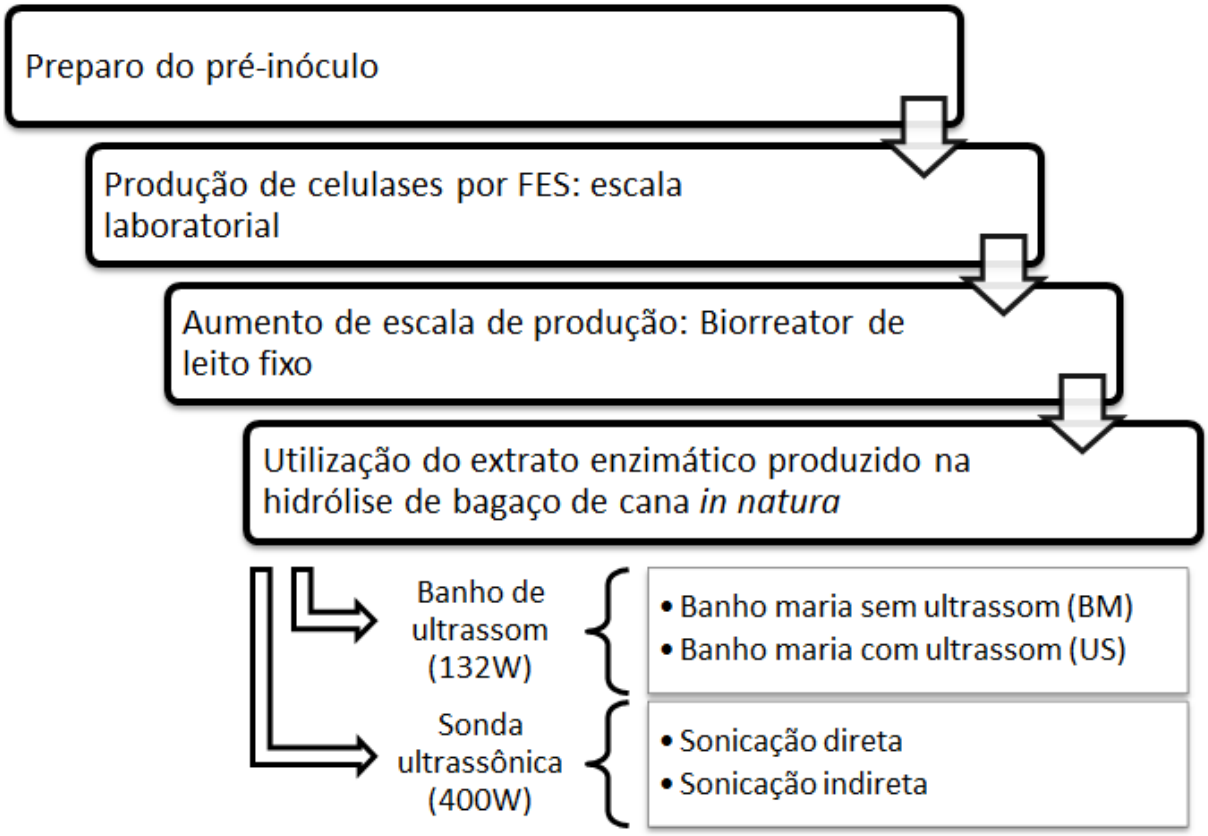

Figura 5: Fluxograma geral do processo de produção de celulases e sua aplicação na hidrólise enzimática de bagaço de cana.

Já a Figura 6 apresenta um fluxograma detalhado do processo, mostrando as etapas experimentais desenvolvidas nos Artigos 1 e 2, detalhando algumas condições de processo, planejamentos experimentais utilizados e as respostas analisadas em cada etapa. Nessa figura, as atividades foram divididas em três grandes etapas: preparo do pré inóculo, produção de celulases em escala de bancada, e produção e aplicação na hidrólise de bagaço de cana.

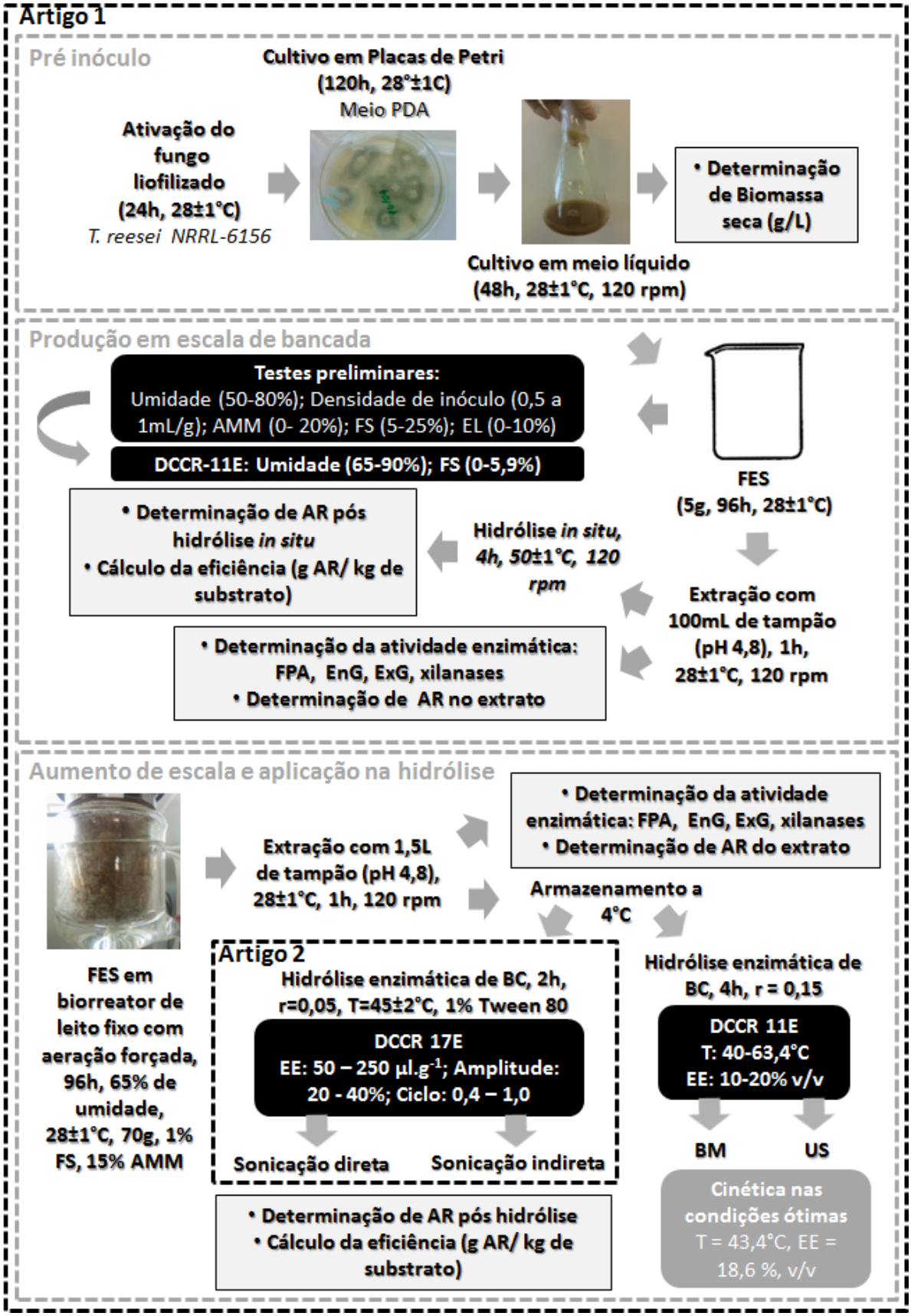

Figura 6: Fluxograma detalhado do processo de produção de celulases e aplicação na hidrólise enzimática de bagaço de cana.
*Todas as siglas apresentadas na figura estão especificadas na lista de abreviaturas e siglas no início do trabalho.

4 ARTIGO 1: Production of cellulolytic enzymes and application of crude enzymatic extract for saccharification of lignocellulosic biomass

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Abstract

In this study the production of cellulolytic enzymes by *Trichoderma reesei* NRRL-6153 in the solid state fermentation was optimized. The experiments were assessed in conical flasks and validated in a packed-bed bioreactor. Afterwards, the crude enzymatic extract obtained in the optimized condition was used for hydrolysis of sugarcane bagasse in water and ultrasound baths. The optimized conditions for cellulases production in bench scale were 28°C, moisture content 68.6 wt.% and soybean bran concentration 0.9 wt.%, and it resulted in a production of 1.42, 3.13, 170.73 and 2.09 U/g of filter paper, exo-cellulases, xylanases and endo-cellulases activities, respectively. After the process scale up, the obtained enzymatic extract had 4.18, 7.34, 1734.82 and 2.50 U/g of the same enzymes. The crude enzymatic extract obtained in the bioreactor was applied for hydrolysis of sugarcane bagasse in water and ultrasound baths, being obtained 224.0 and 229 $g.kg^{-1}$, respectively at temperature of 43.4°C and concentration of enzymatic extract of 18.6%. The obtained yields are comparable to commercial enzymes. These results demonstrated that the cellulolytic enzymes from *T. reesei* can be a good alternative for the lignocellulosic material saccharification.

Key-words**:** Cellulytic enzymes; solid-state fermentation; ultrasound-assisted hydrolysis; crude enzymatic extract.

1 Introduction

The conversion of lignocellulosic biomass into fuels and other chemicals can be achieved using a multi-enzyme system acting in synergy in order to hydrolyze biomass to glucose. It is well established that hydrolytic efficiency is a result of the synergistic actions of a multicomponent enzymatic system containing at least three major groups of enzymes: endoglucanases (E.C. 3.2.1.4), which hydrolyze the cellulose polymer internally, exposing reducing and non-reducing ends, exoglucanases or cellobiohydrolases (E.C. 3.2.1.91), which act on the reducing and non-reducing ends, releasing cellobiose and cellooligosaccharides, and β–glucosidases (E.C. 3.2.1.21), which cleaves cellobiose, liberating two molecules of glucose – the end product (Maeda et al., 2011; Delabona et al., 2013; Gottschalk, 2010). Enzymatic hydrolysis of cellulosic biomass depends on many factors: physical properties of the substrate (composition, crystallinity, degree of polymerization, etc.), enzyme synergy (origin, composition, etc.), mass transfer (substrate adsorption, bulk and pore diffusion, etc.), and intrinsic kinetics (Zhang & Lynd, 2004).

Several industrial and agricultural byproducts such as sugarcane bagasse, rice straw, bermudagrass, soft wood and water hyacinth are proposed as potential sources for the production of bioethanol. Among these, sugarcane bagasse (SCB) is abundantly available and is also rich in polysaccharides. Brazilian sugar and alcohol industries generate 195 million tons of sugarcane bagasse per year, and these have been burnt in mills inefficiently for energy cogeneration and as a way to reduce the bagasse disposal problem. Despite this, a 12% surplus remains unexploited and could be used as a raw material for the production of lignocellulosic ethanol, thereby increasing fuel production per planted area (Ferreira-Leitão et al., 2010; Bon et al., 2008; Gottschalk et al., 2010).

The bottleneck in the cellulosic bioethanol production is the high prices of commercial cellulases in the market, which can achieve around 50% of the production cost. This way, the

development of new technological routes and more efficient equipment for cellulases production is crucial to ride out this problem and make the production of lignocellulosic ethanol, also known as second generation ethanol, economically viable (Rocky-Salimi and Hamidi-Esfahani, 2010).

Cellulose production by solid state fermentation (SSF) can be an alternative to overcome this problem, since it has several advantages in comparison to submerged fermentation (SmF), such as higher production yield, lower residue generation, and energy savings in fermentation process and enzyme purification. Moreover, agroindustrial residues can be used as raw material in SSF, and it is possible to use the crude enzyme extract to hydrolyze cellulosic material, reducing significantly the production process expenses (Latifian et al., 2007). Cellulases can be produced and secreted by filamentous fungi and other microorganisms. The most common enzymes used in the hydrolysis of lignocellulosic biomasses are the cellulolytic complex produced by *Trichoderma reesei*. This microorganism usually produces large quantities of exo- and endoglucosidases but little β-glucosidase activity (Maeda et. al, 2011).

Saccharification breaks the hydrogen bonds present in cellulose and hemicellulose fractions and produces sugars such as hexoses and pentoses, respectively. Enzymatic hydrolysis is more specific but not a cost-effective method because of the high cost associated with the isolation of pure enzymes (Velmurugan and Muthukumar, 2012). Moreover, the production of cellulases by solid state fermentation of lignocellulosic residues and subsequent application of the crude enzyme extract in cellulose hydrolysis can be an alternative to become the saccharification process less expensive. Also, the production of enzymes using the same lignocellulosic material that will be used in the hydrolysis as carbon source has shown that these enzyme preparations can present better performance (Delabona et al., 2012; Jorgensen and Olsson, 2006).

Several authors have shown that saccharification of cellulose is enhanced efficiently by ultrasonic pretreatment. More recently, the effect of ultrasound on lignocellulosic hydrolysis efficiency have been studied. The effect of ultrasound can be based on a direct interaction with molecular species as well as on the cavitation phenomenon. Ultrasound has a direct effect on the enzyme molecules and enhances the mass transfer in the heterogeneous processes by the local turbulences created by acoustic cavitation. Furthermore, mechanical impact produced by the collapse of cavitation bubbles, provide an important benefit of opening up the surface of solid substrates to the action of enzymes (Gogate and Kabadi, 2009; Kwiatkowska et al, 2011; Szabó and Csiszár, 2013).

In this study, the optimal conditions for production of cellulolytic enzymes by *Trichoderma reesei* NRRL-6153 using the solid state fermentation were assessed in conical flasks and validated in a packed-bed bioreactor. Afterwards, the crude enzymatic extract obtained at the optimized experimental condition was used for hydrolysis of sugarcane bagasse in water and ultrasound baths.

2 Material and Methods

2.1. Microorganism, culture medium and solid substrates

2.1.1 Microorganism

Trichoderma reesei NRRL - 6156 was obtained from Agricultural Research Service of the United States Department of Agriculture.

2.1.2 Pre inoculum culture medium

Stock cultures of *T. reesei* were propagated on potato dextrose agar (PDA) using Petri plates at 28 °C for five days. For pre inoculum preparation, the conidia from sporulating plates cultures were suspended in 5 mL of sterile water, and 5 mL of the suspension was transferred to a 250 mL Erlenmeyer flask containing 50 mL of the growth medium, which

was incubated in an orbital shaker (Tecnal, TE-240, Brazil) for 2 days at 28°C and 120 rpm. The medium for pre inoculum was composed by ($g.L^{-1}$): 2.0 $(NH_4)_2SO_4$, 0.5 $MgSO_4$, 1.0 $FeSO_4.7H_2O$, 1.0 $MnSO_4.7H_2O$, 10.0 yeast extract, 10.0 glucose, 150.0 corn steep liquor and 1.0 $mL.L^{-1}$ of Tween 80. All experimental conditions were set based on a literature research and preliminary tests.

2.1.3 Solid substrate

Sugarcane bagasse was obtained in a microdistillery located within the Federal University of Santa Maria. In the laboratory, it was dried at 60 °C during 24 hours, grounded in a cutting mill and sieved with final particle size of 8 mesh. The solid substrate for enzyme production was composed by sugarcane bagasse as main carbon source, supplemented with soybean bran and corn steep liquor.

2.2. Production of cellulolytic enzymes and their assays

In the first step of the work, fermentations were carried out in conical flasks (500 mL) containing 5 g of solid substrate. Afterwards, the solid substrate was supplemented and the moisture content adjusted at specified level. Each flask was covered with hydrophobic cotton and autoclaved at 121°C for 20 min. Preliminary studies showed that no changes in moisture content of the substrate after autoclaving were detected. After cooling, each flask was inoculated as defined above and incubated for 96 h in a chamber with temperature and humidity control.

Firstly, preliminary tests were made for screening the important variables in the fungal enzyme production by solid state fermentation. The evaluated variables were moisture content (50 to 80%), inoculums concentration (0.5 to 1 mL per gram of substrate), corn steep liquor (0

to 20% v/w), yeast extract (0 to 10 wt.%) and soybean bran (5 to 25 wt.%) concentrations. The response evaluated in these preliminary tests was the filter paper unity ($FPU.g^{-1}$).

Based on the analysis of results, a central composite rotational design (CCRD) for two independent variables was conceived to investigate the influence of moisture content (65 to 90%) and soybean bran concentration (0 to 5.9 wt.%) on cellulolytic enzymes production. The cellulolytic enzymes considered in this work were filter paper activity ($FPU.g^{-1}$), exocelulases activity ($U.g^{-1}$), endocelulases activity ($U.g^{-1}$) and xylanase activity ($U.g^{-1}$). Additionally to enzyme activities, the yield of fermentable sugar for *in situ* hydrolysis was also determined, as detailed in the next section. The optimized condition obtained in the CCRD was used to evaluate the production of the cellulolytic enzymes on a fixed-bed bioreactor with forced aeration using 70 g of dry substrate.

2.3 Extraction of the cellulolytic enzymes and assays

At the end of each fermentation, the cellulolytic enzymes were extracted using 100 mL of 50 mM sodium acetate/acetic acid buffer (pH 4.8) in an orbital shaker at 120 rpm and 28°C during 1 hour. Afterwards, 30 mL of the enzyme extract was withdrawn for determination of enzymes activities and the other 70 mL were maintained in the beaker with fermented solid for 4 hours in an orbital shaker at 150 rpm and 50°C to evaluate the yield of fermentable sugar obtained for *in situ* enzyme hydrolysis.

Cellulolytic enzymes activities were determined as described by Ghose (1987), with few modifications. The Filter paper activity assay was carried out using 50 mg of filter paper Watmann n°1, 1 mL of diluted enzyme extract 2 mL of 50 mM sodium acetate/acetic acid buffer (pH 4.8), and the mixture was incubated for 60 min at 50 °C. Exocellulases activity were determined using 50 mg of Sigmacell Cellulose type 20, 20 µm (microcrystalline cellulose, Sigma Aldrich), 1 mL of diluted enzyme extract 2 mL of 50 mM sodium

acetate/acetic acid buffer (pH 4.8), and the mixture was incubated for 5 min at 40 °C. Endocellulases activity was measured using 1 mL of diluted enzyme extract in 2 mL of a 2% Carboxymethyl cellulose (Sigma Aldrich) in a 50 mM acetate/acetic acid buffer (pH 4.8), and the reaction were carried out at 50°C during 30 min. Xylanase activity was measured by adding 0.3 mL of diluted enzyme, 2.7 mL of a 1% Beachwood Xylan (Sigma Aldrich) solution in a 10 mM phosphate buffer (pH 5.2), and it was incubated for 5 min at 50°C. For all enzyme activity measurements, a standard without substrate was carried out to subtract the initial amount of reducing sugars (RS). Reducing sugars were measured by the spectrophotometric DNS method, using glucose as standard for FPU, exocellulases and endocellulases whereas xylose was used as standard for xylanase activity. In all cases the absorbance of samples were measured at 540 nm (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme which forms 1 µmol of glucose or xylose per min under assay conditions. All analysis were made in triplicate and the averages were expressed as response. Blanks were performed when necessary.

2.4. Ultrasound-assisted hydrolysis of sugarcane bagasse using crude cellulolytic enzymes

The cellulolytic enzymes used for the experiments involving the ultrasound-assisted hydrolysis of sugarcane bagasse were produced in the fixed-bed bioreactor at optimized condition of the CCRD. After the fermentation, the enzymes were extracted according to the methodology described above and the crude extract was maintained under refrigeration (4 °C) until the moment of the experiments.

Hydrolyses were carried out in an ultrasonic bath (Unique Inc., model USC 1800A, Brazil, BR) equipped with a transducer having longitudinal vibrations and temperature control (temperature accuracy of ±1.0 °C). The ultrasonic unit has an operating frequency of 40 kHz

and a maximum-rated electrical power output of 132 W. The ultrasonic transducer (surface area of 282.2 cm^2) is fitted at the bottom of the bath horizontally along the length of bath. In the experiments with ultrasound assisted the ultrasonic transducer was maintained on, whereas in the experiments using conventional procedure, the ultrasonic bath was used as thermostatic bath.

The effects of temperature (40.0 – 63.4 °C) and concentration of crude enzymatic extract (10 – 20 wt.% - in relation to the mass of dry solids) on the hydrolysis of sugarcane bagasse were assessed by means of central composite rotational design (CCRD) with 8 runs plus 3 central points. All experiments were carried out using 15 g of sugarcane bagasse, with addition of 1% of Tween 80, to enhance cellulose hydrolysis yields by reducing non-productive enzyme adsorption on the lignin in the substrate, and a final volume of 100 mL (solid/liquid ratio = 0.15) using sodium acetate buffer 0.1 M pH 4.8 during 4 hours. The experiments were performed at a water bath and ultrasound bath. At optimal conditions, it was evaluated the kinetic of hydrolysis in the presence and absence of ultrasound. The results were expressed in terms of gram of fermentable sugars per kilogram of dry solid material, determined by DNS method (Miller, 1959). All analysis were made in triplicate and the average were expressed as response.

2.5 Statistical analysis

All the results were analyzed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 90%.

3 Results and Discussion

3.1 Production of cellulolytic enzymes

Table 1 presents the activities of cellulolytic enzymes as well as the yield of fermentable sugar obtained by *in situ* hydrolysis for the eleven runs of the CCRD. Filter paper activity ranged from 0.61 (run 6) to 3.25 $FPU.g^{-1}$ (run 7); Exo-cellulase activity ranged from 0.22 (run 3) to 6.38 $U.g^{-1}$ (run 10); Xylanase activity ranged from 127.86 (run 4) to 366.81 $U.g^{-1}$ (run 6); Endo-cellulase activity ranged from 0.28 (runs 4 and 7) to 4.14 $U.g^{-1}$ (run 3). Among the enzymes determined in this work, the strain used showed the highest production for xylanase.

Table 1. Matrix of the CCRD to evaluate the influence of independent variables on the cellulolytic enzymes by solid-state fermentation

	Dependent Variables		**Independent Variables**				
Exp.	**Soybean Bran (wt.%)**	**Moisture (wt.%)**	**R1**	**R2**	**R3**	**R4**	**R5**
1	0.9 (-1)	68.6 (-1)	1.42	3.13	170.73	2.09	47.3
2	5.0 (1)	68.6 (-1)	1.31	2.13	172.60	0.95	46.6
3	0.9 (-1)	81.9 (1)	1.31	0.22	184.53	4.14	33.8
4	5.0 (1)	81.9 (1)	0.68	5.03	127.86	0.28	28.7
5	0.0 (-1.41)	75.3 (0)	0.92	5.37	153.96	1.16	44.0
6	5.9 (1.41)	75.3 (0)	0.61	1.12	366.81	2.13	29.1
7	2.9 (0)	65.0 (-1.41)	3.25	6.26	167.75	0.28	44.6
8	2.9 (0)	90.0 (1.41)	1.21	3.58	128.61	1.31	23.3
9	2.9 (0)	75.3 (0)	1.21	5.26	145.38	2.07	36.0
10	2.9 (0)	75.3 (0)	1.21	6.38	148.37	1.16	44.0
11	2.9 (0)	75.3 (0)	1.04	5.26	134.57	2.44	41.8

R1 = Filter paper activity ($U.g^{-1}$); R2 = Exo-cellulases activity ($U.g^{-1}$); R3 = Xylanases activity ($U.g^{-1}$); R4 = Endo-cellulases activity ($U.g^{-1}$); R5 = Yield of hydrolysis ($g.kg^{-1}$)

Data from Table 1 were used to determine the effects of independent variables on each response considering a significance level of 90% ($p<0.1$). These effects will be discussed here, but, for sake of brevity, the data will not be presented. The linear and quadratic effects for moisture content were significant for filter paper activity, where was verified a negative linear effect and positive quadratic effect, which means that the response will be higher as the moisture decrease, but there is a minimum point for moisture where the filter paper activity will be higher. For other enzymes no significant effects of the independent variables were verified in the evaluated range, indicating that independent of the experimental condition used the amount of enzyme produced is statistically the same.

A positive aspect of the results is that all evaluated cellulolytic enzymes were produced by the microorganism, taking into account that the hydrolysis of lignocellulosic materials is dependent of the synergic effect of different enzymes. However, it is difficult to select an experimental condition that maximizes the production of all enzymes. For this reason, it was opted to optimize the process by analyzing the yield of fermentable sugar obtained by *in situ* hydrolysis, enabling the selection of an experimental condition that lead to the maximum synergic action of the enzymes. From Table 1, it is seen that the yield ranged from 23.3 (run 8) to 47.3 $g.kg^{-1}$ (run 1). The yield obtained in run 8 can be considered expressive, taking into account that no purification procedure was applied in the fermented bagasse. Obviously that the yield should be increased for industrial applications, but it is presented a good perspective for future development.

Data referring yield of fermentable sugar for *in situ* hydrolysis were used to determine the effects of the studied variables. The effects were expressed in the form of Pareto chart, which are presented in the Fig. 1. Both linear effects for moisture and soybean bran concentration were statistically significant ($p<0.1$), which presented negative effects

indicating that the increase of moisture content and soybean bran concentration decrease the synergic action of the enzymes. Other terms were not significant in the evaluated range.

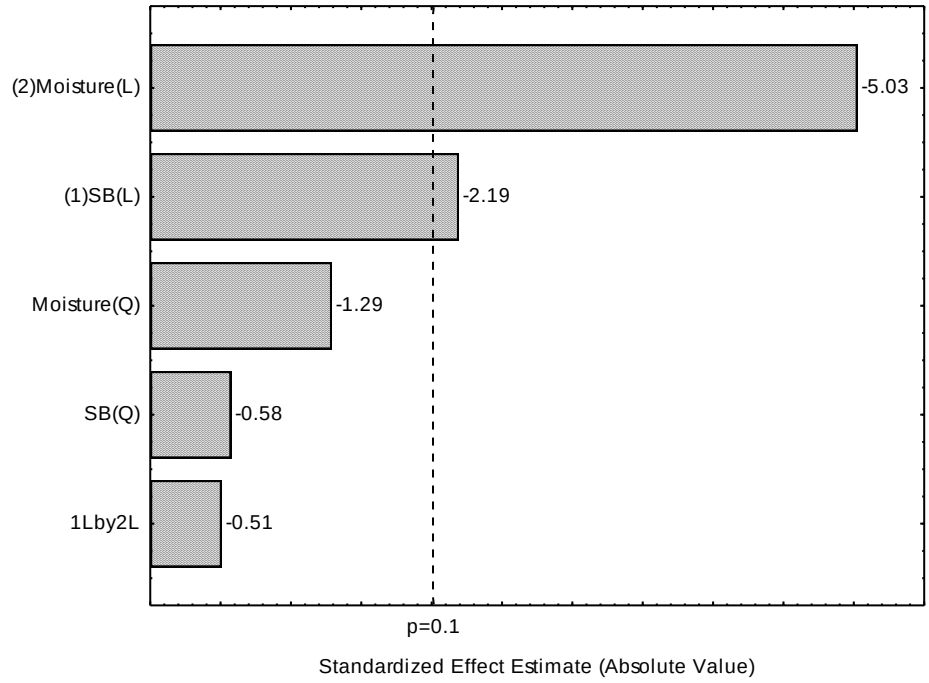


Fig. 1. Pareto Chart showing the effects of linear, quadratic and interaction terms of independent variables on the CCRD for cellulolytic enzymes production by solid-state fermentation.

In order to optimize the experimental condition to obtain maximum yield of fermentable sugar, a quadratic model considering the significant terms ($p<0.1$) was proposed in Eq. 1:

$$FS = 40.58 - 3.36 \cdot SB - 7.70 \cdot M \quad (1)$$

where FS is the yielding of fermentable sugar ($g.kg^{-1}$) obtained by *in situ* hydrolysis, SB and M are the coded soybean bran concentration and moisture content, respectively. This model was validated by analysis of variance (ANOVA). The calculated F-test for Eq. 1 was about 5.6 times greater than the tabulated ones for significance at $p = 0.1$ and the determination coefficient (R^2) was 0.8651. The high value for the determination coefficient indicates good fitting of experimental data, allowing the use of the model to predict process performance as

well as to use them as tool for process optimization. The applicability of this model is explored in Figure 2.

The highest yield of hydrolysis was achieved for moisture content lower than 72 wt.% and soybean concentration lower than 3.4 wt.%. This result is corroborated by responses of run 7, where high production of all enzymes was verified at moisture content of 65 wt.% and soybean bran concentration of 2.9 wt.%. On another hand, low yield of hydrolysis was verified in the run with high moisture content (run 8), where the cellulases production is known to be low because the microorganism does not develop well, and consequently produces less enzyme.

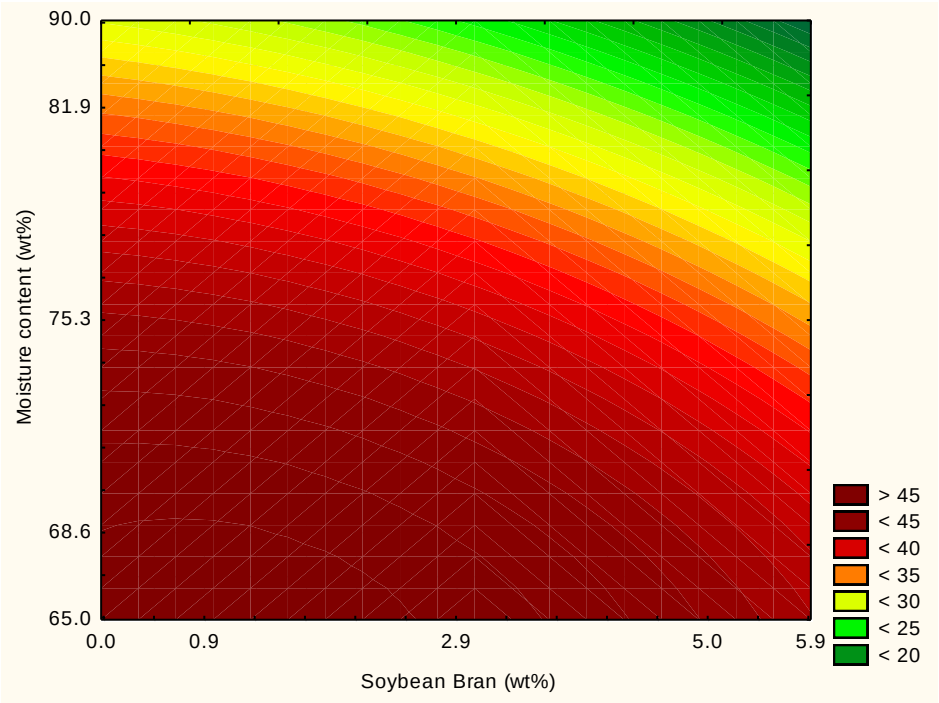


Fig. 2. Contour plots showing the influence of independent variables on cellulolytic enzymes production by solid-state fermentation.

The experimental condition of run number 1 was considered to be optimal for cellulolytic enzymes production (68.6 wt.% moisture content and 0.9 wt.% soybean bran concentration) and it was extrapolated for a scale up test, using a fixed-bed bioreactor with forced aeration. The total used substrate was 70 g, which represent an increase of 18 times. The activities of all enzymes obtained in the bioreactor were 4.18 $FPU.g^{-1}$, 7.34 $U.g^{-1}$,

1734.82 $U.g^{-1}$ and 2.50 $U.g^{-1}$ for FPA, exo-cellulase, xylanase and endo-cellulase, respectively. Comparing these results with those of run 1 of Table 1 it is seen an increase in the production of all enzymes by using a bioreactor with forced aeration. Similar result also was obtained for Mazutti et al. (2010) for the production of inulinase in a packed-bed bioreactor, where the enzyme production in the bioreactor was about two times greater than in the conical flasks. The reason for the increase in the enzyme production in the bioreactor is that in the conical flask the mass-transfer is limited to thickness of substrate layer, whereas the aeration in the fixed-bed bioreactor provides better medium oxygenation and heat exchange, and also helps to maintain the medium moisture, once the inlet air is saturated with water. This way, enzyme extract obtained in the scale up process had significantly higher activities, for all analyzed enzymes and this can be easily explained by the better operational conditions during the experiment in the bioreactor.

3.2 Application of crude enzymatic extract for hydrolysis of sugarcane bagasse

In this section, the crude enzymatic extract produced in the process scale up was used to optimize the enzymatic hydrolysis of sugarcane bagasse in a water bath (WB) and ultrasound bath (US). The results obtained in the CCRD referring to yield of hydrolysis are presented in Table 2. The yield of hydrolysis ranged from 80 to 224 $g.kg^{-1}$ for hydrolysis in the water bath and from 102 to 229 $g.kg^{-1}$ for hydrolysis in the ultrasound bath. The highest yield in water bath was 224.0 $g.kg^{-1}$ in the conditions of experiment 3 (T = 43.4°C; EE = 18.6 %, v/v). For experimental conditions of run 1 (T = 43.4 °C; EE = 11.5 %, v/v), yield of hydrolysis was 199.0 $g.kg^{-1}$ using 38% less enzymatic extract in comparison with run 3, being verified a reduction of 4.8% in the yield of hydrolysis. Comparing the same runs (1 and 3) in the ultrasound bath, it is seen a reduction of yield around 8.7%.

Table 2. Matrix of the CCRD to evaluate the influence of independent variables on the enzymatic hydrolysis of sugarcane bagasse in water and ultrasound baths using the crude cellulolytic enzymes obtained by solid-state fermentation

EXP	T(°C)	EE (% v/v)	Yield in WB ($g.kg^{-1}$)	Yield in US ($g.kg^{-1}$)	Increase/decrease in yield (%)
1	43.4 (-1)	11.45 (-1)	199.0	209.0	5.0
2	60.0 (1)	11.45 (-1)	122.0	147.0	21.0
3	43.4 (-1)	18.55 (1)	224.0	229.0	2.0
4	60.0 (1)	18.55 (1)	128.0	191.0	49.0
5	40 (-1.41)	15 (0)	150.0	157.0	4.0
6	63.4 (1.41)	15 (0)	80.0	102.0	28.0
7	51.7 (0)	10 (-1.41)	187.0	178.0	-4.0
8	51.7 (0)	20 (1.41)	211.0	221.0	5.0
9	51.7 (0)	15 (0)	174.0	201.0	16.0
10	51.7 (0)	15 (0)	184.0	192.0	4.0
11	51.7(0)	15 (0)	184.0	191.0	4.0

In a general way, it was observed a discrete increase in the hydrolysis efficiency with action of ultrasound, but this increase was more expressive for T > 60 °C. The same behavior was observed for Leães (2013), who obtained amylases activities always higher in the presence of ultrasound irradiation than in the absence, for temperatures up to 50 °C. This behavior concerning the temperature is partially explained in the work of Wang et al. (2011), which stated that the sonication of a liquid causes two primary effects: cavitation and heating. Although the heating effect of ultrasound irradiation was eliminated by temperature controlled water bath, the local shear stress caused by the collapse of bubbles can promote a slightly heating that is not measured due to the position of sensor, what can result in a local

increase of temperature, leading to the less pronounced effect of temperature in the enzyme activity in the presence of ultrasound.

The results presented in Table 2 were used to build two quadratic models expressing the yield of hydrolysis as functions of independent variables. Based on statistical analysis of model parameters, two empirical models are presented below. Eq. 2 presents the significant terms ($p<0.1$) concerning the yield of hydrolysis in the water bath, and Eq. 3 represents the yield obtained in the ultrasound bath:

$$Yield_{WB} = 180.62 - 34.06 \cdot T - 30.14 \cdot T^2 + 12.11 \cdot EE^2 \quad (2)$$

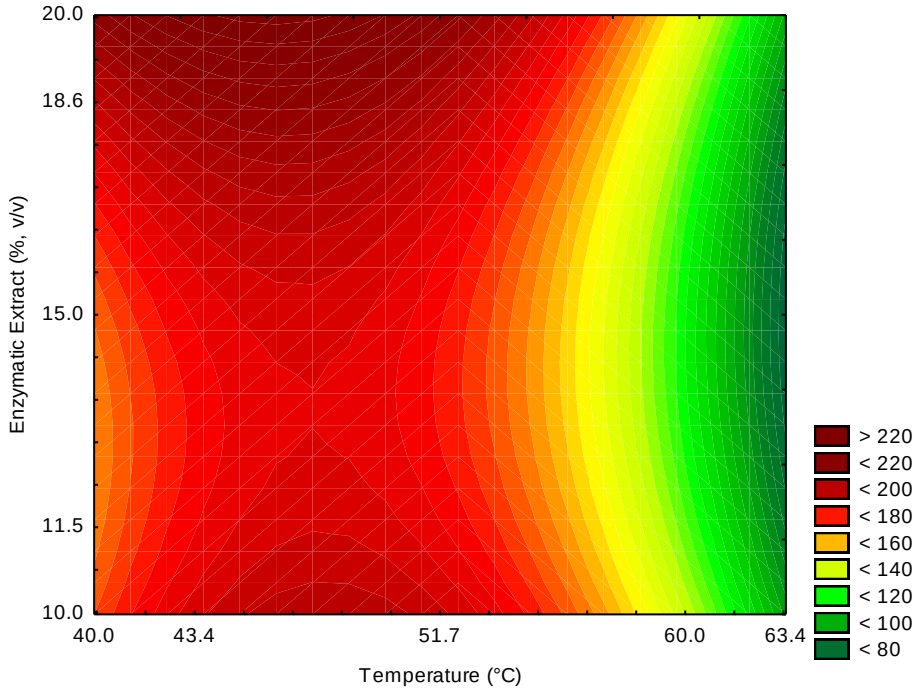
$$Yield_{US} = 203.87 - 22.26 \cdot T - 28.16 \cdot T^2 + 15.63 \cdot EE \quad (3)$$

where $Yield_{WB}$ and $Yield_{US}$ are the yield of hydrolysis expressed as gram of total reducing sugar per kg of dry bagasse ($g.kg^{-1}$) obtained in the water and ultrasound baths, respectively, T is the coded temperature and EE is the coded concentration of enzymatic extract.

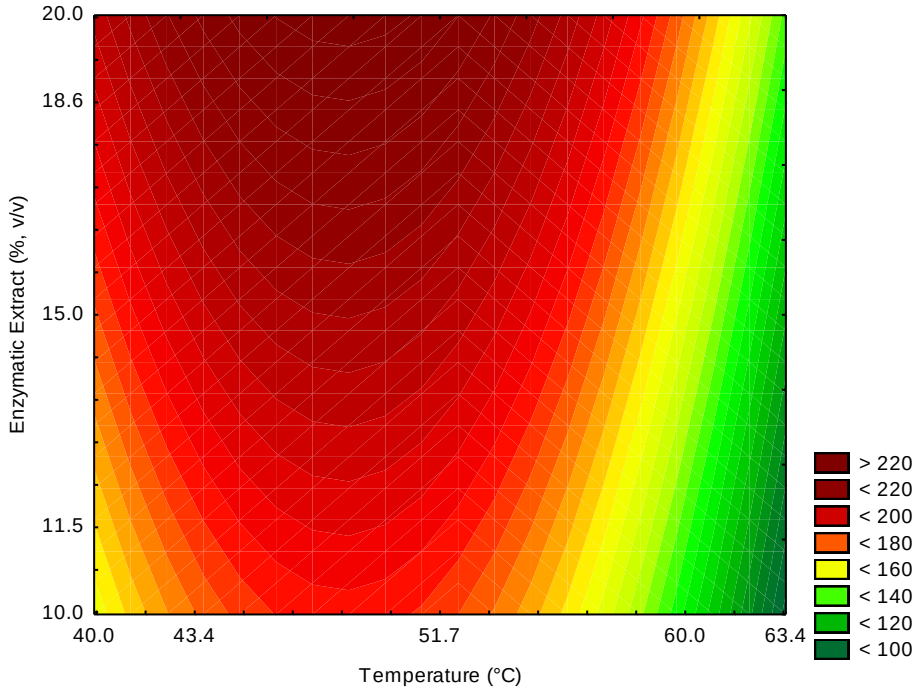
Analysing the terms of empirical models it is seen that linear and quadratic terms for temperature in both models were negative ($p<0.1$). The negative effect of temperature on the yield for both models is related to deactivation of enzymes at high temperatures. From Table 2 it is seen that for temperatures higher than 60 °C, the yield of hydrolysis decrease significantly, indicating that enzymes can be denatured at this temperature. For the concentration of enzymatic extract the quadratic term was positive for hydrolysis in the water bath, whereas for the ultrasound bath only the linear term was statistically significant. Although significant, the terms for concentration of enzyme extract presented lesser influence than temperature, conform can be detected by analysing the magnitude of the each term.

To enable the construction of contour plots, these models were validated by analysis of variance (ANOVA). The calculated F-test for Eq. 2 and 3 were about 8.0 and 3.2 times greater than the tabulated ones for significance at $p = 0.1$, and the determination coefficients (R^2) were 0.9462 and 0.8594, respectively. The high values for the determination coefficient

indicate good fitting of experimental data, allowing the use of such models for optimization purposes.



a)



b)

Fig. 3. Contour plots showing the influence of independent variables on enzymatic hydrolysis of sugarcane bagasse in water bath (a) and ultrasound bath (b).

Figure 3a presents the yield of hydrolysis in the water bath predicted by Eq. 2, whereas Figure 3b presents the yield of hydrolysis in the ultrasound bath predicted by Eq. 3. The optimal temperature for hydrolysis is similar in both systems and ranged from 45 to 50 °C. Considering the concentration of enzymatic extract it is seen that high yield can be obtained for concentrations higher than 18 % (v/v) in both systems. However, for hydrolysis in the water bath, high yield also can be obtained for concentrations around 10 %. Based on the results presented in Figure 3, the optimized condition for both systems was temperature of 43.4 °C and concentration of enzymatic extract of 18.6 %, specifically at experimental condition of run 3.

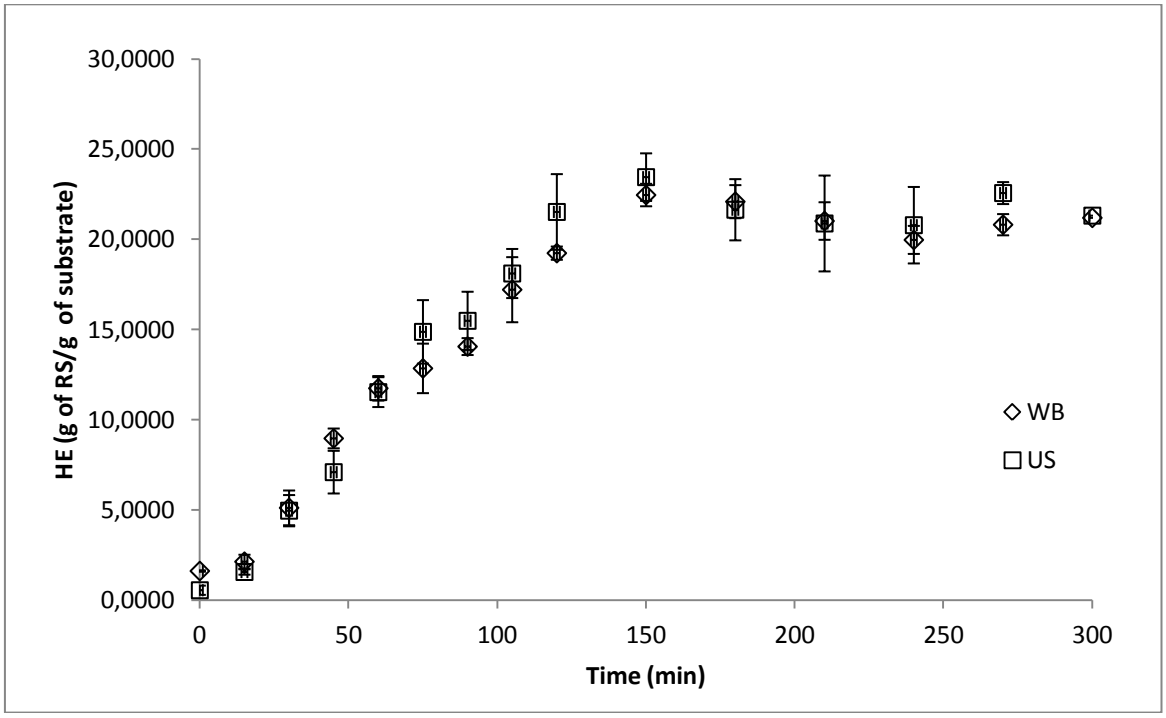


Fig. 4. Kinetic of hydrolysis of sugarcane bagasse using crude enzymatic extract at optimized condition in water and ultrasound baths. All analysis were made in triplicate, the points represents the average and the bars represents the standard deviation.

Figure 4 presents the kinetic profile of yield of hydrolysis obtained at optimized experimental conditions in the water and ultrasound baths. Similar behavior was verified for both kinetics without statistical difference among them ($p<0.05$). The results of Figure 4 also validated the data of run 3 of Table 2, which were very close to the presented here. The time

for the constant reaction rate was about 150 minutes, being obtained the highest yields in this time. For industrial applications, it is desirable that the reaction ends shortly after the constant reaction rate period, since it implicates in the obtainment of high amount of fermentable sugar in a relatively short time (Werle et al., 2013).

Gottschalk et al. (2010) tested the concentration of glucose in the hydrolysate during enzymatic hydrolysis of the steam-treated sugarcane bagasse using the enzymes produced by *T. reesei*, *A. awamori*, and in the enzyme blends of the *T. reesei* and *A. awamori* cellulases at ratios of 25:75, 50:50 and 75:50, obtaining 90 $g.kg^{-1}$, 40 $g.kg^{-1}$, 155 $g.kg^{-1}$, 180 $g.kg^{-1}$, 200 $g.kg^{-1}$, using 10FPU/g and 5 hours of fermentation.

Li et al. (2014) studied the synergistic effect of cellulase, xylanase, and pectinase on hydrolyzing sugarcane bagasse resulting from different pretreatment technologies. Their work evaluated the synergy between four different commercial enzymes, in different concentrations and their effects on hydrolysis of pretreated sugarcane bagasse under three different processes. The best obtained result was for a commercial enzymes mixture, composed of 3.17, 1.63 and 1.2 mg/g of substrate, of Celluclast1.5 L, Novozym 188, and Xylanase, respectively. Evaluating the synergy between cellulases and xylanase, it was obtained approximately $225g.kg^{-1}$ of glucose after 2h of hydrolysis of NaOH pretreated sugarcane bagasse, and approximately $445g.kg^{-1}$ of glucose after 12h of hydrolysis.

This way, considering that in this work, the cellulases were produced and its extract was applied in sugarcane bagasse hydrolysis without any pre-treatment, the obtained hydrolysis yields of 224 $g.kg^{-1}$ (without sonication) after 4h of saccharification can be considered a very reasonable value, comparable with those obtained by Gottschalk et al. (2010) and Li et al. (2014) hydrolyzing a pre-treated sugarcane bagasse. There is still a potential increase in the hydrolysis yields pretreating the substrate. Also, for higher

temperatures, the ultrasound assisted hydrolysis could be an alternative to improve the glucose formation during the reaction.

4 Conclusions

The results obtained in this work allowed concluding that the optimum moisture content and soybean bran concentration for production of cellulolytic enzymes by solid-state fermentation were 68.6 wt.% and 0.9 wt.%, respectively. In addition, 99,1 wt.% of sugarcane bagasse, 15 v.% of corn steep liquor, 0.5 mL of inoculum per gram of substrate, 28°C and 4 days of fermentation are the better obtained condition for cellulases production. After the definition of the operational condition, the production was carried out in a packed-bed bioreactor, increasing the production of all enzymes determined. The crude enzymatic extract was applied for hydrolysis of sugarcane bagasse in water and ultrasound baths, being obtained 224.0 and 229 $g.kg^{-1}$, respectively at temperature of 43.4°C and concentration of enzymatic extract of 18.6%. The obtained hydrolysis yields are comparable to commercial enzymes, and it allows the conclusion that the use of crude *T. reesei* cellulases extract in the saccharification of sugarcane bagasse without any pre-treatment could be a feasible alternative to lower the hydrolysis process costs.

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5 ARTIGO 2: Home-made cellulolytic enzymes for hydrolysis of sugarcane bagasse under direct and indirect sonication

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Abstract

In this work the enzymatic hydrolysis of non-treated sugarcane bagasse under direct and indirect sonication was studied using home-made cellulolytic enzymes produced by solid-state fermentation. Hydrolyses were carried out using a high-intensity ultrasound probe system of 400 W and 24 kHz. The variables investigated were oscillation amplitude, pulse factor and enzyme concentration. Maximum yield of hydrolysis were 31.3 $g.kg^{-1}$ and 60.6 $g.kg^{-1}$ under direct and indirect sonication, respectively. The optimized condition for indirect sonication hydrolysis is enzyme concentration higher than 209.5 $\mu l.g^{-1}$, continuous ultrasound irradiation and oscillation amplitude of 20 % that corresponds to an ultrasound power of 60 $W.cm^{-2}$. The indirect ultrasound irradiation showed to be a more promising technology to be used in enzymatic reaction due to its positive effects on the yield of hydrolysis.

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1 Introduction

Increasing costs of fossil fuels associated with their greenhouse gases emission effects are creating a need to develop cheaper and environment friendly biofuels. An alternative to fossil fuels that have been largely studied in the last years is the production of biofuels from plant biomass, especially agroindustrial residues that is abundant in large countries as Brazil [1]. Lignocelluloses materials are most promising feedstock as natural, abundant, and renewable resource and ethanol produced from this kind of material can potentially provide a long term sustainable fuel supply [2].

The enzymatic route has been receiving a great deal of attention as an alternative technology for hydrolysis of lignocellulosic material for second generation ethanol production due to mild operation condition and minimal formation of degrading compounds. However, large scale economical commercial production of fuel ethanol from lignocellulosic materials has still not been implemented, mainly because the high cost of cellulolytic enzymes used in the feedstock saccharification [1, 3].

The production of cellulases by solid state fermentation of lignocellulosic residues and subsequent application of the crude enzyme extract in cellulose hydrolysis can be an alternative to become the saccharification process less expensive [4, 5]. However, acceptable rates of enzymatic hydrolysis only are possible after the pretreatment of material to reduce the recalcitrance of the substrate [6]. By other hand, several authors have shown that saccharification of cellulose is enhanced efficiently by ultrasonic pretreatment [7, 6, 8, 9]. More recently, the effect of ultrasound on catalytic power of enzymes [10, 11, 12, 13, 14, 15, 16] and lignocellulosic hydrolysis efficiency [17, 7, 18, 19] have been studied.

The effect of ultrasound can be based on a direct interaction with molecular species as well as on the cavitation phenomenon. In the field of biotechnology, low-frequency power ultrasound has recently gained much attention in the intensification of enzyme-aided

processes. Ultrasound has a direct effect on the enzyme molecules and enhances the mass transfer in the heterogeneous processes by the local turbulences created by acoustic cavitation. Furthermore, mechanical impact produced by the collapse of cavitation bubbles, provide an important benefit of opening up the surface of solid substrates to the action of enzymes. However, if the ultrasound intensity is too high, the enzymes can be denatured [20, 21, 11]. Ultrasound applications are based on three different methods: direct application to the product, coupling with the device, submergence in an ultrasonic bath. The most studied method to enhance enzymatic activity is the submergence in an ultrasonic bath, as the equipment has low cost, it is versatile and simple, and in addition has low power sonication [10, 16]. The ultrasound direct application to the product, as it has higher power ultrasound, is widely studied in processes as emulsification, homogenization, extraction, crystallization, dewatering, low temperature pasteurization, degassing, defoaming, activation and inactivation of enzymes, particle size reduction and viscosity alteration [22]. However, the effect of direct and high power ultrasound in the enzymatic activity of cellulases is little studied [15].

In this context, the main objective of this work was to evaluate the enzymatic hydrolysis of non-treated sugarcane bagasse under direct and indirect sonication using home-made cellulolytic enzymes. For this purpose, cellulolytic enzymes were produced by solid-state fermentation and the crude enzymatic extract used for experiments, which were carried out using a high-intensity ultrasound probe system of 400 W and 24 kHz. The variables investigated in direct and indirect sonication were oscillation amplitude, pulse factor and enzyme concentration, and the fixed variables were temperature, pH and solid/liquid ratio of 45°C, 4,8 and 0,05, respectively.

2 Materials and Methods

2.1. Sugarcane bagasse

Sugarcane bagasse was obtained in a microdistillery located within the Federal University of Santa Maria. In the laboratory, it was dried at 60 °C during 24 hours, grounded in a cutting mill and sieved with final particle size of 8 mesh. The solid substrate for enzyme production was composed by sugarcane bagasse as main carbon source, supplemented with soybean bran and corn steep liquor.

2.2. Production of cellulolytic enzymes by solid-state fermentation

Trichoderma reesei NRRL - 6156 was obtained from Agricultural Research Service of the United States Department of Agriculture. Stock cultures of *T. reesei* were propagated on potato dextrose agar (PDA) using Petri plates at 28 °C for five days. For pre inoculum preparation, the conidia from sporulating plates cultures were suspended in 5 mL of sterile water, and 5 mL of the suspension was transferred to a 250 mL Erlenmeyer flask containing 50 mL of the growth medium, which was incubated in an orbital shaker for 2 days at 28 °C and 120 rpm. The medium for pre inoculum was composed by ($g.L^{-1}$): 2.0 $(NH_4)_2SO_4$, 0.5 $MgSO_4$, 1.0 $FeSO_4.7H_2O$, 1.0 $MnSO_4.7H_2O$, 10.0 yeast extract, 10.0 glucose, 150.0 corn steep liquor and 1.0 $mL.L^{-1}$ of Tween 80. All experimental conditions were set based on preliminary tests and literature search.

Solid-state fermentations were carried out using 70 g of substrate (dry basis) in a fixed-bed bioreactor with forced aeration. The solid substrate was composed of sugarcane supplemented with 1 wt.% of soybean bran and moisture content of 65 wt.%. Afterwards, the material was autoclaved at 121 °C for 30 min and microorganism inoculated. The fermentations were carried out at 28 °C during 4 days. Experimental conditions were set in our group previous work. After the end of fermentation, the cellulolytic enzymes were extracted using 1500 mL of 50 mM sodium acetate buffer (pH 4.8) in an orbital shaker at 120

rpm and 28 °C during 1 hour. The crude enzymatic extract was maintained under refrigeration (4 °C) until the hydrolysis experiments.

Cellulolytic enzymes activities were determined as described by Ghose [23], with few modifications. The Filter paper activity assay was carried out using 50 mg of filter paper Whatmann n°1, 1mL of diluted enzyme extract 2 mL of 50 mM sodium acetate/acetic acid buffer (pH 4.8), and the mixture was incubated for 60 min at 50 °C. Exocellulases activity were determined using 50 mg of Sigmacell Cellulose 20 µm – microcrystalline cellulose (Sigma Aldrich), 1 mL of diluted enzyme extract 2 mL of 50 mM sodium acetate/acetic acid buffer (pH 4.8), and the mixture was incubated for 5 min at 40 °C. Endocellulases activity was measured using 1 mL of diluted enzyme extract in 2mL of a 2% Carboxymethyl cellulose (Sigma Aldrich) in a 50mM acetate/acetic acid buffer (pH 4.8), and the reaction were carried out at 50 °C during 30 min. Xylanase activity were measured by adding 0.3 mL of diluted enzyme, 2.7 mL of a 1% Beachwood Xylan (Sigma Aldrich) solution in a 10 mM phosphate buffer (pH 5.2), and it was incubated for 5 min at 50 °C. For all enzyme activity measurements, a standard without substrate was carried out to subtract the initial amount of reducing sugars (RS). Reducing sugars were measured by spectrophotometric DNS method, using glucose as standard for FPU, exocellulases and endocellulases whereas xylose was used as standard for xylanase activity. In all cases the absorbance of samples were measured at 540 nm [24]. One unit of enzyme activity was defined as the amount of enzyme which produces 1 µmol of glucose or xylose per min under assay conditions.

2.3. Enzymatic hydrolysis of non-treated sugarcane bagasse

The experimental system for direct sonication was composed of a jacked reactor (1 L of capacity) connected to a thermostatic water bath (temperature accuracy of ±1.0 °C) for temperature control, a high-intensity ultrasound processor of 400 W, frequency 24 kHz with

amplitude adjustable from 20 to 100% and pulse mode factor between non-operation and acoustic irradiation adjustable between 0 to 100% (Hielscher, Model UP 400S) equipped with a titanium probe of 7 mm (Model H7 Tip 7) presenting an acoustic power density of 300 $W.cm^{-2}$. For indirect sonication the system was the same, except for the reactor which was exchanged for a sonication beaker with capacity for 5 test tubes of 15 ml acting as reactors. The sonication beaker with test tubes was immersed in the thermostatic bath during hydrolysis to maintain constant the temperature and the sonication probe was coupled in the middle of the beaker in direct contact with water while the hydrolysis was carried out in the test tubes, characterizing the process as indirect sonication.

The effects of concentration of crude enzymatic extract (1.25 – 6.25 $mL.g^{-1}$), oscillation amplitude (20 – 40%) and pulse cycle (0.4 to 1.0) on the hydrolysis of sugarcane bagasse were assessed by means of central composite rotational design (CCRD) with 15 runs plus 3 central points. All experiments were carried out at 45 ± 2 °C using sodium acetate buffer 50 mM pH 4.8 during 2 hours. The final volume for direct sonification experiments were 500 mL whereas for indirect sonification were 10 mL, maintaining the solid liquid ratio for all experiments at 0.05 wt.%. All experimental conditions were set based on literature research and preliminary tests. To increase the yield of hydrolysis by reducing non-productive enzyme adsorption on the lignin in the substrate 1 % of Tween 80 [11]. The same run was carried out in direct and indirect sonication for comparison purposes.

After the hydrolysis, an aliquot of solution was filtered by vacuum filtration (Whatman qualitative filter paper, grade 1) and the supernatant was used to determine the amount of fermentable sugars by the 3.5-dinitrosalicylic acid method (DNS) [14]. The results were expressed in terms of gram of fermentable sugars per kilogram of dry solid material, which represent the yield of hydrolysis.

2.4 Statistical analysis

All the results were analyzed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 90%.

3 Results and Discussion

In this work, crude enzymatic extract obtained by solid-state fermentation was used for the enzymatic hydrolysis of non-treated sugarcane bagasse under direct and indirect sonification. The activities of all enzymes determined in this work were 4.2 $FPU.g^{-1}$, 7.3 $U.g^{-1}$, 1734.8 $U.g^{-1}$ and 2.5 $U.g^{-1}$ for filter paper activity, exo-cellulase, xylanase and endo-cellulase, respectively. It is important to point out that crude enzymatic extract obtained has at least four different enzymes acting in a synergic way for the hydrolysis of lignocellulosic materials. As can be seen, the extract presents high xylanase activity, although filter paper activity also presented a satisfactory value.

Table 1 presents the fermentable sugar yields obtained in the seventeen runs of the CCRD for the direct and indirect ultrasound-assisted enzymatic hydrolysis of sugarcane bagasse. The fermentable sugar yield obtained in the enzymatic hydrolysis for direct sonication ranged from 6.3 $g.kg^{-1}$ (run 13) to 31.3 $g.kg^{-1}$ (run 5), whereas the fermentable sugar yield for indirect sonication ranged from 15.9 $g.kg^{-1}$ (run 13) to 60.6 $g.kg^{-1}$ (run 6). The mean yields of fermentable sugar (considering the 17 runs) were 18.3 and 41.4 $g.kg^{-1}$ for the direct and indirect ultrasound-assisted enzymatic hydrolysis, respectively. This result showed that the uses of direct sonication led to a significant (Tukey test at $p<0.05$) decrease in the yield of fermentable sugar and it could happened because the ultrasound high intensity may affect the enzyme structure, leading to its deactivation. In a general way, the yield of fermentable sugar in indirect sonication hydrolysis was always higher than for the direct one. Data of Table 1 were used to determine the effects of the studied variables on yield of

fermentable sugar. The effects were expressed in the form of Pareto chart, which are presented in the Fig. 1.

Table 1. Matrix of the CCRD to evaluate the influence of independent variables on the enzymatic hydrolysis of sugarcane bagasse under direct and indirect sonication

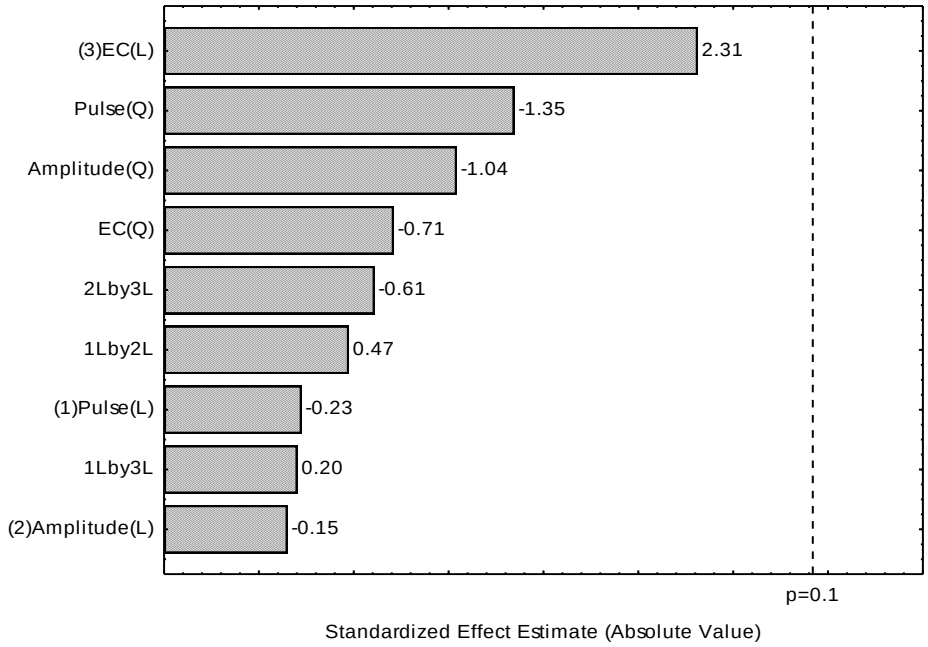
Run	Pulse factor (%)	Amplitude (%)	Enzyme concentration ($\mu l.g^{-1}$)	Yield for DS ($g.kg^{-1}$)	Yield for IS ($g.kg^{-1}$)
1	0.5 (-1)	24 (-1)	90.5 (-1)	19.5	39.6
2	0.9 (1)	24 (-1)	90.5 (-1)	16.9	39.7
3	0.5 (-1)	36 (1)	90.5 (-1)	19.0	39.8
4	0.9 (1)	36 (1)	90.5 (-1)	14.0	32.8
5	0.5 (-1)	24 (-1)	209.5 (1)	31.3	50.4
6	0.9 (1)	24 (-1)	209.5 (1)	23.9	60.6
7	0.5 (-1)	36 (1)	209.5 (1)	18.8	45.1
8	0.9 (1)	36 (1)	209.5 (1)	22.2	56.1
9	0.4 (-1.68)	30 (0)	150 (0)	9.0	41.3
10	1.0 (1.68)	30 (0)	150 (0)	12.8	45.1
11	0.7 (0)	20 (-1.68)	150 (0)	8.3	52.5
12	0.7 (0)	40 (1.68)	150 (0)	16.7	50.4
13	0.7 (0)	30 (0)	50 (-1.68)	6.3	15.9
14	0.7 (0)	30 (0)	250 (1.68)	22.2	43.9
15	0.7 (0)	30 (0)	150 (0)	18.6	27.9
16	0.7 (0)	30 (0)	150 (0)	30.6	29.9
17	0.7 (0)	30 (0)	150 (0)	21.5	32.3

DS – Direct sonication; IS – indirect sonication.

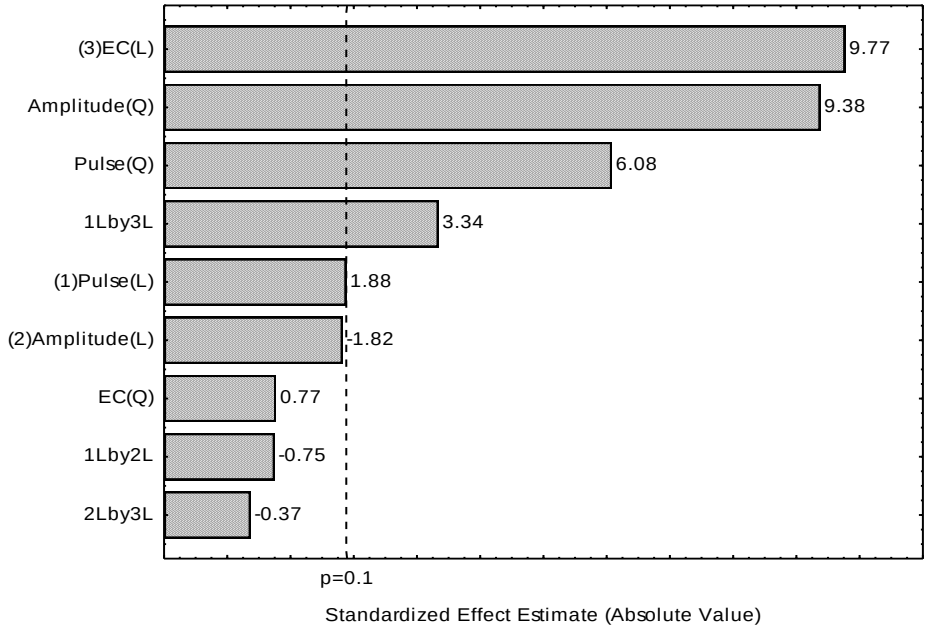
For direct sonication hydrolysis (Fig. 1a), none of the studied variables were statistically significant in the evaluated range. The linear and quadratic effects of oscillation amplitude and pulse factor, although not significant were negative and it may be due the high range of amplitudes tested (20 to 40%) that negatively affected the hydrolysis efficiency, since the enzyme can be denatured under high sonication power. In general, low yield were obtained in these experiments, not exceeding 31.3 $g.kg^{-1}$ and it may be due to the low activity of the crude enzyme extract, combined with the low enzyme concentration range used and the oscillation amplitude negative effect. The linear effect referring to enzyme concentration reached a p-value close to significance ($p = 0.113$) and had a positive effect on the yield, as can be seen on Fig. 1a. Increasing the enzyme concentration it is possible to note an increasing in the yield of hydrolysis. The negative effect of oscillation amplitude and pulse factor indicate that at high power sonication caused irreversible damage in the enzymes structure, corroborating with the positive effect for enzyme concentration, since as high is the enzyme deactivation more enzyme is required to accomplish the hydrolysis.

For indirect sonication hydrolysis (Fig. 1b), the significant variables ($p<0.1$) were linear effects for enzyme concentration, pulse factor and oscillation amplitude, quadratic effects for pulse factor and oscillation amplitude, and the interaction effect between pulse factor and enzyme concentration. Positive linear effects were verified for pulse factor and enzyme concentration that led to a positive effect of interaction between these variables. In other words, increasing the enzyme concentration as well as the pulse factor can be obtained high yields. By other hand, linear effect for oscillation amplitude presented negative effect, indicating that increasing the ultrasound power, decreases the yield, probably due to enzyme deactivation. Quadratic effects for pulse factor and oscillation amplitude were positive, showing the presence of minimum point for these variables in the studied range. The correct interpretation of the quadratic effects is important, because they are indicating that within the

studied range for each variable can be found a point or region on the yield is the lowest. For example, linear effect for oscillation amplitude was negative, but the quadratic one was positive. A direct conclusion that decreasing the oscillation amplitude will lead to increase in the yield is wrong, because between 20 and 40% of oscillation amplitude there is a point where the yield is the lowest.



a)



b)

Fig. 1. Pareto Chart showing the effects of linear, quadratic and interaction terms of independent variables on the yield of fermentable sugars under (a) direct and (b)indirect sonication hydrolysis.

In order to help the selection of independent variables that will lead to maximum yield of fermentable sugar, an empirical model presenting the significant terms ($p<0.1$) concerning to indirect sonication hydrolysis is presented in Eq. 1:

$$FS = 29.82 + 1.52 \cdot P + 5.40 \cdot P^2 - 1.47 \cdot A + 8.31 \cdot A^2 + 7.87 \cdot EC + 3.25 \cdot P \cdot EC \qquad (1)$$

where FS is the yield of fermentable sugar ($g.kg^{-1}$) obtained for the indirect sonication hydrolysis, P, A and EC are the coded pulse factor, oscillation amplitude and enzyme concentration, respectively. This model was validated by analysis of variance (ANOVA). The calculated F-test for Eq. 1 was about 18 times greater than the tabulated one for significance at p=0.1, and the determination coefficient (R^2) was 0,96907. The high values for the determination coefficient indicate good fitting of experimental data, allowing the use of such models to predict process performance as well as to use them as tool for process optimization. The applicability of this model is explored in Figs. 2 to 4.

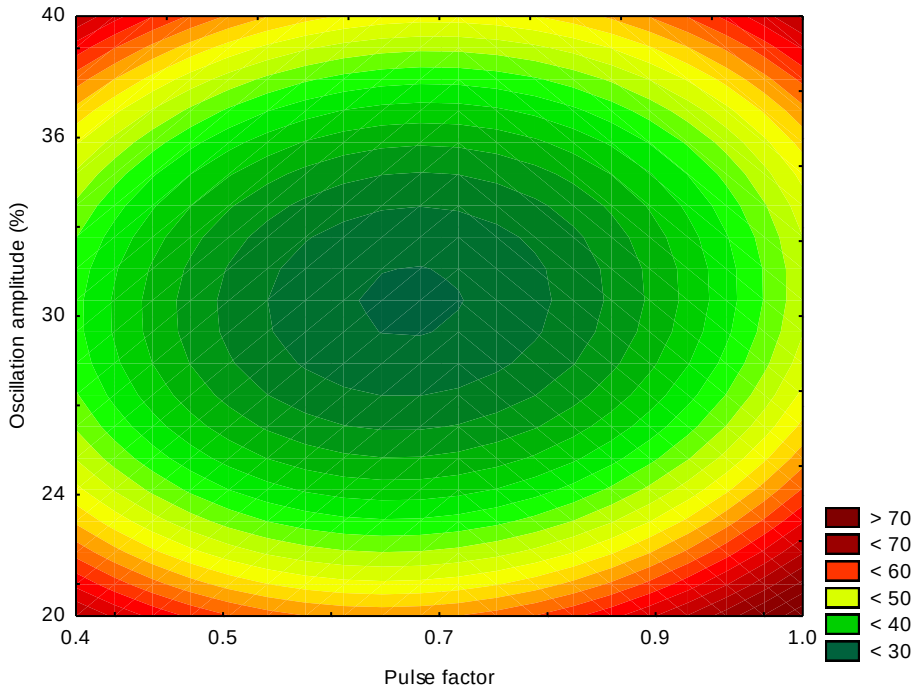


Fig. 2. Contour plots showing the influence of pulse factor and oscillation amplitude on the yield of fermentable sugars under indirect sonication hydrolysis. Enzyme concentration was maintained at the central point of the CCRD.

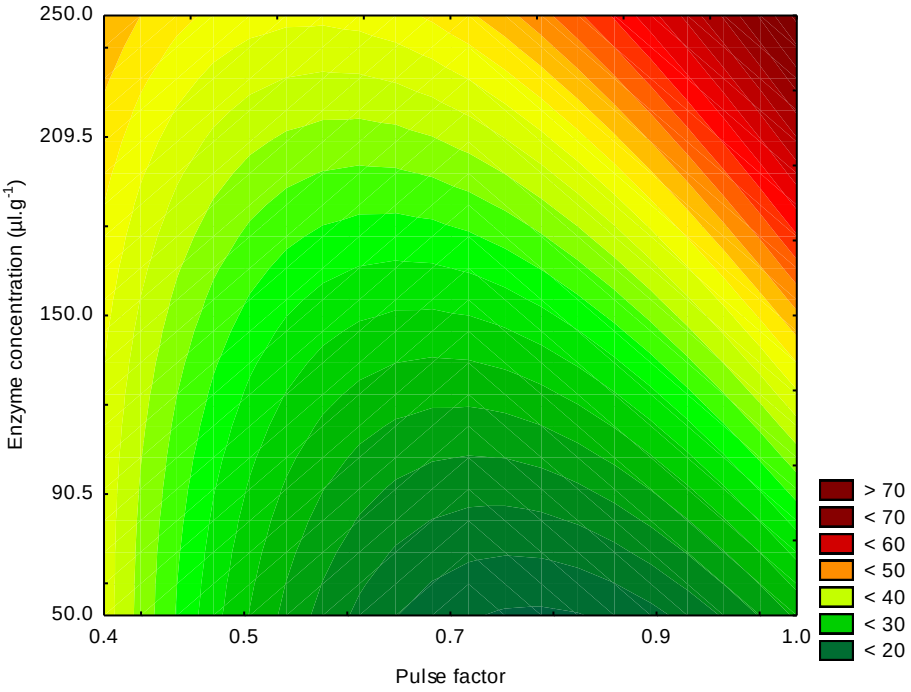


Fig. 3. Contour plots showing the influence of pulse factor and enzyme concentration on the yield of fermentable sugars under indirect sonication hydrolysis. Oscillation amplitude was maintained at the central point of the CCRD

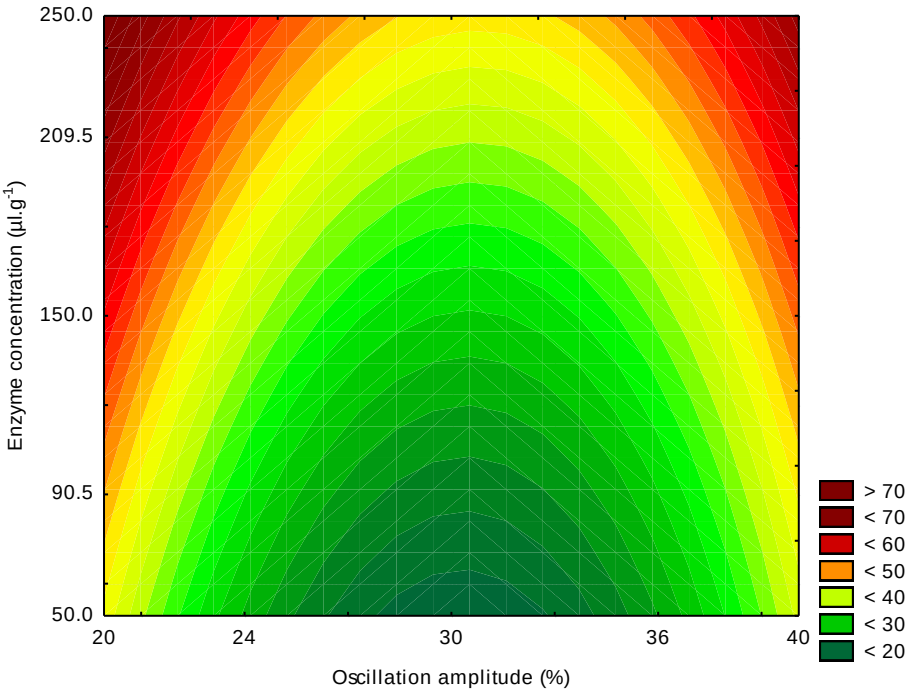


Fig. 4. Contour plots showing the influence of oscillation amplitude and enzyme concentration on the yield of fermentable sugars under indirect sonication hydrolysis. Pulse factor was maintained at the central point of the CCRD

Fig. 2 presents the influence of the pulse factor and oscillation amplitude on the yield of fermentable sugar. The minimum point discussed above concerning pulse factor and oscillation amplitude can be easily detected, which is localized around the central point of the experimental design, where the yield was around 30.0 $g.kg^{-1}$. Maximum yields can be obtained at pulse factor ranging from 0.4 to 0.5 or from 0.9 to 1.0, both at oscillation amplitude around 20%. Fig. 3 presents the influence of the pulse factor and enzyme concentration on the yield of fermentable sugar. Maximum yield was obtained for concentrations higher than 209.5 $\mu l.g^{-1}$ at pulse factor higher than 0.9. It is important to note that decreasing the pulse factor until 0.9 it is necessary to increase the enzyme concentration to obtain high yield, what indicates that continuous indirect ultrasound irradiation improves the catalytic power of the enzymes. Fig. 4 presents the influence of the oscillation amplitude and enzyme concentration on the yield of fermentable sugar. Maximum yield was obtained for concentrations higher than 209.5 $\mu l.g^{-1}$ at oscillation amplitude of 20%, showing that the increase of the ultrasound power lead to deactivation of the enzymes. Considering the nominal power of the ultrasound probe, the hydrolysis is favored for ultrasound power of 60 $W.cm^{-2}$ (300 x 0.2). Based on the analysis of the Figs. 2-4 it is seen that optimized range of the experimental conditions for indirect sonication hydrolysis are enzyme concentration higher than 209.5 $\mu l.g^{-1}$, continuous ultrasound irradiation (pulse mode of 1.0) and oscillation amplitude of 20% that corresponds to an ultrasound power of 60 $W.cm^{-2}$.

In this work, the use of indirect sonication showed to be more promising that direct one. In a general way, the yield was considerably higher when indirect sonication was employed. This result can be associated with deactivation of the enzymes when high ultrasound power is applied in the reaction media. Although the experiments were carried out in the same experimental condition, the effective ultrasound power dissipated in the reaction media under indirect sonication is lower than for direct one. Bashari et al. [25] showed that

for direct sonication the activity of a dextranase decreased considerably for ultrasound power higher than 40 W. Wang et al. [12] reported that the activity of a cellulase increase until ultrasound power of 15 W under direct sonication, decreasing for higher values. By other hand, our research group have verified that the activity of some hydrolytic enzymes (cellulase, amylase, inulinase, xylanase) increased under indirect sonication, using an ultrasonic bath with ultrasound power of 136W [16, 7, 18 e 19].

Referring the hydrolysis, there is few works in literature using ultrasound-assisted enzymatic hydrolysis of lignocellulosic materials. The ultrasound is very used for pre-treatment of biomass, but the number of works using it for intensification of hydrolysis is limited. Silva et al. [18] reported maximum yield of fermentable sugar of 217 $g.kg^{-1}$ from sugarcane bagasse for hydrolysis carried out using pressurized liquefied petroleum gas combined with indirect sonication. Lunelli et al. [19] evaluated the solid-state enzymatic hydrolysis of sugarcane bagasse under indirect sonication and reported twice more fermentable sugar using ultrasound. For other raw materials, the application of ultrasound in the production of glucose from sorghum flour increased percentage of saccharification by about 8% [26].

4 Conclusions

In this work it was demonstrated that there is a huge difference between using direct and indirect sonication in hydrolysis of lignocellulosic material. Considerable increase in the yield of hydrolysis was obtained in experiments under indirect sonication. Maximum yield of hydrolysis were 31.3 $g.kg^{-1}$ and 60.6 $g.kg^{-1}$ under direct and indirect sonication, respectively. These results can be considered reasonable values, since it was used an extremely low concentration of enzyme, and there is a potential to increase these yields by increasing the enzyme concentration. The optimized condition for indirect sonication hydrolysis is enzyme

concentration higher than 209.5 $\mu l.g^{-1}$, continuous ultrasound irradiation and oscillation amplitude of 20% that corresponds to an ultrasound power of 60 $W.cm^{-2}$. The indirect ultrasound irradiation showed to be more promising technology to be used in enzymatic reaction due to its positive effects on yield of hydrolysis.

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6 CONSIDERAÇÕES FINAIS

Os resultados obtidos nesse trabalho, apresentados na forma de dois artigos submetidos para publicações em diferentes revistas, são produto de um único e extenso trabalho que vem sendo desenvolvido desde o início de 2012, no Laboratório de Bioprocessos do Programa de Pós Graduação em Engenharia de Processos da Universidade Federal de Santa Maria.

Esse trabalho teve como proposta o desenvolvimento de um processo para produção de celulases utilizando uma cepa do fungo filamentoso *Trichoderma reesei*, para posterior utilização do extrato enzimático produzido na hidrólise enzimática de bagaço de cana, a fim de avaliar os efeitos do ultrassom de baixa e de alta intensidade no processo.

O processo otimizado de produção das celulases consistiu em cinco dias de crescimento do pré-inóculo em placas de Petri, seguido de dois dias de crescimento meio líquido otimizado, e quatro dias de FES de BC suplementado com 0,9% de farelo de soja (FS) e 15% de água de maceração de milho (AMM), 68,6 % de umidade, 28±1°C e densidade de 0,5mL de inóculo por grama de substrato. Essa condição experimental em escala de bancada (5g de substrato seco) resultou em uma produção de 1,4 FPU/g, e esse valor foi aumentado em aproximadamente três vezes (4,7FPU/g) com o aumento de escala de produção (70g de substrato seco), em um biorreator de leito fixo com aeração forçada.

Nos testes de hidrólise enzimática do bagaço de cana sem tratamento prévio, assistida de banho de ultrassom, a condição que atingiu melhores eficiências (229 $g.kg^{-1}$) foi de 43,4±2°C e 18,5% (v/v) de concentração de enzima, que corresponde a 1,2 $mL.g^{-1}$, e foi observado um aumento médio de 12% na eficiência de hidrólise naqueles experimentos em que houve exposição aos efeitos do ultrassom. Os rendimentos obtidos são comparáveis às enzimas comerciais.

Para os testes de hidrólise utilizando a sonda ultrassônica, foi utilizada a temperatura ótima determinada anteriormente (43,4°C), a concentração de enzima foi reduzida consideravelmente, e os testes foram feitos para avaliar os efeitos da sonicação direta e indireta durante a sacarificação do bagaço de cana sem pré-tratamento. Os resultados demonstraram que há uma enorme diferença entre o uso de sonicação direta e indireta na hidrólise de material lignocelulósico. Os resultados utilizando sonicação indireta durante a sacarificação tiveram um aumento considerável, em média, 158% maiores que aqueles utilizando sonicação direta. O rendimento máximo de hidrólise foram 31,3 $g.kg^{-1}$ e 60,6 $g.kg^{-1}$

sob sonicação direta e indireta, respectivamente. O estado otimizado para a hidrólise de sonicação indireta é a concentração de enzima maior que 209.5 µL.g $^{-1}$, a irradiação de contínua ultrassom e amplitude de oscilação de 20%, o que corresponde a uma fonte de ultrassom de 60 W.cm^{-2} . Dessa forma, conclui-se que a utilização de sonicação indireta é mais indicada como auxiliar nas hidrólises, uma vez que a sonicação direta pode causar desnaturação da enzima e diminuir a eficiência do processo.

7 CONCLUSÕES E SUGESTÕES

O presente trabalho, viabilizou o desenvolvimento de um processo otimizado de produção de enzimas celulolíticas de *Trichoderma reesei*, utilizando bagaço de cana como principal fonte de carbono, suplementado com farelo de soja e água de maceração de milho por meio da tecnologia de fermentação em estado sólido, em escala de bancada. Um aumento de escala, utilizando um biorreator de leito fixo com aeração forçada proporcionou a produção de um complexo enzimático com atividades celulolíticas consideravelmente maiores. Sugere-se, então, um estudo mais detalhado do aumento de escala utilizando essa configuração de biorreator, envolvendo otimização das condições de operação e também estudo da cinética de produção de enzimas celulotíticas, bem como a utilização de novas configurações de biorreatores e maiores escalas de processo, a fim de viabilizar a produção dessas enzimas industrialmente.

A utilização direta do extrato celulolítico produzido para hidrólise enzimática de material lignocelulósico sem tratamento prévio mostrou-se uma alternativa viável, uma vez que as eficiências obtidas nas hidrólises em banho-maria e em banho com ultrassom foram próximas às encontradas em outros trabalhos utilizando enzimas comerciais, para concentrações de enzima de 1,2 mL por grama de bagaço. Além disso, constatou-se que o efeito do ultrassom, apesar de pouco expressivo, é positivo nas sacarificações do bagaço de cana não tratado, sendo que as eficiências obtidas foram sempre um pouco maiores naqueles experimentos sob efeitos da sonicação.

O estudo de sacarificações de material lignocelulósico exposto à sonicação de maior intensidade, cujos efeitos eram até então desconhecidos, mostrou que a sonicação direta a intensidades maiores que 60 $W.cm^{-2}$ resultam numa diminuição considerável na eficiência de hidrólise, resultado de uma possível modificação estrutural e, portanto, perda do poder catalítico dessas enzimas. Já a sacarificação com a exposição indireta ao ultrassom mostrou um aumento considerável na eficiência de hidrólise, para todas as condições experimentais, permitindo a conclusão de que a hidrólise de material lignocelulósico sob sonicação indireta, de média intensidade, é uma alternativa promissora para a melhoria da eficiência desses processos hidrolíticos. Além disso, é sabido que o ultrassom tem também efeitos sobre a estrutura da matéria prima. Logo, sugere-se, para trabalhos futuros, um estudo mais detalhado desta estrutura para auxiliar nas conclusões a respeito dos efeitos da exposição ao ultrassom.

Por fim, pode-se dizer que o desenvolvimento desse trabalho proporcionou um aprendizado contínuo e bastante sólido, principalmente pelas muitas dificuldades enfrentadas no decorrer das atividades experimentais. As etapas iniciais de cultivo do micro-organismo e fermentação em estado sólido foram críticas, tendo em vista as diversas limitações em controlar as variáveis de processo encontradas. Foram necessários inúmeros testes preliminares, em diversas condições distintas, para a obtenção das condições ótimas de processo e a produção do extrato enzimático em maior escala, exigindo muitas vezes paciência e, principalmente, persistência para que os objetivos do trabalho fossem alcançados. Mesmo assim, as dificuldades foram superadas, permitindo a obtenção de resultados bastante satisfatórios, fazendo com que todo o esforço tenha sido válido.

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ANEXOS

1. Artigo: Technological prospection on solid-state fermentation

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Abstract: Solid-state fermentation has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products. It has gained renewed attention not only from researchers but also from industry, especially in the last two decades. A review of patents and scientific articles is presented to cover the application of solid-state fermentation on the most diverse areas, including process development, substrate and microorganism preparation, process modeling and design of bioreactors.

Keywords: solid-state fermentation; bioreactor design, process and products, microorganisms.

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INTRODUCTION

Solid-state fermentation (SSF) is defined as any fermentation process occurring in the absence or near-absence of free water, employing a natural substrate as above, or an inert substrate used as solid support [1]. This process is known from ancient times in Asiatic countries, but it had nearly been ignored in western countries after 1940, due to emergence of submerged fermentation (SmF) technology, which was driven by the important development of penicillin in SmF that time [2].

In recent few years, SSF have been received more interest from researchers and industries worldwide, since several studies in different areas, particularly in solid waste management, biomass energy conservation and its application to produce high value–low volume products, have shown that SSF can give higher yields or better product characteristics than SmF [3]. Another great advantage of SSF compared to SmF is the lower capital and operating costs due to the utilization of low cost agricultural and agro-industrial wastes as substrates. The low water volume used in SSF has also a large impact on the economy of the process mainly because of the smaller fermenter-size, the reduced downstream processing, the reduced stirring and lower sterilization costs [4].

Uses of SSF have been continuously increasing for the development of bioprocesses, such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, biopulping, and production of value-added products [5].

There are various important factors to be considered for the development of a successful bioprocess under SSF conditions. Some of the most important include the selection of a suitable microorganism and solid support to be used. A diversity of microorganisms, including fungi, yeasts and bacteria can be used in SSF processes. Due to the low moisture

content in the SSF media, fungi are the most commonly used microorganisms because of their ability to growth in these environments. The selection of the microorganism to be used in a SSF process also depends on the desired end product [1, 6].

Despite the processing and biological advantages SSF has over SmF technology, there are some problems in scale up, purification of end products and biomass estimation that researchers have to overcome. Scale up in SSF has been a limiting factor since long because of the build-up of gradients in temperature, pH, moisture, oxygen, substrate and inoculums, but recently with advent of biochemical engineering a number of bioreactors have been designed which could overcome the problems of scale up and to an extent also the on-line monitoring of several parameters, as well as heat and mass transfer [5].

In this sense, this article brings the reader an analysis of various patents and scientific articles worldwide in the important and fast growing area of SSF. Aiming a better organization of this paper, patents will be presented focusing aspects such as product/processes development, biomass/substrate, and bioreactors in general.

RECENT PATENTS ON SOLID STATE FERMENTATION

Searching in patent databases is always harder than searching for scientific articles. This happens because most of inventors use the less known synonyms in their patent titles to intentionally hinder the search and prevent that other people or companies may use it for their own benefit. This way, all searches in this paper was made trying to overcome these difficulties and involve the largest possible number of patents in solid state fermentation.

A search done in the European Patent Office (EPO) [7] using the worldwide database, which includes a collection of published application from more than 90 countries, using the following keywords (“solid state fermentation” or “solid substrate fermentation”) in title or

abstract resulted in 399 records. Another search done among scientific manuscripts in the Web of Knowledge [8] including all databases, using the same keywords used for the patents search resulted in 3832 records. These numbers are probably much lower than the real number of patents and articles in this area, since some authors may use different terms to express solid-state fermentation. However, this search can give a great estimation of publications on solid state fermentation field.

Fig. 1 presents the estimated number of records concerning patents and scientific manuscripts on solid-state fermentation per year since 2000. From Fig. 1, it is possible to notice that the number of scientific articles published is much higher than patents, which was expected since a lot of studies and researches are necessary before the patent can be granted.

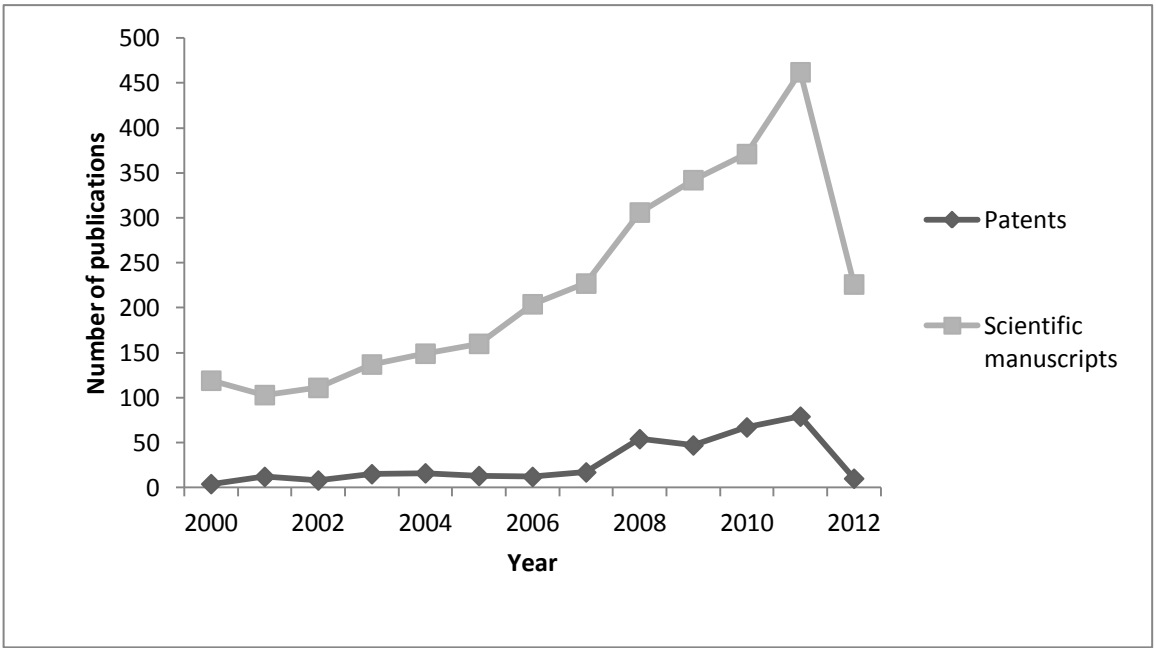

Fig. (1). Estimated number of publication on solid state fermentation per year.

Among all patents in SSF, almost 90% (approximately 354 patents) were published after the year of 2000. These statistics show the growing interest in all SSF area, especially in the last five years when the number of published patents per year increased faster, as showed in Fig. 1. Another important data to highlight is that almost 70% of all patents in SSF are Chinese (CN), others 2% are from Korea (KR) and around 1% are from Taiwan (TW),

whereas only 8% are from European countries (EP) and United States together (US), demonstrating that Asiatic countries have been leading the development of bioprocess using SSF.

The large annual number of patents on product/process development is depicted again in Fig. 2, which presents the distribution of patents among the above-mentioned three categories on a cumulative basis. Some patents are included in more than one category. Evidently, the number of patents on product/process is larger than the sum of patents on biomass and on bioreactors.

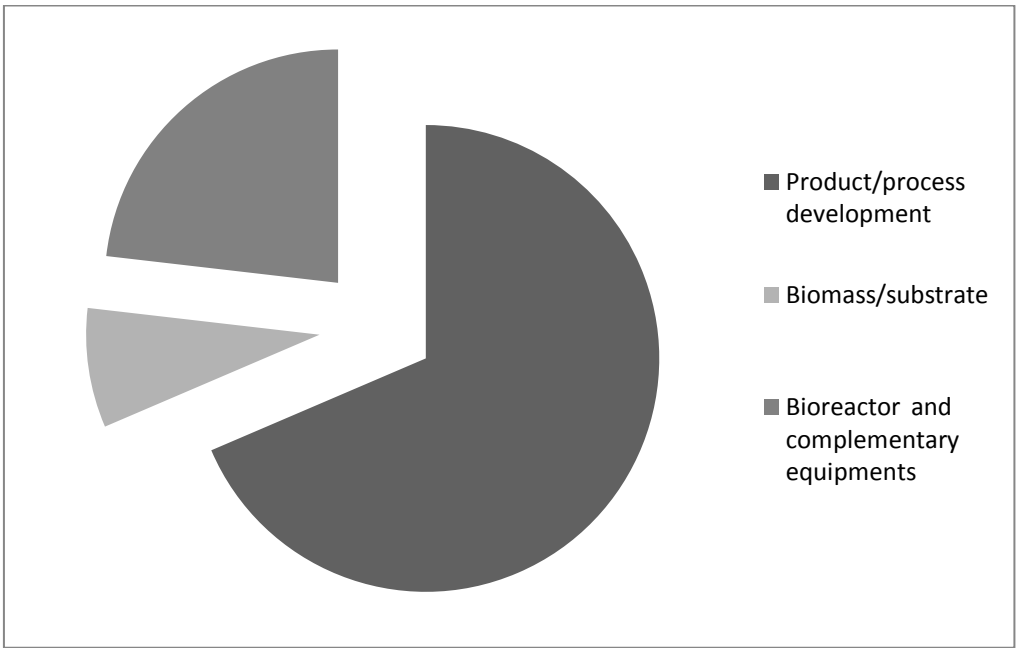

Fig. (2). The distribution of patents between the three major categories (Product/process development, Biomass/substrate and Bioreactor and complementary equipments) on a cumulative basis

It was verified that the first patent involving the application of SSF technology in a production process, for the solid-state fermentation of glucidic substrates [9], and also the first patent involving the preparation of biomass, which consisted in a method of a mold microorganism immobilization [10], were granted in 1985, whereas that involving the design

of a bioreactor for SSF was granted in 1984, and consisted in a recirculation tower bioreactor for solid-state fermentation [11].

Analyzing the patents applicants, it is possible to note that the Institute of Process Engineer of Chinese Academy of Sciences (abbreviated as INST PROCESS ENG CAS or INST OF PROCESS ENGINEERING OF CHINESE ACADEMY in the Espacenet website) has the greatest number of applied patents, totalizing 52 in all SSF field. Some of them involve methods for product development, some involve bioreactors in approximately the same proportion and also there are few patents that describe a method and a device for product development. There is only one patent comprising a preparation of a microorganism. Following this applicant, it is possible to point out Tsinghua University (UNIV TSINGHUA) with eight applied patents, and five of these involve ethanol production, and also Nanjing University (UNIV NANJING), with five applied patents.

Other interesting information to point out is that there is not any company dominating the patents on SSF field. The companies that have the greatest number of applied patents are Tianjin Shengji Group Co Ltd, with patents for feed preparation, and Tianjin Beiyang Biotrans Biote Co Ltd, with patents for organic acids production, and they have only three patents each.

In general terms, it can be seen that the great majority of applicants are universities or academic institutions, while only less than 20% of all granted SSF patents applicants are companies (CO or LTD). It illustrates the fact that there are still several researches being done to overcome the technological bottlenecks on scaling up solid state fermentation processes, and few companies were already able to apply solid state fermentation technology into an efficient industrial process.

Process Patents

The recent years have witnessed an unprecedented increase in the use of solid-state fermentation (SSF) for the development of bioprocesses in different areas, such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, biopulping, and production of value-added products. Table 1 summarizes the main patents on process/product deposited or granted, being presented a brief description of process. Among value-added products obtained by solid-state fermentation it is possible to point out biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, enzymes, organic acids, biopesticides (including mycopesticides and bioherbicides), biosurfactants, biofuel, aroma compounds, etc [1].

Table 1: Some of recent patents on process/ product development patents

Ref.*	Product; Subgroup	Brief process description
[29]	Fermentable sugars; Biofuels/Food, Feed or Beverage	It describes compositions and methods for the conversion of lignocellulosic material to fermentable sugars (hydrolysis or saccarification step) and to products produced therefrom, such as ethanol or foodstuff (fermentation step).
[16]	Seasoning products: food additives; Food, Feed or Beverage	A method for preparing a food seasoning product comprising a solid state fermentation step, an hydrolysis step and a thermal reaction step, wherein the solid state fermentation step, hydrolysis step and thermal reaction step are carried out in the same reaction vessel.
[49]	Forage grease; Others	A method for producing forage graese by solid state fermentation of maize starch and wheat bran by *Mortierella isabellina*. The substrate in a 1:3 ratio and 60% of water is inoculated after two days of fermentation, adjusting initial pH to 6.0, performing double temperature culture and fermentation for 7 days.
[12]	Mushroom soy; Food, Feed or Beverage	This method comprises in washing fresh mushrooms, cutting, mixing the fresh mushrooms with soybeans after pretreatment, steaming, cooling to room temperature, adding wheat flour and inoculating for solid-state fermentation.

[37]	Docosahexanoic acid; Organic acids	The invention comprises a process of producing polyunsaturated fatty acids from *Thraustochytriales* at high availability of oxygen by solid state fermentation or submerged fermentation in a batch, semi-continuous or continuous mode.
[22]	Beta-glucanase for feeding; Enzymes	This invention describes a method for Beta-glucanase production by solid state fermentation, which comprises the following steps of: (A) preparation of strain (B) preparation of culture medium (peanut hull meal and bran as main raw materials). The culture is convenient, the growth rate is high, and the fermentation period is greatly shortened (only 37 hours);
[47]	Microbial Organic fertilizer; Biopesticides and Biofertilizers	The present invention relates to the antagonistic bacteria for controlling the *Fusarium* wilt of continuous cropping banana and their microbial organic fertilizer. It belongs to technology of intensive agricultural production. The present invention separates two antagonistic bacteria NJN-6 and NJN-11 and produces the microbial organic fertilizer through inoculating the two said strains into pig manure compost and rapeseed cake compost to conduct solid-state fermentation.
[48]	*Bacillus thuringiensis* Biological pesticide; Biopesticides and biofertilizers	The invention provides a solid-state fermentation production process of a *Bacillus thuringiensis* biological pesticide by utilizing food wastes to co-culture Bacillus thuringiensis and Bacillus subtilis. The production process comprises the following specific steps of: inoculating a certain amount of dry food wastes with mixed strain, and fermenting for 40-50 hours to obtain the Bt biological pesticide raw powder.
[13]	*Lucid ganoderma* fermentation rice; Food, Feed or Beverage	The invention provides a method for producing *Lucid ganoderma* fermentation rice by utilizing solid state fermentation of rice; the *Lucid ganoderma* mycelia grows in the rice through a solid state fermentation manner to obtain the rice rich in the mycelia. The method comprises the following steps: 1. manufacturing a liquid strain; 2. manufacturing a rice solid state fermentation culture medium; and 3. inoculating and fermenting.
[23]	Bioethanol; Biofuels	The invention discloses a method for producing ethanol by continuous solid state fermentation of restaurant-kitchen garbage, with the following basic steps of: (1) steam explosion pretreatment on the restaurant-kitchen garbage; (2) addition of saccharification enzyme, cellulose, and active dry yeast into the treated raw materials; (3) raw materials continuous delivery to a reactor under set conditions to perform continuous ethanol fermentation and extraction; and (4) fermentation residue automatic discharge out of the reactor, and drying the fermentation residue in a continuous dryer to form an organic fertilizer.
[24]	Biobutanol; Biofuels	The method for butanol production from restaurant-kitchen garbage comprises the following steps: (1) liquid-solid separation on the substrate by using a centrifugal dehydrator; (2) steam explosion pretreatment; (3) saccharification enzyme addition into the treated substrate, and delivery of cellulase to a continuous solid state fermentation reactor; (4) continuous inoculation of the cultured *Clostridium acetobutylicum* seed solution to a culture medium; (5) raw materials continuous delivery to the reactor under set conditions to perform continuous butanol fermentation and extraction; (6) The condensed solvent rectification to obtain pure products of acetone, ethanol and butanol; and (7) further drying the fermentation residue to prepare an organic fertilizer.

[25]	Hydrogen and Methane; Biofuels	The method for producing hydrogen and methane through continuous solid state fermentation of kitchen garbage comprises the following steps: (1) dewatering substrate; (2) steam explosion; (3) mixing sludge and initiating continuous solid state fermentation; (4) separately feeding the kitchen garbage after fermentation is initiated, and simultaneously inoculating newly fed kitchen garbage subjected to steam explosion with bottom fermentation residue on the top; (5) agitating the materials by using circulating wind in the fermentation process; (6) automatically extracting and separating the materials when the gas pressure is higher than 0.1 MPa; and (7) further drying the fermented residue to be used as an organic fertilizer.
[38]	Resveratrol: nutritional supplement; Medicine	The invention provides a method for preparing resveratrol by converting polydatin by combining steam explosion and biological conversion methods: after the polydatin treatment by steam explosion, the cell walls of it are broken, thereby being favorable for subsequent biological conversion by solid state fermentation utilizing cellulase or microorganisms capable of generating the cellulase, thus the polydatin can be completely converted into the resveratrol.

*Ref. = Reference

Among the three major groups of patents defined previously in this paper, the process is undoubtedly the vaster group, including patents for bioprocess or product development in all diverse areas where SSF can be applied. For this reason, the category was subdivided according the applicability in different industries, as can be seen in Fig. 3.

Refining the search done previously in the European Patent Office (EPO) by adding the following keywords (process* or method or product*), it resulted in 290 records.

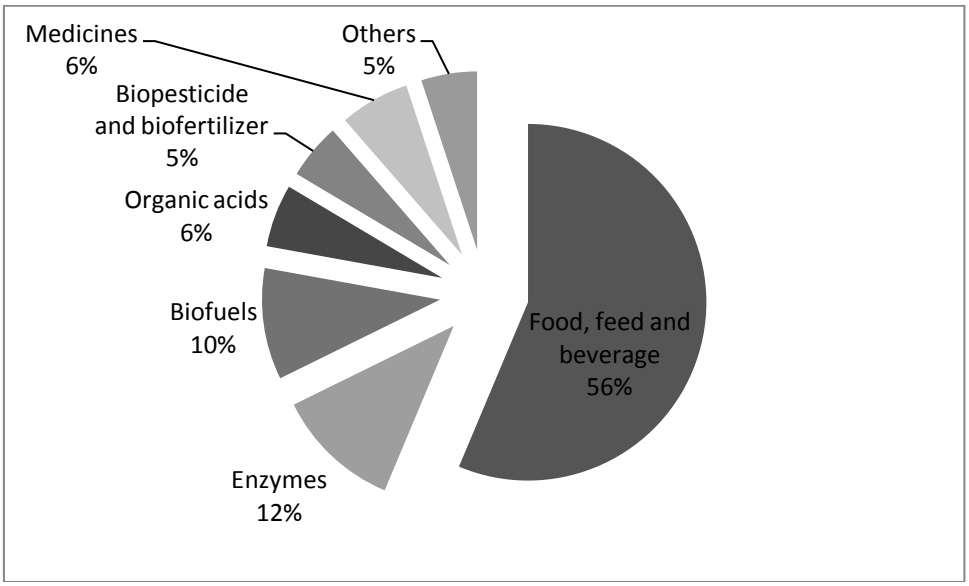

Fig. (3). The distribution of Product/process development patents in different subareas according the applicability in different industries

Due to the large number of records in this refined search, it is difficult give an explanation about each patent. For this reason, some of the most important patents, according to the authors' criteria, as well as a brief description of each process are presented in Table 1. Two criteria were used by the authors to choose the patents in this table: at least one patent of each subgroup should be presented, and the most recent patents should be preferred from others. Also, the most interesting processes according to the authors' will be discussed in the following paragraphs.

As can be seen in Fig. 3, the great majority of patents involves a process or product development to be applied on animal feed, food (production of sauces, vinegar etc.) or beverage industries (brewing, white spirit, alcoholic liquor and wine), such as feed additives (protein, peptide, essences, flavors etc.) and enzymes in general. As enzymes can also be applied in other industries, it was classified as a separated subgroup (Fig. 3).

Among the food, feed and beverage group, there are various patents involving the preparation process of Asian food. Wang [12] invented a process for mushroom soy production, which after be processed by the fermentation method it become rich in vitamin and protein and has a health-care effect. Another interesting patent that can be pointed out is one invented by Wang et al. [13], who invented the production process of *Lucid ganoderma* fermentation rice, a product used as food that contains various nutritive components and mineral elements to improve the immunity of the organism, inhibit tumors, stabilize the blood pressure and improve the fat metabolism. The same author had also published patents for production of *Grifola frondosa* and *Cordyceps militaris* fermentation rice [14; 15], using the same process methodology except the microorganism, and resulting in a product with similar properties for a healthy people diet. As these three last production processes cited above [13,

14 and 15] are equals, only the patent for *Lucid ganoderma* fermentation rice production is presented in Table 1.

One of the most recent patents published this year and invented by Helge et. al [16] describes a method for preparing seasoning product, such as sauces, pastes and others flavoring agents to be applied in the food industry (Table 1).

Among enzymes production, there are patents for production of lipase, cellulase, xylanase, α-amylase, β-glucanase, etc. as well as some complex of multi-enzymes [17; 18; 19; 20; 21; 22]. One of these patents is presented in Table 1, invented by Liu et. al (2011), and summarize a method for production of β-glucanase from solid state fermentation of peanut hull meal by *Aspergillus niger*. The β-glucanase for feeding has the advantages of high enzyme activity, good heat resistance and good application effect [22].

Another area that has a significant number of patents is the biofuel production, which includes mainly ethanol and butanol process development. Chen and Li [23, 24, 25] have published three interesting patents in this area last year, for production of ethanol, butanol and hydrogen and methane through continuous solid-state fermentation of restaurant kitchen garbage. It was noted that by adopting the continuous solid state fermentation process in these cases, wastewater treatment is avoided, the treatment efficiency is increased, the energy consumption is reduced, and the production cost of both biofuels is reduced.

Despite biofuels subgroup has not the largest amount of processes patents, it is one of the areas with a big potential to grow rapidly in the coming years due to the large number of researches mainly for production of ethanol from agroindustrial residues by solid state fermentation combined with the development of cheaper processes to produce enzymes to be used in the enzymatic hydrolysis step for ethanol production. In this context, a few paper reviews discussing the potential for biofuels production from agroindustrial residues [26] and enzyme production for biofuels [27, 28] can be cited. The most recent published patent in this

year describes a process for the conversion of lignocellulosic material to fermentable sugars [29], which comprises the first step for lignocellulosic ethanol production. There are also some other patents involving the ethanol production by lignocellulosic [30, 31, 32, 33, 34] and starchy materials [35, 36].

Regarding patent applicants for ethanol production, it is possible to note that Institute of Process Engineer of Chinese Academy of Sciences and Tsinghua University have applied seven of the total twelve published patents.

According to Figure 3, around 6% of the patents found in this search involve a process for organic acids production. One of these patents, invented by Rakesh R et. al and published in 2011, describes a method for production of docosahexanoic acid, which is a polyunsaturated fatty acid [37]. This process is also briefly presented in Table 1.

In a lower number of granted patents, it is possible to notice the pharmaceutical area, including some antibiotics and other kind of drugs, especially components to be used as nutritional supplements or drug components. As example, it can be pointed out a patent describing a new process for resveratrol production (Table 1), which is a natural phenol with properties of life extension that is usually sold as nutritional supplement. This invention has the advantages of easy control of process, stable products and lower costs and according to inventors it is also suitable for industrial production [38].

Biopesticides and biofertilizers subgroup also have lower number of patents, but it is due to the fact that the research in this area have been developed in the last few years. As well as biofuel area, it has a great potential to grow fast because of the increasing interest in non-polluting technologies to be used in agriculture combined with reuse of residual materials [39, 40, 41]. It can be noted that the first patent in this area was granted in 2008 [42], and all others only in 2011 [43, 44, 45, 46], which proves that is a new area and still have to be developed. The most recent patent in this area, invented by Gong et. al and also published in

2011, describes a method for a *Bacillus thuringiensis* biological pesticide production (Table 1). Compared with the Bt (*Bacillus thuringiensis*) biological pesticide prepared by Bt fermentation, the Bt biological pesticide prepared by co-culturing Bs (*Bacillus subtilis*) and Bt using the food wastes as substrate has higher titer, and can be directly used for agricultural production. According to the patent authors', this production process improves the utilization efficiency of the food wastes and the product titer, and realizes the resource treatment of the food wastes and the popularization of the pollution-free Bt biological pesticides [48].

Biomass/Substrate Patents

There are several important factors, which affect SSF processes. Among these, selection of a suitable strain and substrate and selection of process parameters (physical, chemical and biochemical) are crucial. While efforts largely continued to exploit filamentous fungi and yeast for the production of various products, attempts also have been made to explore the possibilities of using bacterial strains in SSF systems [1].

Refining the EPO patent search by adding the following keywords (biomass or strain or fungi or bacteria or yeast or substrate or mold or microorganism), 21 records were found. However, most of those results are also included in the other two categories and among those records, only around eight patents can be classified on Biomass/substrate category. On the other hand, the majority of patents that should be classified in this category are not included in those 21 records because the authors used the microorganism or substrate name in the patent title, instead of using one of the keywords used to refine the search. This way, it was necessary to look all the initial search records, one by one, to find more patents in this group, and it resulted in adding more 27 patents to this group. Finally, there are around 35 patents in the Biomass/substrate category. Some of these patents are summarized on Table 2.

Table 2: Some important patents in Biomass/Substrate preparation

Reference	Classification	Brief patent description
[50]	Biomass/ Product	The invention provides a new multi-strain (*Monascus rubber, Aspergillus oryzae, Aspergillus niger*) starter propagation process for producing soy sauce by using rice dregs as raw materials.
[51]	Biomass/ Product	The invention relates to an organic synthesizing method of traditional Chinese medicinal herbs, microbes and inorganic micro elements, in particular to a preparing method of solid fermentation for a traditional Chinese medicinal herb yeast selenium.
[52]	Biomass/ Substrate preparation	The invention discloses a fermented feed. The fermented feed is prepared by the following steps of: performing amplification culture on *Lactobacillus acidophilus* ACCC10637 and *Bacillus subtilis* ACCC10619 respectively to obtain amplification culture solution by taking soybean whey as a raw material of a liquid culture medium, and performing amplification culture on *Candida utilis* ACCC20060 to obtain an amplification culture medium by a semi-solid culture medium;
[53]	Biomass/ Product	The invention relates to a rapid preparation method of fluid inoculum for solid-state fermentation production of industrial microorganism, an application method and a device thereof.
[54]	Substrate/ Biomass	The present invention also provides SSF media for fungal cultivation. Although the SSF method provided in the present invention can be used in growing most fungi, the best list of fungi includes *Cordyceps sinensis, Ganoderma lucidum, Antrodia camphorata, Trametes versicolor, and Agaricus blazei*.
[55]	Substrate/ Biomass	A process of preparing a mushroom growth medium from a cellulosic material is provided in which the cellulosic material used in the solid-state fermentation process is chemically ammoniated.

Almost all patents covering substrate or biomass preparation also include a subsequent process for production of some specific product [50, 51, 52, and 53]. It was expected since the microorganism and substrate preparation are mandatorily the initial steps for any bioprocess. It is also important to highlight that patents in this group generally use agricultural products or agroindustrial residues as substrates, as can be noted in patents described on Table 2. Among these, it is possible to cite a Chinese patent published in 2011 by Hu et al. [50], which invention provides a new multi-strain (*Monascus rubber, Aspergillus oryzae, Aspergillus niger*) starter propagation process for producing soy sauce by using rice dregs as raw materials. The greatest novelty in this process is that bean pulp serving as a main raw material

in the conventional brewing process is replaced by the rice dregs serving as discarded resources in sugar industry, monosodium glutamate industry and rice vinegar industry, so the production cost of the soy sauce is reduced greatly. Simultaneously, the saccharifying enzyme, neutral protease and acid protease of the prepared yeast rice are higher than those of the yeast rice of the conventional starter propagation, so this new process is opened up for the recycling of the rice dregs and the production of the high-quality soy sauce.

Also, there are few patents specifically about preparating substrates or propagating microorganisms. One of these was invented for Pei-Jung and Chung-Guang [54] and published in 2002. This invention provides a solid-state fermentation method for propagating fungi, which is effective for both small and large-scale and also this invention provides a SSF media for this propagation. According to the authors, this method not only produces high yield of fungi, but also stimulates the production of fungal metabolites, particularly the kinds with pharmaceutical and medicinal activities. Other patent that can be cited was granted in 1992 by Dunn Coleman Nigel. It describes a method for production of mushroom cultivating substrates using a chemically ammoniated process. The chemical ammonization step reduces composting time, particularly preconditioning time, while providing a compost and a mushroom bed material comparable to that achieved by commercial composting processes, which do not use ammoniated straw [56]. This was the first granted patent specifically about substrate preparation.

Bioreactor Patents

Today, significant advances have been made towards the development of quantitative scale-up strategies for SSF bioreactors, through mathematical modeling of the biological phenomena and mass and heat transfer phenomena which occur [56]. Mathematical modeling

is an essential tool for optimizing bioprocesses not only because it can guide the models design and operation of bioreactors, but also it can provide an understanding into how the various phenomena within the fermentation system combine to control overall process performance.

In the last ten years, according to a search made on the Web of Knowledge (keywords: "solid state fermentation" or "solid substrate fermentation", from 2003 to 2012), there were over 2900 scientific publications about development or modeling of process and devices involving solid-state fermentation in general. Among these, more than 140 are specifically related to designing or modeling solid-state bioreactors [keywords: "solid state fermentation" or "solid substrate fermentation" and (bioreactor or reactor or equipment or device or tank or apparatus), from 2003 to 2012].

Regarding patents, another refined search was done on European Patent Office (EPO), adding to the original keywords the following (bioreactor or reactor or equipment or device or tank or apparatus). This search resulted in 98 records, including different types of bioreactor and complementary devices, such as control systems to be applied on solid-state bioreactors.

The increasingly number of scientific publications and patents on bioreactor field clearly highlights the effort that the scientific community and the industry have been doing to develop novel bioreactors designs to overcome the scale up problems on solid state fermentation. Three types of bioreactors are commonly used in SSF processes: packed-bed, rotating drum and fluidized bed, but all three has their particular problems that have to be overcome. Packed bed bioreactors, which typically have static substrate beds, are suited to SSF processes in which mixing is deleterious, such as the production of fungal spores. They allow better control of fermentation parameters than is possible in trays. In large trays problems arise with high temperatures and lack of oxygen in the center. In packed beds these problems are partially overcome by forced aeration, but high temperatures can still be reached

near the air outlet. Rotating drum comprises continuously mixed SSF bioreactors with air circulation and the biggest problem of this type of bioreactor is that heat, mass and oxygen transfers is greatly reduced by the agglomeration of mycelium and the substrate particles, particularly starchy and sticky materials. When the rotation rate of the drum is increased, it can affect the mycelium growth presumably because of shear effects. This problem also can affect fluidized bed bioreactor performance, since it is also a continuously mixed bioreactor [56, 57]. This way several novel types of bioreactor have been invented trying to overcome these problems.

The first SSF bioreactor patent was applied by Kingston University in 1984 and it consists in several perforated trays, each one containing a layer of substrate and loaded one above another in a recirculating tower, with air moving upwardly through the reactor and nutrient medium circulating countercurrent to the air. This equipment was developed to produce single cell proteins from lignocellulosic materials, but, according to the patent inventors, it can also be used to produce others valuable products from solid materials [11].

After that, several other bioreactor configurations were developed, always trying to improve the efficiency and solve the operational problems to make SSF industrially feasible. Some technological patents describing different bioreactors configurations are presented on Table 3.

Table 3: Some bioreactors patents for SSF

Reference	Brief patent description
[11]	A method and apparatus for producing single cell proteins or other products from a substrate made up of solid lignocellulosic materials, such as wood chips, wherein a plurality of perforated trays each containing a layer of substrate are loaded one above another in a recirculating tower reactor. After innoculating the layers of substrate with a white rot fungi, such as *Polyporus anceps*, a nutrient solution is recirculated downwardly through the reactor, at a rate sufficient to keep the microorganism active and the layers of substrate moist, and air is injected upwardly through the reactor at a rate sufficient to maintain aerobic fermentation and about 2-3% carbon dioxide in the exhaust gases.

[58]	A fermentation device is described whereby a single fermentation vessel is used to conduct deep bed solid state fermentation of microorganisms to generate useful products. The device incorporates an assembly for agitation of the fermentation substrate which allows injection of fluids into the substrate bed to maintain homogenous culture conditions throughout the substrate regardless of the depth of the substrate bed.
[59]	The invention discloses an enclosed solid state fermentation bioreactor. The bioreactor comprises a solid state fermenter, an air inlet system, a humidification system and a tail gas detection and collection system. The solid state fermenter is a sealed container, and a central shaft of the fermenter is provided with a reverse double spiral stirring paddle which allows materials to carry out bidirectional movement, so an adhesion problem which appears in stirring solid materials can be solved and a mixing effect can be enhanced. A top tail gas outlet of the fermenter is connected with an evacuation tube and a CO2 collector, and a fermentation process and a fermentation state are displayed through detecting the CO2 content. A jacket is arranged on the outside the fermenter, a bypass is arranged on a humidifier, and the fermentation bed temperature can be automatically adjusted through feeding back information to a control system by a thermocouple which is on an upper part of the fermenter to adjust solenoid valves of jacket cooling water and the humidifier bypass. The humidifier temperature and periods and times of stirring and spraying can be preset according to material fermentation characteristics.
[60]	The utility model provides the solid-state fermentation reactor, which comprises a fermentation tank body and a stirring and rotating mechanism, wherein the stirring and rotating mechanism comprises a spindle; the spindle comprises an inner spindle positioned in the fermentation tank body and an outer spindle positioned outside the fermentation tank body; the inner spindle is provided with stirring paddles; the spindle and the stirring paddles are of the hollow pipe bodies; air ports are arranged on the stirring paddles; an inoculating pipeline and an air pipeline are arranged on the outer spindle; and the stirring and rotating mechanism also comprises an electric motor that drives the fermentation tank body to rotate along the spindle.
[64]	The invention relates to a ventilation-humidification coupled solid-state fermentation tank which comprises a main tank body the outer side of which is provided with a temperature control jacket, a tank cover and a material tube. The top surface of the tank cover is a screen mesh type tank cover surface, and the tank cover and the main tank body are fastened through a fastening buckle arranged at the edge of the top of the tank cover; a screen mesh type material tank bottom is arranged on a supporting ring stand and is provided with a first rubber ring washer for sealing; and a ventilation-humidification coupled unit is arranged at the bottom of the main tank body and comprises branched vent pipes, a sterilized water layer and vent pipes, and the branched vent pipes are distributed inside the sterilized water layer and are connected with the vent pipes. According to the solid-state fermentation device, the dust in the air is filtered off through introducing the air into the sterilized water layer, and the air containing water molecules is introduced into materials, so that air sterilization inside the tank is realized while the material humidity is maintained, and the redundant heat generated during the solid state-fermentation is dissipated; solid-state fermentation of aerobic bacteria is realized; and the ventilation humidification coupled solid-state fermentation tank has the advantages of novel principle, simple structure and low manufacturing cost, and is convenient in operation, generalization and production.

[65]	The invention belongs to the technical field of fuel ethanol production through microbial fermentation, in particular to a solid-state fermentation process and a device for preparing fuel ethanol. The process mainly comprises two steps that: strain adding and continuous solid-state fermentation, i.e. fermentation strains are added into fermentation raw materials before crushed materials enter a continuous fermentation tank body, and then, the mixed fermentation materials are continuously fermented in the continuous solid-state fermentation tank so that fermentable sugar is converted into ethanol. The continuous solid-state fermentation is adopted in the invention, the fermentable sugar content in straws can be sufficiently utilized, the use of preservatives is avoided, the yield of the ethanol is improved, the continuous solid-state fermentation device is adopted, the traditional production mode is changed, the continuity of the solid-state fermentation process is really realized, the rotating speed of the fermentation tank body and the plate shoveling angle can be regulated, and the adjustability and the controllability of the production are enhanced.
[66]	The invention discloses a peristaltic solid-state fermentation technology and device for traditional Chinese medicine. The technical scheme is to lay 2-8 silicone rubber gasbags at the bottom of a fermenter, wherein, the gasbags are presented Tai Chi shape, U-shape, M-shape, semicircle shape or bar shape; on the gasbags, a bubble prominence is arranged; the gasbags are connected with a compressor to periodically open or close a solenoid valve through a controller; the gasbags go up or down during the solid-state fermentation of the traditional Chinese medicine, thereby realizing a periodic peristalsis stimulation, playing a role in mixing, loosening and resisting agglomeration of the traditional Chinese medicine agglomeration during solid-state fermentation, strengthening the heat and mass transfer during solid-state fermentation, shortening the fermentation period, and improving the fermentation efficiency and the yield.
[67]	The invention discloses a bioreactor for enhancing mass transfer and heat transfer by using a microtubule system, and designs a bioreactor which has pipelines similar to plant roots in the reactor by simulating the characteristic that the soil and the plant roots exchange substances. The pipeline consists of a plurality of rigid main pipelines and flexible micro-pipelines, wherein one end of the main pipeline is fixed on the wall of the bioreactor, while the other end extends inward the bioreactor; the micro-pipelines are communicated with the main pipelines; a sac capable of contracting and expanding outside the reactor is communicated with a big pipeline; the contraction and expansion of the sac controls the pressure of fluid or gas in the pipeline; and the pipeline wall is made of a microfiltration membrane, an ultrafiltration membrane or a nano-filtration membrane, so that substances and energy inside and outside the pipeline can be exchanged through the pipeline wall. The bioreactor can realize static culture, overcome the defect of shearing stress of a stirred reactor on cell growth, saves stirring energy, can be used for anaerobic fermentation, aerobic fermentation, liquid-state fermentation and solid-state fermentation, and is also applicable to cell culture of animals and plants.

James [58] invented in 2001 a fermentation comprising a single fermentation vessel used to conduct deep bed solid-state fermentation of microorganisms to generate useful products. The device incorporates an assembly for agitation of the fermentation substrate which allows injection of fluids into the substrate bed to maintain homogenous culture conditions throughout the substrate regardless of the depth of the substrate bed.

Xu et al. [59] had invented an bioreactor that, according to the authors, can overcome the bed overheating problems, which appears in a fermentation peak period and is difficult to solve in solid fermentation processes, by removing heat of fermentation through a plurality of modes such as jacket cooling water adjusting, cold air accessing, atomized water spraying, material stirring and the like. Their bioreactor comprises a solid-state fermenter, an air inlet system, a humidification system, a tail gas detection and collection system. The humidifier temperature and periods and times of stirring and spraying can be preset according to material fermentation characteristics. Also, this invention has a reverse double spiral stirring paddle which allows materials to carry out bidirectional movement, so an adhesion problem which appears in stirring solid materials can be solved and a mixing effect can be enhanced.

Another bioreactor invented by Fang et al. [60] in 2010 also promises to solve some of the problems existing in the traditional solid-state fermentation device, such as that the fermentation is not complete, the fermentation process parameter is difficult to monitor, the fermentation is easy to be polluted by bacteria, etc. The utility model provides the solid-state fermentation reactor, which comprises a fermentation tank body and a stirring and rotating mechanism, wherein the stirring and rotating mechanism comprises a spindle; the spindle comprises an inner spindle positioned in the fermentation tank body and an outer spindle positioned outside the fermentation tank body; the inner spindle is provided with stirring paddles; the spindle and the stirring paddles are of the hollow pipe bodies; air ports are arranged on the stirring paddles; an inoculating pipeline and an air pipeline are arranged on the outer spindle; and the stirring and rotating mechanism also comprises an electric motor that drives the fermentation tank body to rotate along the spindle.

Among patents for bioreactor control system, there are two temperature control device for solid-state fermentation: the first was invented by Fan [61] and published in 2009 and the second one was invented by Lu et al. [62] and published in 2011. Both were designed to

control the temperature of biological feed. Also, Rong-Yuan [63] had invented a precisely monitoring equipment for beneficial fungi solid-state fermentation, which includes a control system to ensure uniform inner temperature and humidity. There is also bioreactors configurations for enhancing ventilation and substrate humidification [64], enhancing mass and heat transfer by using a microtubule system [67] and many others configurations for different purposes.

Technological prospection on Brazil

Searching patents in the Brazilian database (INPI) [68] using the same keywords used before, 16 records were found. Among these records, 14 patents describe the most diverse production processes in SSF, including ethanol, esters, lipids, enzymes, biopolymers, organic acids, pigments, food additives etc., while only 2 involve bioreactors design. An interesting information highlight is that 87.5% of all granted patents in Brazilian database have universities or a physical person as applicant, as well as was noted in the worldwide search, and all few patents applied by companies are from other countries (China and Germany). Also, there is no Brazilian patent on worldwide database. From these data is possible to note that Brazil is still emerging in SSF field, and despite it has a significant number of granted patents, there is still no industrial application for these processes.

RECENT SCIENTIFIC APPROACH ON SOLID-STATE FERMENTATION

In recent years, several excellent reports have appeared providing a great deal of knowledge and understanding of the fundamental aspects of solid-state fermentation. The number of scientific manuscript in this area is large, as can be seen in Fig. 1, and it is also growing fast. For this reason, it is difficult to review all solid-state fermentation aspects in

only one paper. This way, this work will analyze and discuss the published review articles on solid-state fermentation.

From the beginning of 2000 until now, there are several published reviews discussing the most different aspects of solid-state fermentation. Modeling is the most crescent area on solid-state fermentation field due to its importance to processes scale up and for this reason the majority of published reviews discuss it. Pandey [69] had discussed general aspects, biochemical engineering aspects (modeling and design of bioreactors), and application of solid state fermentation in general. Singhania et al. [5] had updated Pandey's work, focusing on solid state fermentation process and product developments mainly from 2003 to 2009.

Pandey et al. [1] had reviewed the different bioprocesses based on solid-state fermentation, as well as the variety of products obtained by solid state fermentation, with no coverage on engineering and modeling aspect. They had recognized that there has been much development on application of solid-state fermentation in various areas such as bioremediation and biodegradation of hazardous compounds, biological detoxification of toxic agro-industrial residues, biotransformation of crops and crop residues for nutritional enrichment, biopulping, etc., and product-developments such as biologically active secondary metabolites, including antibiotics and other drugs, enzymes, organic acids, biopesticides, including mycopesticides and bioheribicides, biofuels (ethanol and other organic solvents), biosurfactants, food flavour compounds, etc.

On the other hand, Mitchell et al. [56] had focused their review on the advances in understanding how to design, operate and scale-up solid-state fermentation bioreactors, reviewing the modeling of micro scale and macro scale phenomena as well as different types of bioreactors and control systems. Lenz et al. [70] have also discussed modeling on solid-state fermentation, including modeling and simulation of microbial growth as well as heat and mass transfer on both microscale and bioreactor scale, guidelines to industrial scale-up

derived from models of bioreactor performance, new techniques for spatial mapping of environmental factors and biomonitoring, parameter optimization using statistical and heuristical methods as well as advanced approaches in process control. Durand [57] had reviewed the various reactor designs and focused on the differences between lab-scale and industrial-scale designs.

There are also some other reviews covering all the engineering aspects of solid-state fermentation, such as mass and heat transfer, design, scale-up, monitoring and control [6, 71]. Some reviews discuss specific aspects of solid state fermentation. Gervais and Molin [72], for example, had studied deeply some physical parameters, relating to the water state of the medium and implicated in the variations of metabolism for solid-state media. Then, the mechanisms of the action of water at the cellular level had discussed for specific works. In another review, Hölker et al. [73] discuss solid state fermentation specifically with fungi, focusing on production of enzymes, as well as secondary metabolites and spores. Banerjee and Bhattacharyya [74] had focused their review in a tool of optimization for solid state fermentation called Evolutionary Operation (EVOP).

FINAL CONSIDERATIONS

There have been significant developments in application of SSF technology over past few years in various areas. From this review it is possible to point out that the product and process development is the widest area among published patents and the major applicability of solid-state fermentation technology occur in feed and food industry, followed by enzymes and biofuel industries. Also, it can be observed that there are a lower number of patents for biomass and substrate preparation and most of them are attached to a process methodology. Regarding bioreactors it is evident the hard effort that researches and companies have been

doing to achieve larger scales on solid state fermentation systems as it offers a viable alternative for many operations including waste disposal and value-added products from these wastes, as well as reduction of operation costs when compared to submerged fermentation systems. Solid-state fermentation is already an important technology for the development of bioprocess and if this area continues the trend to grow fast it may be well developed at par with submerged fermentation technology in a near future.

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