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PRODUÇÃO DE CUTINASE POR *Fusarium oxysporum* UTILIZANDO SUB-PRODUTOS AGROINDUSTRIAIS

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RESUMO

Cutinases são enzimas amplamente encontradas na natureza, podendo ser obtidas de fontes vegetais ou microorganismos. Trata-se de uma esterase que atua tanto como esterase quanto como lipase, capaz de hidrolisar uma ampla variedade de ésteres sintéticos e triglicerídeos. Este perfil de atividade oferece-lhe um número de aplicações importantes nas indústrias como detergentes de roupas e louças, alimentos, fármacos, agroquímicos e cosméticos.

Utilizando a fermentação em estado sólido (FES) é possível a produção de uma enzima com características interessantes de catálise, com baixo custo de produção e bom rendimento, e que possa ser empregada na indústria de modo geral. Este trabalho utilizou FES para produção de cutinase por *Fusarium oxysporum* utilizando sub-produtos agrícolas visando à seleção dos melhores meios e indutores para produção da enzima (utilizando fontes de carbono e minerais), otimização das condições de cultivo (avaliando os parâmetros temperatura e quantidade de água do meio além do estudo do inóculo), produção de cutinases utilizando diferentes substratos (farelo de trigo, casca de soja, farelo de arroz e produzida por meio líquido) e comparação entre eles, além da aplicação das enzimas em reações de esterificação para separação enantiomérica de substâncias racêmicas como o 2-octanol e o Ibuprofeno.

De um modo geral o trabalho mostrou a produção de enzima de grande interesse comercial usando substratos diferentes e seu comportamento frente a reações de esterificação. Três meios sólidos foram selecionados (farelo de trigo, casca de soja e farelo de arroz) e suas condições de cultivo otimizadas (temperatura 28ºC e quantidade de água do meio 100%). Nos testes de aplicação das cutinases, para resolução enantiomérica do 2-octanol todas as cutinases tiveram bons valores de esterificação e enantioseletividade com destaque para farelo de trigo (76,4% de esterificação após 200 horas) e casca de soja (71,9% de esterificação após 200 horas). Para resolução do fármaco racêmico Ibuprofeno as cutinases mais efetivas foram as produzidas por farelo de arroz (5,1% de conversão após 200 horas) e meio líquido (2,9% de conversão após 200 horas).

Esses resultados mostraram a diferença entre o comportamento de cutinases, do mesmo microorganismo, produzidas em diferentes meios, e a grande possibilidade de utilização de cutinases para processos de interesse industrial.

ABSTRACT

Cutinases enzymes are often found in nature and may be obtained from plant sources or microorganisms. This is an enzyme that acts both as esterases and as lipases, capable of hydrolyzed a several variety of synthetic esters and triglycerides. This activity profile gives a number of important applications in industries such detergents, clothes and dishes, foods, drugs, chemicals and cosmetics.

Using solid state fermentation SSF it is possible production of an enzyme interesting of catalysis with low production cost and good yields, and it can be employed in the industries. This work used SSF for the cutinase production by *Fusarium oxysporum* using agro-industrial sub-products to find selection of the best media and inducer to enzyme production (using sources of carbon and minerals), optimization of fermentation conditions (modifying the parameters temperature and water amount and after the inoculum study), cutinases production using different substrates (wheat bran, soy rind, rice bran and produced by liquid medium), comparison among them and enzymes application in esterification reactions to enantiomeric separation of racemic substances such 2-octanol and drug Ibuprofen.

In general the work showed enzyme production of great commercial interest using various substrates and their behavior in esterification reactions. Three solid media were selected (wheat bran, soy rind, and rice bran) and their fermentation conditions optimized (temperature 28ºC and water amount 100%). In tests of cutinases application for enantiomeric resolution of 2-octanol all cutinases had good values to esterification and enantioselectivity and the best results was using wheat bran (76.4% of esterification after 200 hours) and soy rind (71.9% of esterification after 200 hours). The test using racemic drug Ibuprofen the more

effective resolution was found with cutinase produced by rice bran (5.1% of esterification after 200 hours) and liquid medium (2.9% of esterification after 200 hours). These results showed the difference among behavior of cutinases using same strain, produced by different media, and the great possibility of cutinases with interesting catalysis characteristics and it can be employed in the industry.

CAPÍTULO I – INTRODUÇÃO GERAL

Cutinase é uma enzima que catalisa a hidrólise de um biopoliéster insolúvel, a cutina, o componente estrutural da cutícula das plantas. Há alguns fungos fitopatogênicos que crescem em cutina como única fonte de carbono, excretando a cutinase como uma enzima hidrolítica induzida. As cutinases representam uma ponte entre as esterases, as quais atuam em substratos solúveis, e as lipases, que exibem ativação interfacial, necessitando de interface água/lipídeo para a sua atividade. São utilizadas para diferentes tipos de reação: hidrólise, esterificação, inter ou intra-transesterificação do triglicerídeo eficientemente. Este perfil de atividade dá-lhe um número de aplicações importantes nas indústrias que variam daquelas que tratam dos detergentes aos alimentos, e aos produtos químicos.

A procura por esterases mais robustas, cuja aplicação em sistemas não aquosos e/ou heterogêneos seja mais eficiente, é uma necessidade eminente da indústria química, farmacêutica e alimentícia. As cutinases são estudadas há poucos anos e a literatura mostra resultados promissores neste sentido, por esse motivo há grande necessidade de encontrar novas técnicas de produção da enzima, que consiga alta produtividade, estabilidade prolongada dos produtos além de baixo custo na produção.

Como alternativa a processos convencionais, a fermentação em estado sólido (FES) tem sido aplicada com grande êxito a processos fermentativos. O termo fermentação sólida ou fermentação semi-sólida aplica-se ao processo de crescimento de microrganismos sobre substratos sólidos sem a presença de água livre circulante, de modo geral são todos os processos que utilizam como

substrato partículas sólidas. Trata-se de um processo que requer energia relativamente baixa conseguindo alta produtividade e baixo período de tempo.

Dentro desta perspectiva é que este trabalho foi realizado com estudos para produção de cutinase por fermentação em estado sólido, procurando conciliar alta produtividade em baixo custo, garantindo maiores rendimentos em reações em condições brandas, o que proporciona metabólitos estáveis e de qualidade em processos de baixo custo, além de reduzir a ocorrência de sub-produtos agroindustriais, minimizando a poluição ambiental e agregando valor a materiais antes descartados.

Em trabalhos anteriores foi realizado o isolamento e identificação de uma linhagem produtora de cutinase (um tipo especial de esterase) identificada como *Fusarium oxysporum* (Macedo & Pio, 2005) seguindo com otimização da produção e condições em fermentação líquida (Pio & Macedo, 2007).

O uso de FES para produção de metabólitos comerciais ainda hoje é pouco explorado, o uso da fermentação líquida convencional prevalece por ser uma técnica familiar, porém através da FES é possível ter concentrações maiores dos produtos, muitas vezes em menor período de tempo, tornando-se cada vez mais competitivo e mais viável para a produção industrial de metabólitos secundários (Robinson *et al*., 2001).

O objetivo principal deste trabalho foi obter a cutinase produzida por *Fusarium oxysporum* em fermentação em estado sólido partindo de sub-produtos agroindustriais, avaliar os principais parâmetros de fermentação em escala laboratorial e obter enzima com potencial de aplicação, avaliando seu comportamento na resolução enantiomérica de substâncias racêmicas como o ácido 2-octanol e o fármaco Ibuprofeno.

A escolha dos melhores substratos ocorreu avaliando a produção de cutinase durante a cinética de fermentação sendo escolhidos para estudos posteriores farelo de trigo, casca de soja e farelo de arroz. A adição de sais e óleos como fontes de carbono e minerais resultaram na inibição da produção da enzima pelo microrganismo, mostrando que a cutinase como metabólito

secundário, não precisa ser suplementado com outros nutrientes usando meio sólido, sendo economicamente positivo para produção da enzima.

O controle dos parâmetros pode ser usado para modificar a produção metabólica ou excreção dos microrganismos. A transferência de massa é uma grande dificuldade em FES, pois grandes quantidades de calor podem afetar crescimento, formação de esporos além da formação de produtos. Outro parâmetro importante é a atividade de água (a_w) do substrato, que determina a influência da atividade microbiana (Robinson *et al*., 2001). Por esse motivo a temperatura e quantidade de água do meio foram estudados a fim de otimizar o meio de produção para o três meios sólidos selecionados.

Nesta etapa os meios mostraram ter mesmo comportamento frente à variação de temperatura e quantidade de água do meio, sendo obtido máximos valores de atividade de cutinase na faixa de 28-30ºC e 100-150% de água em todos os tempos estudados. Nesta fase, foi realizado um estudo sobre a influência do pré-inóculo mostrando que produção da enzima tem maiores rendimentos quando menores valores de inóculo são utilizados.

Em seqüência os meios selecionados e as melhores condições de cultivo foram utilizados para a produção da cutinase. As enzimas produzidas em meio sólido foram comparadas à cutinase produzida em meio líquido descrita por Pio & Macedo, 2007. Os valores de atividade enzimática em meio líquido foram superiores aos produzidos em meio sólido com pouca diferença em relação ao meio sólido com maior produção de cutinase; assim essas enzimas foram comparadas em relação a seu comportamento frente à separação enantiomérica de substâncias racêmicas.

Um grande investimento em pesquisas tem sido feito para desenvolver diferentes processos para obtenção da forma enantiomérica ativa destas substâncias. O produto opticamente puro tem valor comercial maior que o racemato, mas a produção tem sido limitada por dificuldades legais e de processo (Carvalho *et al.*, 2005).

Tendo em vista o potencial da cutinase em catálises em meio orgânico, foi testado o poder de esterificação enantiomérica para o álcool primário 2-octanol e posteriormente usando o fármaco racêmico Ibuprofeno.

Todas as cutinases apresentaram bons valores de conversão de esterificação, porém apenas as cutinases produzidas pelos substratos farelo de trigo, casca de soja e farelo de arroz foram enantioseletivas, sendo a cutinase de farelo de trigo a com maior potencial enantioseletivo. A cutinase produzida em meio líquido apresentou alto valor de conversão, porém baixa enantioseletividade.

Quando as cutinases foram submetidas à reação de esterificação do fármaco Ibuprofeno os melhores valores de esterificação foram obtidos com cutinase de farelo de arroz e produzidos por meio líquido; as cutinases produzidas por farelo de trigo e casca de soja não apresentaram bons resultados.

Este trabalho foi importante por comparar as características funcionais de enzimas produzidas por meios diferentes, utilizando reações de esterificação na resolução de fármacos racêmicos, sendo uma possibilidade extremamente promissora, que ainda se encontra em fase de estudos.

A apresentação da dissertação será em forma de artigos. O capítulo I apresenta a introdução geral do trabalho. A revisão bibliográfica, resultados e discussão são apresentados nos capítulos II, III, IV e V no formato de artigos científicos submetidos a revistas indexadas. O capítulo VI traz a conclusão geral do trabalho e o capítulo VII as referências bibliográficas.

CAPÍTULO II

CUTINASES FÚNGICAS: PROPRIEDADES E APLICAÇÕES

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REVIEW

Cutinases (EC 3.1.1.74) also known as cutin hidrolases, are enzymes able to degrade cutin, the biopolyester that forms plant cuticle, which is composed of hydroxy and epoxy fatty acids. Cutinases present catalytic properties of both lipases and esterases, making them interesting as biocatalysts in several industrial processes. Hydrolysis, esterification and trans-esterification are catalyzed by cutinase with high efficacy in diverse reaction media, allowing its application in different areas such as the food industry, cosmetics, fine chemicals and waste management. The present review describes the characteristics, applications and perspectives of these new enzymes.

Keywords: Cutinase; *Fusarium oxysporum*, esterificação; transesterificação.

INTRODUÇÃO

Biocatalisadores ou catalisadores bioquímicos são proteínas cuja principal função é catalisar reações nos organismos. Os biocatalisadores são utilizados em química orgânica como alternativa aos processos químicos clássicos por apresentarem inúmeras vantagens. Dentre essas destacam-se elevada velocidade de reação, utilização de condições brandas, compatibilidade com substratos sintéticos, em alguns casos podem catalisar as reações nos dois sentidos e podem ainda, apresentar alguma seletividade quanto ao tipo de reações que catalisam[1].

As enzimas hidrolíticas (proteases, celulases, lipases, amilases e cutinases) são as mais freqüentemente usadas na química orgânica. Entre as várias razões que as tornam opção particularmente atrativa, pode-se citar ampla disponibilidade, baixo custo, condições suaves de síntese, facilidade de uso porque não necessitam cofatores, e especificidade para substratos[2].

No início da década de 70, foi descoberto que muitos fungos fitopatogênicos produziam enzimas capazes de hidrolisar a cutina. Várias dessas cutinases fúngicas foram purificadas e caracterizadas. Está claro atualmente que as cutinases possuem papel importante na penetração de superfícies intactas de

plantas por certos fungos, baseado em estudos com inibidores químicos, anticorpos específicos para cutinase e uso de técnicas de biologia molecular[3].

Cutinases são enzimas que catalisam a hidrólise de biopoliéster insolúvel, a cutina, que forma o componente estrutural da cutícula das plantas[4]. Este poliéster é composto dos seguintes ácidos graxos: ω-hidróxi ácidos graxos, ácido dihidroxidróxi palmítico, e ácidos 18-hidróxi-9,10,-epóxi C18 saturados e Δ12 insaturados, e ácidos 9,10,18-trihidróxi C18 saturados e Δ12 monoinsaturados. A composição da cutina é dependente da espécie do microorganismo, porém, em geral, em plantas de crescimento rápido predominam os ácidos de 16 carbonos, particularmente dihidroxipalmitato, enquanto em plantas de crescimento lento uma mistura de ácidos de 16 e 18 carbonos é encontrada. A cutinase libera hidroliticamente todos os tipos de monômeros a partir do polímero[5].

Além da hidrólise, as cutinases também são capazes de catalisar reações reversas, como esterificação, trans-esterificação (interesterificação, alcoólise e acidólise) e aminólise, sendo que a atividade de água do meio reacional é um dos fatores determinantes para cada classe de reação [6, 7, 8].

Segundo Brenda[9], a cutinase é classificada como EC 3.1.1.3 com nome recomendado de triacilglicerol lipase, e nome sistemático de triacilglicerolacilhidrolase. Também é classificada, pela mesma fonte, como 3.1.1.74 com nome recomendado de cutinase e nome sistemático de cutina hidrolase, ou seja, é a princípio esterase do tipo que hidrolisa cutina, podendo agir e ser classificada também como lipase.

O presente artigo descreve as propriedades e potenciais aplicações das cutinases fúngicas em processos industriais.

Propriedades da Cutinase

Estrutura

Cutinase é um dos menores membros da família serina hidrolase, que tem a tríade catalitica clássica composta por serina, histidina e um grupo carboxil. A sub-família das cutinases geralmente consiste em base de 20 membros baseado numa

seqüência de aminoácidos similares. Sua estrutura e dinâmica molecular é conhecida em detalhes após extensivos estudos de cristalografia por raios X e NMR. Em todas serina hidrolases, o sítio ativo está invariavelmente localizado na extremidade C-terminal de uma das cadeias β[10].

Trata-se de molécula compacta que consiste em 197 resíduos, é uma α/β proteína com núcleo hidrofóbico que compreende uma folha β central rodeada por 5 α hélices ligeiramente torcidas e contém um único resíduo triptofano (Trp_{69}) que está localizado na vizinhança de uma ponte dissulfeto[11] (Cys_{31}-Cys_{109}).

Segundo Borreguero *et al.*[12], o sítio catalítico da cutinase não é protegido por alça (tampa) helicoidal anfipática, a qual está caracteristicamente presente nas lipases. O sítio oxiânion, que nas lipases é formado sobre a ligação com o substrato, é pré-formado nas cutinases. Estes dois fatores poderiam ser responsáveis pela ausência da ativação interfacial detectada no comportamento catalítico da cutinase.

Melo *et al*.[13] descrevem, em algumas cutinases, a presença de um sítio de conformação que abre e fecha, o que indicaria ser a enzima preferencialmente lipase do que esterase. Este movimento de “mini-tampa” da cutinase cobrindo seu sítio ativo poderia ser responsável por sua adaptação a diferentes solventes.

Aplicações da Cutinase

A cutinase apresenta-se como enzima versátil demonstrando várias propriedades úteis para a aplicação em produtos e processos industriais (Tabela 1). Em anos recentes, a atividade estereolítica da cutinase tem sido largamente explorada. Fazendo uso de sua atividade cutinolítica em vivo, uma preparação enzimática contendo cutinase tem sido desenvolvida para aumentar o efeito farmacológico de químicos agrícolas. Uma aplicação particularmente importante, na qual estas enzimas possuem grande potencial é o manejo de resíduos gerados pelo processamento de frutas e vegetais. Cascas de maçã, cítricos e tomate são utilizados através da hidrólise da cutina para obter materiais importantes na indústria, como o ácido ricinolêico, importante na indústria farmacêutica, cuja fonte principal atualmente é o óleo de mamona[14].

Tabela 1: Aplicações biocatalíticas descritas para cutinases

Número da Publicação	Data da Publicação	Inventor	Nome	Referência
WO8809367	01-12-1988	Kolattukudy, *et al.*	Composições e métodos do uso de cutinase em limpeza	(1)
JP3088897	15-04-1991	Eruuseido, *et al.*	Método de utilização de lipases e surfactantes de cutinases úteis para lavagem	(1)
NZ337239	28-09-2001	Rainhard and Henrik.	Método de degradação enzimática de polímeros biodegradáveis.	(1)
EP1694903	30-08-2006	Cavaco-Paulo, *et al.*	Método de modificação de fibras de poliacrilonitrila e de poliamida.	(1)
JP200505828	10-03-2005	Yoshinobu, *et al.*	Método de produção de ésteres na ausência de solventes orgânicos.	(1)
CA 2480912	23-10-2003	Salmon, *et al.*	Método para melhorar a resistência a abrasão e força de tensão a materiais celulósicos como o algodão.	(2)
CA 1262860	14-11-1989	Yuichi, *et al.*	Método para aumentar o efeito de biocidas em agricultura.	(2)
CA 2060510	10-01-1991	Ayrookaran, *et al.*	Método para aumentar a permeabilidade da superfície de frutas e vegetais.	(2)
CA 2465250	15-05-2003	Shi, *et al.*	Método para remover a tintura adicional de materiais têxteis tingidos.	(2)

(1) http://ep.espacenet.com/?locale=en_ep
(2) http://patents1.ic.gc.ca/fcgi-bin/patquery_eo_el

Cutinases apresentam, *in vitro*, atividade hidrolítica contra grande variedade de ésteres, desde ésteres sintéticos solúveis (como *p*-nitrofenil ésteres) a triglicerídeos insolúveis de cadeia longa, como trioleína e tricaprilina, bem como triacilgliceróis emulsificados[10].

A cutinase pode também ser utilizada na síntese de triglicerídeos estruturados, polímeros, surfactantes, ingredientes para produtos de higiene pessoal, química farmacêutica e agroquímicos contendo um ou mais centros quirais [15].

A cutinase tem sido aplicada como enzima lipolítica na composição de detergentes de roupas e louças. Algumas vantagens foram obtidas quando a cutinase foi comparada com lipase comercial (Lipolase ™) para remoção de triacilgliceróis em processo de lavagem simples, pois a cutinase é capaz de hidrolisar gorduras na ausência do cálcio [16].

Indústria de Laticínios e Óleos

Trans-esterificação de gorduras e óleos ou esterificação estereoseletiva de álcoois podem ser obtidas em baixa atividade de água utilizando cutinase[15].

Nos últimos anos têm havido crescente interesse na tecnologia de modificação de óleos e gorduras[17,18,19,20]. Esta tendência pode ser atribuída principalmente ao fato desses materiais serem obtidos de fontes naturais e empregados como importantes matérias-primas para a indústria química, farmacêutica e alimentícia. Existem casos em que se torna necessário modificar as características desses materiais, para adequá-los a determinada aplicação. Portanto o setor industrial de óleos e gorduras tem desenvolvido diversos processos para manipular a composição das misturas de triglicerídeos[21, 22, 23].

A estrutura básica dos óleos e gorduras pode ser redesenhada, por meio da modificação química dos ácidos graxos (hidrogenação), pela reversão da ligação éster (hidrólise) e reorganização dos ácidos graxos na cadeia principal do triglicerídeo (interesterificação)[17].

O enfoque biotecnológico vem se apresentando como alternativa atraente para a exploração na indústria de óleos e gorduras, principalmente quando são consideradas algumas das vantagens dessa abordagem, tais como maior rendimento do processo, obtenção de produtos biodegradáveis, menor consumo de energia, redução da quantidade de resíduos e introdução de processos mais econômicos de produção[23, 24, 25].

A hidrólise parcial da gordura do leite é uma transformação tipicamente dependente de enzimas, intrínseca à produção de diversos lacticínios. Tal processo pode ser realizado por enzimas pertencentes à microflora nativa (como ocorre na produção de queijo a partir de leite cru), ou pela adição deliberada de enzimas exógenas. Dependendo da extensão e especificidade de tal hidrólise, a gordura láctea enzimaticamente modificada irá exibir diferentes aromas e sabores: desde tons livres de acidez até sabor de creme queijo ou manteiga. Regado *et al*.[26] avaliaram o uso de dez lipases microbianas e da cutinase de *F. solani pisi* na lipólise parcial de gordura láctea. Os ácidos graxos liberados no meio de reação

foram analisados por HPLC. Os sistemas testados apresentaram nível de modificação enzimática equivalente aos sistemas comerciais já empregados.

Compostos de Aroma

Ésteres terpênicos de ácidos graxos de cadeia curta são óleos essenciais com grande aplicação na indústria alimentícia, cosmética e farmacêutica como aromatizantes e flavorizantes. Dentre estes, os acetatos, propionatos e butiratos de álcoois terpênicos acíclicos (geraniol e citronelol) são os mais importantes[27]. Tradicionalmente, tais ésteres são obtidos por métodos como síntese química, extração de fontes naturais e fermentação[28]. Contudo, tais métodos são onerosos e apresentam baixo ganho de reação. Com o crescente interesse por produtos naturais, a indústria procura no uso da biotecnologia produzir aromas naturais, em particular por métodos enzimáticos[29]. O uso de enzimas como catalisadores industriais em meio orgânico apresenta numerosas vantagens[30]. Em tais sistemas, as enzimas hidrolíticas podem ser empregadas para realizar reações de síntese desde que o equilíbrio químico da reação seja alterado no sentido do produto de síntese[31,32]. Síntese de ésteres terpênicos por esterificação direta e transesterificação em meio com baixo conteúdo de água foram descritos[33, 34, 35, 36, 37]. Etil ésteres de ácidos graxos também são importantes ingredientes aromáticos. Avanços recentes em biocatálise empregando meios não convencionais tornaram possível a utilização de enzimas hidrolíticas para catalisar a síntese de compostos aromáticos[38, 39]. Barros *et al.*[40] estudaram os parâmetros de reação referentes a esterificação do etanol com ácidos graxos de cadeia curta (C_2 a C_6) em meio orgânico utilizando cutinase, demonstrando aumento de 35% na conversão molar, com o aumento de 1,4% para 2,1% na concentração da enzima.

Produção de Compostos Fenólicos

Os compostos fenólicos têm sido muito estudados devido a sua influência na qualidade dos alimentos. Englobam uma gama enorme de substâncias, entre elas os ácidos fenólicos, que por sua composição química, possuem propriedades antioxidantes. Os ácidos fenólicos possuem outras propriedades biológicas muito

interessantes (quelantes, filtro UV, antimicrobianas, remoção de radicais livres), mas devido a sua baixa solubilidade em meio aprótico, sua aplicação em produtos oleosos é muito limitada. A esterificação de sua função ácido carboxílico com álcool graxo aumenta sua hidrofobicidade e resulta em molécula anfifílica multifuncional. Tal reação é conhecida como lipofilização, e pode ser obtida quimicamente ou enzimaticamente. A lipofilização enzimática de ácidos fenólicos é atualmente estudada utilizando diferentes lipases, feruloilesterases, tanases e cutinases. Stamatis *et al*.[41] realizaram a esterificação de ácido ferúlico com 1-octanol utilizando diferentes enzimas, dentre as quais a cutinase de *F. solani.* Estas enzimas também se mostraram capazes de catalisar a esterificação de ácido cinâmico, p-cumárico e p-hidroxifenil propiônico a taxas relativamente altas.

Degradação de Inseticidas

Os inseticidas organofosforados são largamente utilizados para o controle de pragas na agricultura e no tratamento de parasitas na pecuária. Tais compostos permanecem no ambiente, principalmente em substâncias lipídicas, sendo rapidamente absorvidos por praticamente todas as vias, incluindo o trato gastrointestinal, pele, membranas mucosas e pulmões[42, 43, 44].

Kim *et al*.[45] estudaram a biodegradação e detoxificação do organofosforado malation utilizando cutinase de *F. oxysporum* e esterase de levedura. A taxa de degradação do inseticida pela cutinase foi cerca de 60% da quantidade inicial durante os primeiros 30 minutos e cerca de 50% foi decomposto nos primeiros 15 minutos. Com o uso da esterase de levedura, cerca de 65% do malation permaneceu não decomposto mesmo após dois dias de tratamento. A composição química final após dois dias foi significativamente dependente da enzima utilizada. A cutinase fúngica resultou em MDA como principal produto de degradação. Contudo na degradação do malation pela esterase de levedura, um isômero do MMA foi produzido em abundância. Efeitos tóxicos do malation e de seus produtos de degradação foram investigados utilizando bactérias recombinantes bioluminecentes. Os produtos de degradação obtidos utilizando esterase (incluindo MMA) causaram severo dano de membrana e inibição da síntese

protéica nas bactérias. Com o uso da cutinase fúngica, o malation foi degradado principalmente para o produto não tóxico MDA.

Indústria Têxtil

Na indústria têxtil as fibras sintéticas representam quase 50% de todo o mercado mundial. E a produção de fibras de poliéster é maior que a de fibras de algodão. Devido à importância das fibras sintéticas, existe grande interesse em pesquisa relacionada à melhora dos processos de produção, bem como melhora das características do produto.

Características como hidrofobicidade, que torna as fibras pouco adequadas para o contato com a pele humana e baixa reatividade que dificulta o tratamento com agentes finalizantes como corantes, levam ao tratamento com agentes alcalinos fortes pode melhorar a hidrofobicidade e reatividade química de tais fibras, mas tal tratamento é de difícil controle, resultando em níveis inaceitáveis de perda de resistência do tecido[46, 47, 48]. Além disso, a grande quantidade de hidróxido de sódio e as altas temperaturas necessárias tornam o processo pouco atraente do ponto de vista ambiental. A modificação das propriedades das fibras em busca de maior hidrofobicidade deve limitar-se à superfície sem alterar as propriedades do interior. Atualmente existe grande interesse no uso de enzimas como lipases e cutinases, as quais têm mostrado melhorar a hidrofilicidade de poliésteres através da hidrólise de ligações éster. Devido ao tamanho dessas enzimas, sua atuação ocorre apenas na superfície das fibras. As reações ocorrem sob condições mais brandas e não há necessidade de grande maquinaria para a realização do processo, o qual apresenta baixo nível de resíduos[48, 49, 50, 51].

Silva *et al*.[52] utilizaram a cutinase de *F. solani pisi* para modificar a superfície de fibras sintéticas como poliéster, poliamida e acrílicos. A cutinase foi escolhida para atuar na modificação de fibras devido a sua característica hidrofóbica e devido a sua ação em biopoliésteres presentes na cutina das plantas. Foram monitoradas alterações na superfície das fibras após ação enzimática e formação dos grupos hidroxil nas fibras de poliéster e acrílico e grupos amino nas fibras de poliamida. Em tecnologia têxtil a reação precisa de

tempos maiores que 24 horas e a cutinase demonstrou poder hidrolisar ligações amidas tendo maior atividade em poliamida que em poliéster.

Degani *et al*.[53] empregaram cutinase para melhorar a umectabilidade de fibras de algodão. O efeito da cutinase foi estudado de forma isolada e em co-reação com pectinaliase. A combinação de cutinase e pectinaliase apresentou efeito sinérgico. O uso de detergentes também aumentou a eficiência da reação.

Degradação de Polímeros

Na indústria de polímeros, novos paradigmas estão surgindo em virtude da escassez de fontes de matéria-primas tradicionais, bem como do manejo de resíduos. Algumas enzimas em meio não aquoso têm se mostrado ativas em ampla gama de reações de síntese de poliésteres e policarbonatos. A maioria de tais enzimas são da família das lipases, sendo a lipase B de *Cândida antarctica* a mais utilizada. Recentemente cutinase de *Humicola insolens* (Novozymes®) demonstrou atividade promissora em reações de polimerização[54]. A maioria da investigação em biotransformações catalisadas por cutinase tem se concentrado na degradação de poliésteres e na esterificação ou transesterificação de pequenas moléculas [55].

Mais de meio século atrás, os polímeros sintéticos passaram a substituir os materiais naturais em quase todas as áreas, e atualmente os plásticos se tornaram indispensáveis à vida moderna. A estabilidade e durabilidade dos plásticos têm sido melhoradas continuamente, fazendo desse grupo de materiais sinônimo de resistência a influências ambientais, principalmente microbianas. A grande produção de plásticos durante as últimas décadas resultou em problemas de manejo dos resíduos desses materiais. Devido ao seu curto tempo de existência na natureza, a evolução não pôde desenhar novas estruturas enzimáticas capazes de degradar polímeros sintéticos. Cerca de 20 anos atrás os cientistas começaram a pesquisar a possibilidade de produzir plásticos vulneráveis ao ataque microbiano, tornando-os biodegradáveis em meio ambiente microbiano ativo, mas ainda mantendo suas propriedades favoráveis[56]. Desde o início ficou evidente que polímeros com heteroátomos na cadeia principal tais como,

poliésteres, poliéteres, poliamidas e poliuretanos podem ser degradados por microorganismos, e o desenvolvimento de plásticos biodegradáveis foi dominado predominantemente por materiais derivados de poliésteres[57, 58].

Plásticos baseados em poliidroxidecanoatos são biodegradáveis, porém seu custo de produção é muito alto [59]. Policaprolona é um poliéster sintético que é degradado por uma variedade de microorganismos, mas suas propriedades físicas limitam suas aplicações. Murphy *et al*.[60] demonstraram que a cutinase de *F. solani pisi* tem a capacidade de degradar a policaprolona.

Fitalatos são plastificantes utilizados na produção de cloreto de polivinil e freqüentemente pinturas laquês e cosméticos[61, 62]. Diexilfitalato (DHP) é um dos ésteres de fitalato comumente utilizados, empregado em brinquedos, luvas de vinil, sapatos e em embalagens de alimentos. Os fitalatos incluindo o DHP têm sido considerados poluentes ambientais, e mesmo sendo controlados em vários paises, os seres humanos estão expostos a eles diretamente através de ingestão, inalação e exposição dérmica[63]. Kim *et al*.[45] estudaram a eficácia da cutinase de *F. oxysporum* e esterase de *Candida cylindracea* na degradação do DHP. A cutinase atua de forma muito mais rápida e precoce que a esterase, com alta estabilidade da atividade hidrolítica. A taxa de degradação do DHP pela cutinase foi cerca de 70% do DHP inicial dentro de 4 ou 5 horas, enquanto que mais de 85% do DHP permaneceu inalterado com o uso da lipase, após 3 dias. A toxicidade dos produtos de reação foi avaliada utilizando bactérias bioluminescentes recombinantes. Os produtos de hidrólise da cutinase não causaram nenhum dano celular observável, o mesmo não ocorrendo com a esterase.

Reações de Esterificação Enantiosseletiva

A importância do uso de enzimas em biocatálise tem se mostrado cada vez mais evidente. Grande versatilidade de reações catalisadas, condições brandas de reação, natureza regio, quimio e enantiosseletiva são algumas de suas vantagens[64]. Devido a sua enantiosseletividade, algumas cutinases têm sido

empregadas em vários processos deste tipo por meio de hidrólise em meio aquoso ou de síntese em meio orgânico.

Mannesse *et al*. [65] sintetizaram análogos de triglicerídeos de cadeias de tamanhos variados com enantiômeros R e S para estudar a influência do comprimento da cadeia na posição 1-, 2-, e 3- na atividade de cutinase e estereopreferência. A enzima hidrolisou preferencialmente o enantiômero R, mas essa preferência foi fortemente dependente do comprimento da cadeia acil, com atividade R: S de 30: 1. A enantiosseletividade foi encontrada em três diferentes sistemas.

Borreguero *et al*. [66] testaram a regio e enantiosseletividade de cutinase recombinante de *Fusarium solani pisi* em três racêmicos e um proquiral fenil-alcanodiol por transesterificação irreversível com vinil acetato. Foi demonstrado que o aumento da estereosseletividade da acilação do grupo primário hidroxil, foi obtido com a pré-incubação da enzima em presença do substrato diol 1, entretanto, não houve correlação com o tempo de incubação.

Indústria Alimentícia

Os ácidos graxos mais importantes contidos nos óleos marinhos são o eicosapentanóico (EPA) e docosaexanóico (DHA) que apresentam aplicações terapêuticas no tratamento de enfermidades inflamatórias auto-imunes e doenças cardiovasculares, e não podem ser obtidos por métodos convencionais de aquecimento, sem sofrer decomposição substancial[67]. Da mesma forma, o ácido γ-linolênico, um importante nutriente encontrado em sementes oleaginosas pode ser obtido pela hidrólise catalisada por lipases sob condições moderadas de temperatura[68].

Na indústria de panificação, a lipase aumenta o volume do pão, melhora a textura do miolo e confere cor mais branca. Os lipídeos do trigo são degradados pela lipase, modificando sua interação com o glúten, permitindo que este apresente rede mais forte e mais elástica[69].

A aplicação de enzimas hidrolíticas tem sido também preconizada na degradação biológica e remoção de efluentes industriais gerados em frigoríficos, abatedouros, laticínios e indústrias de alimentos em geral[70, 71, 72].

Estas indústrias produzem elevado teor de resíduos líquidos e sólidos, com odores desagradáveis, que prejudicam intrínseca e extrinsecamente as unidades industriais. Um tratamento preliminar desses efluentes por meio da ação de enzimas reduz o teor de lipídeos, o diâmetro das partículas de gorduras em até 60% e o tempo de residência do efluente nas lagoas de estabilização[73, 74].

As cutinases apresentam diversas características interessantes para aplicações em uma ampla gama de produtos, variando de detergentes a alimentos[75]. Intensa pesquisa abordando sua estrutura, função, purificação e aplicações têm sido desenvolvidas, visando o desenvolvimento de novos processos empregando este grupo de enzimas[4].

CONCLUSÕES

Cutinases (EC 3.1.1.74) são enzimas versáteis que possuem estrutura e propriedades intermediárias entre lipases e esterases. Diversas características destas enzimas as tornam muito interessantes para aplicação em processos industriais envolvendo hidrólise, esterificação e trans-esterificação.

O potencial da biotecnologia para melhorar as propriedades de matérias-primas naturais e submetê-las a transformações profundas, bem como para o tratamento de resíduos industriais e degradação de produtos tóxicos ou pouco biodegradáveis já foi comprovado, em áreas tão variadas como alimentos, cosméticos, química fina e tratamento de resíduos, entre outras. A demanda industrial por tecnologia enzimática vem aumentando gradativamente. As cutinases podem tornar-se importantes protagonistas nesse contexto.

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CAPÍTULO III

CUTINASE PRODUCTION USING AGRO-INDUSTRIAL BY-PRODUCTS BY *Fusarium oxysporum*

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Abstract

Fusarium oxysporum was cultivated on ten different combinations of solid agricultural by-products for cutinase (E.C. 3.1.1.74) production. Significant differences in growth and enzyme productivity were observed on the different media studied. Several carbon and nutrients sources were investigated to find a supplemented inducer to enzyme production. A maximum production of 27.5 U/mL was reached using wheat brain as solid support; 18.8 U/mL using rice bran and 11.9 U/mL using soy rind after 120h of incubation. Among the supplements tested, all contribuited to decrease of cutinase activity, showing that the cutinase as secondary metabolite is inhibited by an excess of nutrients and confirming that the solid media tested provide the nutritional needs of the microorganism. These results are promising because showed to be flexible to produce significant quantities of cutinase using agricultural by-products, resulting in an environmental positive process.

Key Words: cutinase, solid state fermentation, *Fusarium oxysporum*, agro-industrial by-products.

1. Introduction

Solid State Fermentation (SSF) is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absence of free flowing liquid. Both food and agricultural by-products are produced in huge amounts and since they are rich in carbohydrates and other nutrients, they can serve as substrate for production of bulk chemicals and enzymes using SSF technique (Couto & Sanroman, 2006). The use of SSF technology has been used successfully for the production of enzymes and secondary metabolites added value products before discarded (Robinson *et al*., 2001).

Cutinase (E.C. 3.1.1.74) is an enzyme that catalyzes the hydrolysis of cutin, a non-soluble biopolyester which makes up the structural component of the plant cuticle. Cutin is polymer consisting of the following fatty acids: ω-hydroxy fatty

acids, dihydroxy palmitic acid, 18-hydroxy-9,10,-epoxy C18 saturated and Δ12 unsaturated acids, and 9,10,18-trihydroxy C18 saturated and Δ12 monounsaturated acids. The precise composition of cutin depends on the species, but as a rule, cutin of fast-growing plants seems to consist mainly of 16 carbon acids, especially dihydroxypalmitate, whereas the slow-growing plants show both 16- and 18- carbon acids. Cutinase enzymatically removes all types of monomer from the polymer (Kerry & Abbey, 1997; Walton & Kollattukuy, 1972).

Cutinase is a versatile enzyme showing several interesting properties for application in industrial processes. An enzymatic preparation containing cutinase has been developed to increase the pharmacological effect of agricultural chemicals and it has also been applied as a lipolytic enzyme for fat removal in laundry and dishwashing detergents. Degradation of plastics such as the synthetic polyester polycaprolactone, into water-soluble products, has been achieved using cutinase (Egmond & Vlieg, 2000).

The objective of this work was to select agricultural solid by-products as substrate for the cutinase production by *Fusarium oxysporum*. It was used ten solids substrates being selected the best for the tests of supplementation with carbon and minerals sources. It was also to determine the best media and inducers to optimise the process of cutinase production.

2. Methods

2.1. Microbial strain and inoculum

The fungal strain employed in this work was *Fusarium oxysporum* obtained from soil and plants collected in different regions of Brazil (Macedo & Pio, 2005), it was maintained on potato dextrose agar (PDA) slants and stored at 4℃. A suspension of spores was obtained before incubated for 72h at 30ºC and the spores prepared by adding 5 mL distilled water to each slant. It was added 1 mL of inoculum and cultivated in greenhouse at 30ºC. After culture development, to each vial was added 100 mL of distilled water and the system was homogenized in a

rotary shaker under 100 rpm for 1 hour. The mixture was filtered and collected for the analysis of cutinase activity.

2.2. Media composition

The inicial SSF media used for cutinase production were made using agricultural by-products donated by Campinas-SP industries: soy rind, pie and bran; sesame pie; sunflower pie; rice bran; wheat bran; sugar cane bagasse; citric pulp and rapessed pie. The residues were sieved in particle sizes between 0-0.8mm, then oven dried at 60ºC for 24 hours and autoclaved at 121℃ for 20min. SSF was perfomed essentially as described by Christen *et al*., 2000.

2.3. Assay techniques

Generally, the media were cultivated since 48 to 120 hours in greenhouse at 30℃ with controlled humidity. The humidity of medi a was determined by equipment Karl Fisher and the best media compositions were chosen to assay forthcoming.

It was added 20g of the medium composed by solid substrate and distilled water at 1:1 (w/w). The humidity was measured in different concentrations of water rating: 50%, 100% and 150%, from 24 to 120 hours and the cutinolytic activity was measured after 120 hours.

To study the effect of salt solution in the medium for cutinase production was compared using solution containing 0.06% $NaNO_3$, 0.06% K_2HPO_4, 0.02% $MgSO_4$, 0.02% KCl, 0.01% $FeSO_4.7H_2O$, pH 7.2; in rate of 100% and distilled water in rate of 100%.

Strain of *Fusarium oxysporum* proved to be induced in previous studies in liquid media (Macedo & Pio, 2005), new composites mainly solution of oligo-elements and fatty acids (soy oil, rice oil, olive oil, flaxseed oil, palm oil) and water amount were tested to study inducible the cutinase synthesis in different concentrations using strategy of experimental design for election of significant variable.

Cutinase activity was measured using spectrophotometric method following the hydrolysis of p-nitrophenylbutyrate (Pnpb) at 405 nm. An aliquot (0.070 mL) of the culture supernatant was added to 3.43 mL of reaction mixture with the following composition: 1.12 mM Pnpb dissolved in 50 mM phosphate buffer pH 7.2, also containing 0.2% (N/P) Triton X-100 and 0.43 M tetrahydrofuran. The reaction was monitored for 15 minutes against blank solution (Calado *et al.*, 2002). One unit of cutinolytic activity was defined as the amount of cutinase required to release one micromole of p-nitrophenyl in one minute under the specified conditions. All assays were done independently in duplicate.

3. Results and discussion

3.1 Solid state substrate fermentation selection

Agro-industrial by-products are generally considered the best substrates for the SSF processes; the substrate selection for enzyme production depends upon several factors, mainly related with cost and availability, and thus may involve screening of several agro-industrial by-products (Pandey *et al*., 1999).

The cutinase production using different solid substrates and initial humidity of media were shown in Table 1. The three best media were chosen and studied. Rapessed pie, sugar cane bagasse and citric pulp are not suitable for cutinase production. Cutinase activity varied from 0.8 to 27.5 U/mL and the best results were found using soy rind (11.9 U/mL), rice bran (18.8 U/mL) and wheat bran (27.5 U/mL) after 120 hours of fermentation.

The initial humidity of media ranged from 3.7 to 11.8% of water and this variation was not directly related to enzymatic activity in the solid media. The comparison of results of enzymatic activity of the media was carried out through the application of *Tukey* test with 95% confidence and a significance level of $p \leq 0.05$. It was observed that the cutinase production to each media differ statistically when compared to different times studied.

Rao *et al*., 1993 tested rice bran for optimization of the lipase production by C*andida rugosa* supplemented with urea, maltose and rice oil. Lipase reached 22.6 U/mL in 48 hours of fermentation.

Benjamin & Pandey, 2000 tested the production of three different forms of extracellular lipase produced by C*andida rugosa* using wheat bran, the total activity of the supernatant was 48.61 U/mL being lipase A 11.62 U/mL; lipase B 1.15 U/mL and lipase C 1.75 U/mL.

Christakopoulos *et al.*, 1998 studied the production of esterase by *Fusarium oxysporum* under optimal conditions reporting a yield of 7.3 U/mL in a liquid growth medium and 19.4 U/g in a solid medium.

Table 1: Averages of cutinase production from 48 to 120 hours of fermentation and initial humidity of media, submitted to analysis by *Tukey* test at a 95% confidence interval.

Medium	**Cutinase Activity (μmol/min^{-1}.mL^{-1})**				**Initial Humidity**
	48 hours	72 hours	96 hours	120 hours	**(% of water)**
Rapessed pie	1.3_a	0.7_a	1.2_a	0.8_a	7.5
Sugar Cane Bagasse	1.2_a	1.4_a	1.6_b	1_a	9.7
Citric Pulp	5_a	4.9_a	5.7_b	4.6_a	4.5
Sesame	6.8_a	6.3_a	7_b	7.8_c	3.7
Soy Bran	3.4_a	7.9_b	8.5_c	8.5_c	8.0
Soy Pie	7.6_b	3_a	6.8_b	11.8_c	6.9
Sunflower	2.4_a	6.6_b	11.7_c	11.5_c	7.5
Soy Rind	2.8_a	7_b	7.5_b	11.9_c	8.0
Rice Bran	2.1_a	7.94_b	11.9_c	18.8_d	5.2
Wheat Bran	2.5_a	7.2_b	15.3_c	27.05_d	11.8

* Letters equal on the same line did not differ significantly ($p < 0.05$).

3.2 Media water amount rate during fermentation

The substrates selected were submitted to different water concentrations and water amount variation of media was followed during all fermentation as shown in Figure 1. The difference among humidity of media can be observed when different concentrations of water were added and the kinetic of humidity of media was different along the time, showing that each media had a different absorption of water varying with the time and these influence to enzyme production.

The cutinase activity was measured in all substrates after 120 hours to comparison among the behavior of *Fusarium oxysporum* to enzyme producer in different water amount. The value of maximum activity was found using 150% water with wheat bran reached up 18 U/mL with 70.3% humidity of meduim. The

same was not observed when was added 50% of water with 13.5 U/mL being humidity of medium 62.7%.

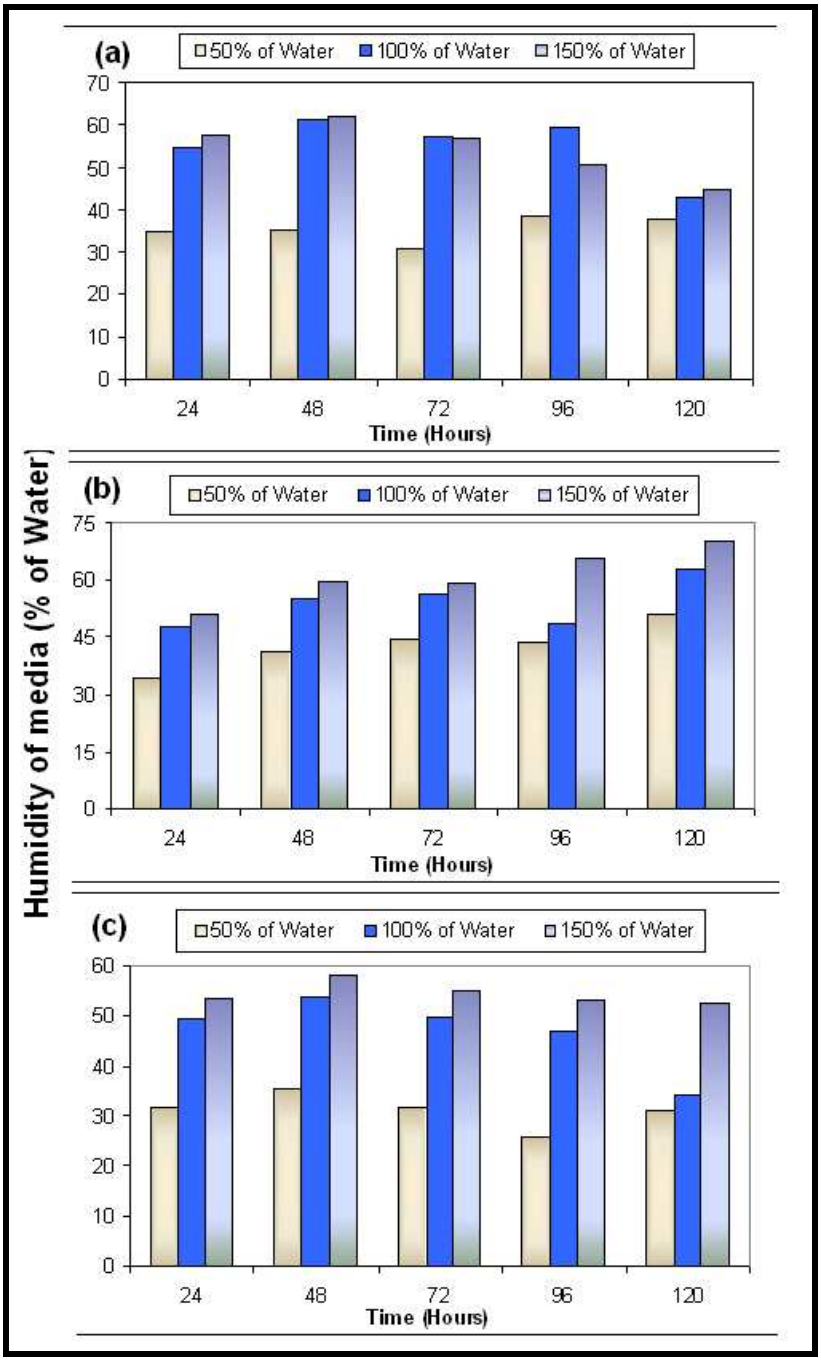

Figure 1: Changes of water amount of three best media cutinase producers in different concentrations of water in greenhouse at 30ºC. (a) Soy Rind; (b) Wheat Bran, (c) Rice Bran.

The substrate soy rind had a decrease of humidity in 120 hours when compared to all times ago, the cutinase activity using 150% of water was 16.3 U/mL and 44.95% humidity of medium; when it was used 50% of water the cutinolitic activity reached only 11.3 U/mL with 37.8% humidity of medium.

The rice bran was the medium with less humidity, when it was used 50% of water reached up 31.1% humidity of medium the cutinase activity was 19.5 U/mL and when it was used 150% of water the cutinase activity decreased with 11.1 U/mL and 52.5% humidity of medium.

Among three concentrations of water studied, 150% was better to cutinase production using wheat bran and soy rind allowed to obtain the best results after 120 hours. The cutinase production using rice bran was the better results when lower water concentrations were used increasing cutinase activity using 50% of water.

Among the several factors that are important for microbial growth and enzyme production the water would play an important role on process in solid state fermentation (Couto & Sanroman, 2006).

Sadhukhan *et al*., 1999 optimized secondary metabolic using wheat bran and distilled water to get an initial misture content of 70%. Rodriguez *et al*., 2006 produced an extracellular lipase with solid state fermentation with humidity of medium around 75%.

3.3 The effect of additional minerals solution

The study was performed by addition of minerals in medium supplemented with carbon sources in the growth of the microorganism. The salts tested correspond to mineral basic solution described by Pio & Macedo, 2007.

Cutinase production was higher in tree media using water compared to the salt solutions in all times of fermentation as it can be seen in Figure 2. The enzyme activity with medium containing wheat bran was about 43% higher than medium supplemented with salt solution. When rice bran and soy rind were used the cutinase activity reached about 31% more with water compared to salt solution in

similar conditions. The mineral solution did not increased the cutinase production when replaced the water in the system.

Rivera-Munõs *et al.,* 1991 tested solid media culture composed of wheat bran humidified with salt solution with magnesium sulphate and potassium phosphate and represent low values of lipase activity for the *Aspergillus niger* and *Geotrichum candidum.*

Pio & Macedo, 2007 measured the cutinase production by *Fusarium oxysporum* in presence of several carbon and nitrogen sources. The greatest cutinolytic activity was obtained in liquid mineral medium supplemented with flaxseed oil after 48 hours of fermentation. The same medium mineral added to the solid medium may be due to catabolic repression for the cutinase production reducing enzyme activity in all times tested. Kuhad *et al*., 1998 described that addition of glucose to the medium containing xylan produced catabolite repression of xylanase production by *Fusarium oxysporum*.

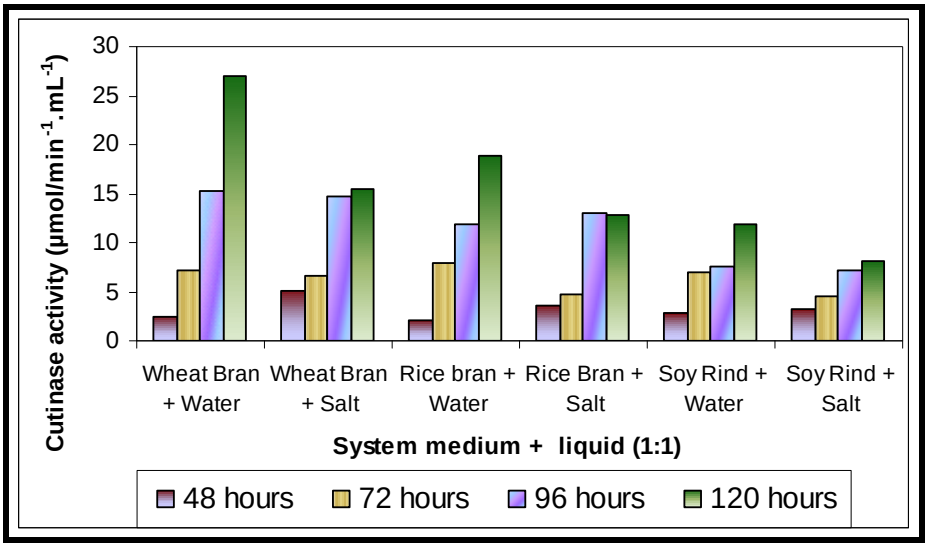

Figure 2: Comparison among cutinase production using water and salt solutions

3.4 Effect of the carbon and oligo-elements source

The variables and levels of the experimental design are shown in Table 2. The first strategy was a 2^{7-3} fractional design plus 4 central points, resulting in a

total of 20 assays to each medium composition, which were analysed after 120 hours of fermentation. Using such strategy, it was possible to analyze the main effects and indications of the independent variables and the best range of values for each variable.

Table 2: Variable and levels for fractional factorial design 2^{7-3}, analysed for cutinase production after 120 hours of fermentation.

Variables (%)	**-1**	**0**	**+1**
Micronutrients*	0	20	40
Soy oil	0	10	20
Rice oil	0	10	20
Olive oil	0	10	20
Flaxseed oil	0	10	20
Palm oil	0	10	20
Water Amount	150	200	250

*Micronutrients solution (g/L): 1.98 $MnCl_2.4H_2O$; 2.81 $CoSO_4.7H_2O$; 0.25 $CuSO_4.5H_2O$; 0.29 $ZnSO_4.7H_2O$; 10 EDTA (Rodriguez *et al*., 2006).

Cutinase production varied greatly with different combinations of the media components and solid media. The main effects and significant variables are shown in Figure 3.

The Fractional factorial design was used to calculate the main effects and indicate which one is significant to increase the enzyme activity.

Although there were some significant variables ($p<0.1$), all of them had a negative effect on cutinase activity. The statistically significant variables were marked with asterisk showing that they influenced the enzyme production reducing the enzyme activity. The correlation coefficient is explained by 65% to wheat bran, 70% to rice bran and 77% to soy rind, being less significant to enzyme production. The experimental design showed lack of fit and that the variables, under the conditions studied; do not contribute to increase cutinase production.

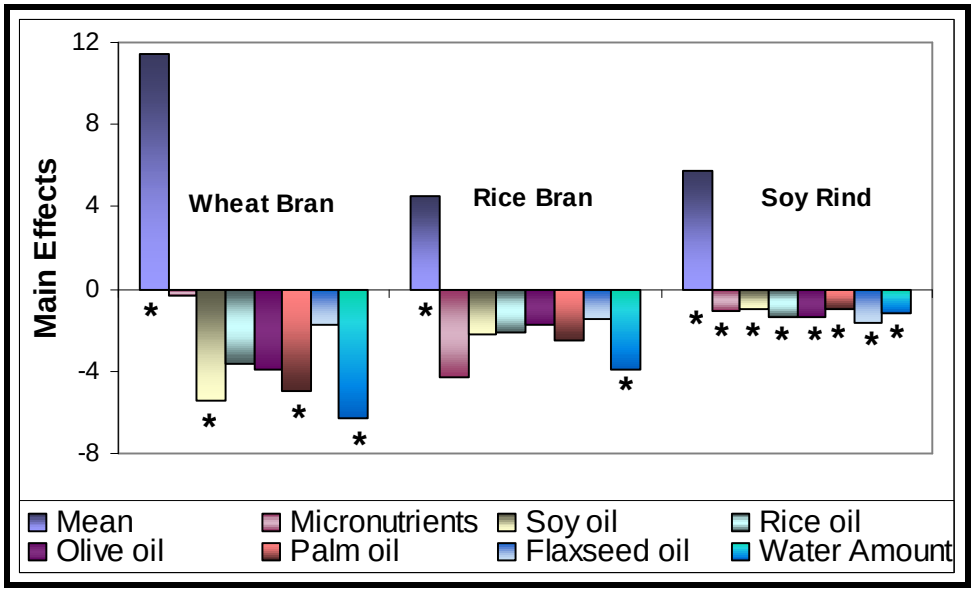

Figure 3: Study of main effects of the variables at 90% level of significance ($p<0.1$), according to fractional factorial design, analysed after 120 hours of fermentation. The (*) represents statistically significant variables.

Christen *et al*., 2000 tested different compositions to supplementation of the solid medium to esterase production, and observe that soy bean oil did not increase the enzyme production.

Rodriguez *et al*., 2006 improved the lipase production by modification of media nutrients; urea, olive oil and oligo-elements produce high lipase production. These nutrients in the same concentrations did not have the same influence for the cutinase production in solid state fermentation.

Topakas *et al*., 2007 produced esterases with *Fusarium oxysporum* in solid state fermentation which does not necessarily require monosaccharides and disaccharides such as glucose, xylose, lactose, maltose and xylitol. This may be due to glucose catabolic repression and/or the mechanism that has not been completely elucidated.

Pandey *et al.,* 1999 reported that various c-sources inhibited protease synthesis using wheat bran, indicating the presence of catabolic repression of protease biosynthesis. The same authors described the use of rice bran and wheat bran, with different concentrations of rice bran oil used as the substrate. Rice bran supplemented with oil showed higher lipase yields.

The substrate that provides all nutrients necessary to the microorganisms growth should be considered as the ideal substrate (Pandey *et al*., 1999).

4. Conclusions

This study showed that agro-industrial by-products used as substrates could be rapidly transformed by *Fusarium oxysporum* to produce cutinase. Large differences were found, in terms of production, depending of the medium used with differences statistically significant among substrates.

Among the three media studied the humidity showed variation between the media during fermentation. The substrates had different behavior when the cutinase production was compared in different concentrations of water.

The results showed that the cutinase produced in solid state fermentation with wheat bran, rice bran and soy rind do not need to be suplemented with other carbon and minerals sources utilized in this study. The supplements tested under the studied concentrations showed inhibitor effect for the enzyme production, showing that the cutinase is a secondary metabolite. This result showed that these media do not need to be supplemented with minerals, being economically positive for enzyme production.

The solid substrate had the best yields using wheat bran, followed rice bran and soy rind. The cutinase activity was better results using 150% of water to wheat bran and soy rind and 50% of water to rice bran. Furthermore, to optimize the culture conditions of these cutinases has been completed.

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CAPÍTULO IV

OPTIMIZATION OF CUTINASE PRODUCTION IN SOLID STATE FERMENTATION BY *Fusarium oxysporum*

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ABSTRACT

Wheat bran, rice bran and soy rind were employed for cutinase production in Solid State Fermentation (SFF). The production of an extracellular cutinase from *Fusarium oxysporum* was improved by modification of fermentation conditions: temperature and water amount. Kinetic of production were carried out change fermentation conditions and a correlation between them was reached using response surface methodology. The best conditions to cutinase production were: temperature 28-30ºC; water amount 150-170% and incubation time 72 hours for all media tested. Cutinase production showed a statistically significant relationship with the inoculum size, when the concentrations of spores decrease, the activity increased around 22%.

Key Words: cutinase, *Fusarium oxysporum*, solid state fermentation, wheat bran, rice bran, soy rind

1 INTRODUCTION

Cutinase (E.C. 3.1.1.74), a small lipolitic enzyme, is the smallest member of the α/β- hidrolase fold family, to which the other lipases belong (13).

The versatility of cutinase relative to a variety of possible substrates makes it an attractive biocatalyst for numerous applications (3,8). Non- aqueous enzymology has been one such area, where cutinase, in similarity with lipases, has proved to be an efficient biocatalyst for organic synthesis (8).

Cutinase is a versatile enzyme showing several interesting properties which can be applied to industrial processes. Its natural function is to catalyses the hydrolysis of cutin, the insoluble lipid polyester matrix that covers plants surfaces. However, cutinase also catalyses triglicerides hydrolisis, esterification, inter and intra-transesterification reactions efficiently. This activity profile gives a number of important applications in industries ranging from those dealing with detergents to foods and chemical (5).

Solid state fermentation (SSF) is defined as fermentation involving solids in absence (or near absence) of free water; however, substrate must possess enough moisture to support growth and metabolism of microorganism (16). Fungal species are easily cultured in solid state fermentation. This culture mode offers many advantages over commonly used submerged fermentation (SmF) processes (16-10-22-18), such as higher productivities, lower capital and operating costs, and simpler equipment and media. On the other hand, some limitations by what means temperature can be reached might cause significant thermal enzyme desnaturation during the fermentation (19) and maintain water support with water addition (1,20) and to control relative air humidity (20).

The parameters control can be used to modify the metabolic production or excretion of microorganisms. If SSF parameters are controlled correctly and the purity of the product is defined, then SSF may be a viable option for the industrial production of secondary metabolites (17).

In the present work, the objective was to produce cutinase of *Fusarium oxysporum* under SSF conditions using agricultural by-products and increase production yield by optimization of fermentation conditions and pre inoculum.

2 MATERIALS AND METHODS

2.1 Reagents and media

Pnpb (p-nithophenylbutytate) was purchased from Sigma-Aldrich Brasil Co. (São Paulo, SP, BR). Triton X-100, tetrahydrofuran, components from culture media and other reagents were purchased from Merck (São Paulo, SP, BR). The agricultural by-products were used as solid fermentation support by different industries, donated in Campinas, SP, Brazil.

2.2 Microorganism

A strain of *Fusarium oxysporum* isolated from soil and plants (15), was used in the present investigation. The stock culture was maintained on potato dextrose agar (PDA) slants and stored at 4℃.

2.3 Cutinase production

Different solid supports were tested for cutinase production (data not shown). The three best support were selected (wheat bran, soy rind and rice bran) and the fermentation conditions studied for each one. The effect of temperature was studied by incubating the flasks in a greenhouse and water amount was defined using proportional solid medium and distilled water (1:1) for 20g of culture medium. The cutinolytic activity was measured after 72, 96 and 120 hours of fermentation.

2.4 Cutinase assay

Cutinase activity was measured using spectrophotometrically method following the hydrolysis of p-nitrophenylbutyrate (Pnpb) at 405 nm. An aliquot (0.070 mL) of the culture supernatant was added to 3.43 mL of a reaction mixture with the following composition: 1.12 mM Pnpb dissolved in 50 mM phosphate buffer pH 7.2, also containing 0.2% (N/P) Triton X-100 and 0.43 M tetrahydrofuran. The reaction was monitored for 15 minutes against a blank solution (2). One unit of cutinolytic activity was defined as the amount of cutinase required to release one micromole of p-nitrophenyl in one minute under the specified conditions. All assays were done independently in duplicate.

2.5 Experimental design

A five level, two variable central composite rotatable design (CCRD) was adopted for optimizing of fermentation conditions. This study required 12 experiments for each medium tested. The variables studied in the process were temperature (to 28.3 from 39.7ºC) and water amount (to 29.3 from 170.7% of water). The independent variables, their levels and real values are presented in Table 1.

Table 1: Variable and levels for central composite design 2^2 for cutinase production

		Coded variable levels				
Variables	**Symbol**	**-1.41**	**-1**	**0**	**1**	**+1.41**
Temperature (ºC)	T	28.3	30	34	38	39.7
Water Amount (% of water)	WA	29.3	50	100	150	170.7

2.6 Effect of inoculum size

The pre inoculum preparation was standardized employing aliquots of 18.95; 7.3 and 2.65 x 10^7 spores for each PDA slant inoculated with *Fusarium oxysporum*. The best conditions of temperature and water amount were chosen for the testing. Aliquots containing 1 mL of pre inoculum was added to 20g of the correspondent medium. All the tests were carried in duplicate.

3. RESULTS AND DISCUSSION

3.1 Fermentation conditions optimization

Application of experimental design was used to optimize cutinase production in solid state fermentation. The media selected were studied separately, by measuring the activity of the enzyme after 72, 96 and 120 hours of fermentation. Using this strategy the effects of the variables can be analyzed and set the best time of fermentation. The results obtained are showed separated for each medium (Tables 2, 3 and 4). Table 2 shows the results observed for cutinase production using wheat bran, Table 3 shows the equivalent using soy rind and Table 4 shows the equivalent using rice bran.

Table 2: Central composite design and responses for cutinase production by *Fusarium oxysporum* using wheat bran.

			Cutinase Activity (U/mL)		
Runs	***T (ºC)***	***WA (% of water)***	***72 h***	***96 h***	***120 h***
1	-1 (30)	-1 (50)	4.9	4.9	13.5
2	1 (38)	-1 (50)	1.01	1.5	1.3
3	-1 (30)	1 (150)	13.1	14.3	18.6
4	1 (38)	1 (150)	1.3	1.05	1.5
5	-1.41 (28.3)	0 (100)	15.5	16.9	21.7

6	1.41 (39.7)	0 (100)	1.5	1.7	2.1
7	0 (34)	-1.41 (29.3)	2.3	1.6	1.6
8	0 (34)	1.41 (170.7)	7.9	8.1	10.6
9	0 (34)	0 (100)	6.6	4.4	5.2
10	0 (34)	0 (100)	3.7	7.2	3.6
11	0 (34)	0 (100)	4.9	5.6	3.9
12	0 (34)	0 (100)	3.7	6.6	5.2

Table 3: Central composite design and responses for cutinase production by *Fusarium oxysporum* using soy rind.

			Cutinase Activity (U/mL)		
Runs	***T (ºC)***	***WA (% of water)***	***72 h***	***96 h***	***120 h***
1	-1 (30)	-1 (50)	5.5	10.0	11.3
2	1 (38)	-1 (50)	1.3	1.1	1.0
3	-1 (30)	1 (150)	9.9	11.6	16.3
4	1 (38)	1 (150)	0.6	0.7	1.02
5	-1.41 (28.3)	0 (100)	11.7	12.5	17.5
6	1.41 (39.7)	0 (100)	1.6	1.3	1.4
7	0 (34)	-1.41 (29.3)	1.1	1.7	1.2
8	0 (34)	1.41 (170.7)	6.4	6.5	7.5
9	0 (34)	0 (100)	2.3	1.7	2.4
10	0 (34)	0 (100)	1.8	2.8	6.1
11	0 (34)	0 (100)	1.9	1.9	3.3
12	0 (34)	0 (100)	3.2	1.9	2.9

Table 4: Central composite design and responses for cutinase production by *Fusarium oxysporum* using rice bran.

			Cutinase Activity (U/mL)		
Runs	***T (ºC)***	***WA (% of water)***	***72 h***	***96 h***	***120 h***
1	-1 (30)	-1 (50)	7.3	13.9	19.5
2	1 (38)	-1 (50)	1.5	0.9	2.2
3	-1 (30)	1 (150)	12.9	8.2	11.1
4	1 (38)	1 (150)	1.7	1.6	1.7
5	-1.41 (28.3)	0 (100)	10.5	15.01	21
6	1.41 (39.7)	0 (100)	1.9	1.05	1.5
7	0 (34)	-1.41 (29.3)	3.4	2.3	5.6
8	0 (34)	1.41 (170.7)	9.8	6.5	10.2
9	0 (34)	0 (100)	6.7	10.5	17.8
10	0 (34)	0 (100)	9.2	10.1	16.8
11	0 (34)	0 (100)	8.8	7.3	10.8
12	0 (34)	0 (100)	8.4	7.5	13.5

The cutinase production had the best yields with wheat bran, followed by rice bran and soy rind. The maximum yield observed was 21.7 U/mL after 120 hours of fermentation at 28.3ºC and 100% of water (run 5, Table 2) and all substrates had better results in the same conditions (run 5). The worst results were obtained with highest temperature and smallest water amount. Low growth of microorganism was observed when tested on temperature of 38℃, that explains the low values of enzyme activity in all substrates when submitted to such conditions. When the solid substrates were submitted to low concentrations of water, low enzyme activity was also observed.

In terms of cutinase productivity, analyzing the three times studied, 72 hours was considered the most appropriate, reaching good yields in short time. Thus, 72 hours was chosen for further statistics analysis for all solid substrates.

The analysis of variance (ANOVA) reproduced on Tables 5, 6 and 7 shows that the models were significant admitting a confidence interval of 95%. The pure error was very low, indicating good reproducibility from the all data obtained. The correlation coefficients and the *F-test* were good indicators for a model representative of the actual relationship between response of cutinase production and variables. The quadratic model (coded equation) was calculated for maximum cutinase activity with each medium after 72 hours, eliminating the statistically insignificant terms (p>0.05).

Table 5: Analysis of variance (ANOVA) for cutinase production with wheat bran after 72 hours

	Sum of squares	**Degrees of freedom**	**Mean squares**	**F-test[a]**
Regression	221.9	4	55.5	27.6
Residual	14.2	7	2.0	
Lack of fit	8.5	4		
Pure error	5.7	3		
Total	236	11		

[a] $F_{(4;\ 7;\ 0.05)} = 4.12$; $R^2 = 0.94$ and p-value < 0.05

$$Y = 4.53 - 4.44\ T + 1.51\ T^2 + 2.05\ WA - 1.98\ T \times WA$$

Table 6: Analysis of variance (ANOVA) for cutinase production with soy rind after 72 hours

	Sum of squares	Degrees of freedom	Mean squares	F-test[a]
Regression	141.6	4	35.4	39.3
Residual	6.3	7	0.9	
Lack of fit	5.1	4		
Pure error	1.2	3		
Total	147.9	11		

[a] $F_{(4;\ 7;\ 0.05)} = 4.12$; $R^2 = 0.96$ and p-value < 0.05.

$$Y = 2.7 - 3.47\ T + 1.86\ T^2 + 1.4\ WA - 1.28\ T \times WA$$

Table 7: Analysis of variance (ANOVA) for cutinase production with rice bran after 72 hours

	Sum of squares	Degrees of freedom	Mean squares	F-test[a]
Regression	140.3	3	46.8	17.3
Residual	21.9	8	2.7	
Lack of fit	18.2	5		
Pure error	3.7	3		
Total	162.2	11		

[a] $F_{(3;\ 8;\ 0.05)} = 4.07$; $R^2 = 0.87$ and p-value < 0.05.

$$Y = 7.5 - 3.65\ T - 0.98\ T^2 + 1.87\ WA$$

The surface (Fig. 1) plot represents the predicated model to the best medium: wheat bran, indicating the variable levels for an optimal process. An amount of *Fusarium oxysporum* cutinase around 28.3-30ºC and 150-170.7% of water increased the enzyme production. The optimal conditions for all substrates were temperature 28.3ºC and water amount of 100% obtaining 15.5 U/mL using wheat bran, 11.7 using soy rind and 10.5 using rice bran after 72 hours of fermentation.

The different activity and optimal fermentation conditions of the substrates could be explained by the different digestibility of these carbon sources (21).

Han *et al.* (9) used wheat bran and soybean as substrates for determining the temperature and humidity of medium for optimal proteases production. The best results were found after 48h at 25ºC and humidity 95-97%. The data obtained

in this study demonstrate that growth and enzyme production were influenced by incubation temperature, humidity, and time.

Lu *et al.* (14) optimized xylanase production testing the temperature and water activity of medium using wheat bran and soybean meal. The authors found the ideal condition for temperature of 30-35ºC and 35-45% of water (w/w).

Gracia- Garza *et al.* (6) investigated sporulation of *Fusarium oxysporum* using soybean and rice flour. The authors confirmed that different media caused different sporulation responses at constant humidity. The best results were found at 100% humidity for all media.

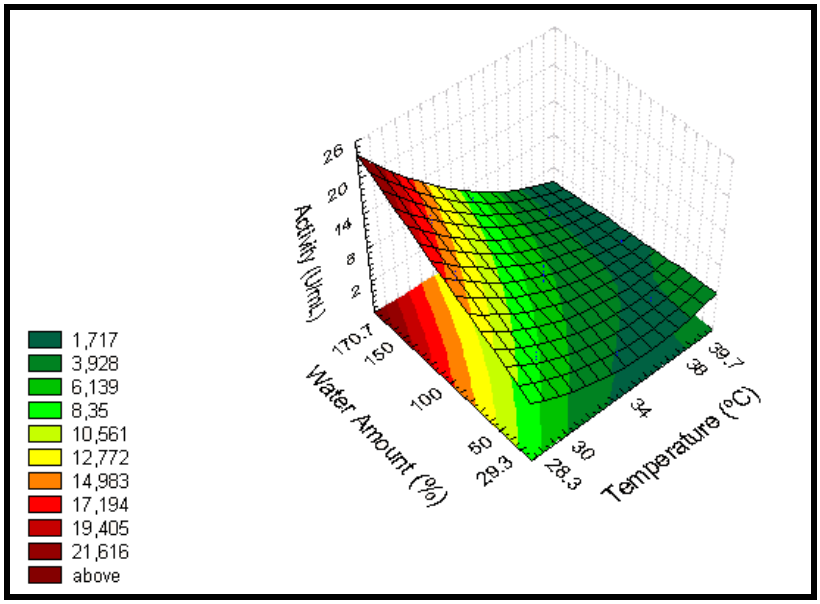

Figure 1: Response surface for cutinase activity obtained by wheat bran after 72 hours of fermentation.

3.2 Effect of inoculum size

The effect of inoculum size on cutinase production by *Fusarium oxysporum* was studied in the basal medium containing 20g of the correspondent medium and 100% of water. Erlenmeyer flasks (250mL) containing the mixture were inoculated with spores and incubated for 72 hours at 28ºC and demonstrated a significant difference among three spores concentrations in the cutinase production (Table 8). For all media tested, there was significant difference among the inoculum size and

the enzyme production observed with a decrease of inoculum level from 18.9 $x10^7$ to 2.6 $x10^7$ spores.

The cutinase activity obtained with less spores concentrations suggesting that the smaller quantity of spores added to the same substrate concentration is better to obtain higher levels of cutinase activity during fermentation. Cutinase activity increase was observed with 19.3% to wheat bran, 25.95% to soy rind and 21.6% to rice bran when the concentration of spores in the media was decreased.

Table 8: Effect of inoculum size on cutinase activity (U/mL) by *F. oxysporum* submitted to analysis by *Tukey* test at a 95% confidence interval.

Substrate	***18.9 x10^7***	***Std.Dev.***	***7.3x10^7***	***Std.Dev.***	***2.6 x10^7***	***Std.Dev.***
Wheat Bran	13.6_a	0.09	14.9_b	0.12	19.2_c	0.11
Soy Rind	7.04_a	0.15	11.7_b	0.05	15.8_c	0.16
Rice Bran	6.2_a	0.08	10.6_b	0.23	13.4_c	0.19

* Letters equal on the same line did not differ significantly ($p < 0.05$).

This fact could be related to a typical phenomenon found in mycology, self inhibition of fungal spore germination. Many fungal spores exhibit a crowding effect (7) with the spores contain a prepackaged self-inhibitor that prevents germination under crowded conditions (11). Another effect observed was that fungi with small inoculum sizes produced a transient mycelial stage with the mycelium length inversely proportional to the inoculum size (12). This effect was also obtained in the production of esterase by *Trichoderma reesei* Rut C-30, in which the average dimension of pellet seems to be inversely size proportional to the inoculum (4).

4. CONCLUSION

The use of CCRD permitted the optimization of the conditions for production of cutinase. The best yields occurred after 72 hours of fermentation at: 28ºC of temperature and 100% of water. The variation of temperature and water amount occurred according of the media type.

The temperature and water amount of the substrate are very critical in SSF as it ultimately affects the growth of the microorganism, spore formation and germination, and product formation (16).

The solid state fermentation using wheat bran, rice bran and soy rind showed to be effective for cutinase production by *Fusarium oxysporum* getting good yields in a simple process and low cost using as substrate by-products of the industry with low commercial value.

In this study, it was observed a direct relationship between the inoculum concentration and cutinase activity. It was possible to increase the cutinase activity changing the concentration of inoculum to all substrates tested.

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CAPÍTULO V

ENANTIOSELECTIVE BEHAVIOR OF FOUR CUTINASES PRODUCED BY *Fusarium oxysporum*

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Abstract

Four microbial cutinases from *Fusarium oxysporum* isolated from Brazilian soil samples were produced using different media (wheat bran, rice bran, soy rind and obtained from liquid medium). The esterification potential and enantioselectivity were compared using racemic substrates in organic medium. Resolution of (R,S)-2-octanol enantiomers was higher cutinase produced in wheat bran after 200 hours reaching total conversion at 76.4%, ee_s 52%, ee_p 16% and E=2.1; in this case, the enzymes preparation showed preferentially catalyses the esterification of (R)-2-octanol for all enzymes tested. When it was tested the resolution of (R,S)-Ibuprofen other cutinase reached better results, cutinase produced in rice bran obtained in terms of enantiomeric excess of the (S)-Ibuprofen acid (ee_s= 16%) and conversion value (c= 5.1%) and E=1.6 after 200 hours of reaction. These results confirmed that cutinases produced in different media using the same strains can present different behavior when submitted to same reactions and conditions. The interest to found new sources of cutinases, mainly substrates economically viable as using agricultural sub-products enabling the discovery of an enzyme with interesting characteristics of catalysis and it can be employed in the industry.

Key Words: cutinases, *Fusarium oxysporum*, enantioselectivity

1. Introduction

Cutinase (E.C. 3.1.1.74) is an enzyme that belongs to serine hydrolase family. They are involved in the degradation of the plant cuticle. The main structural component of cuticle is cutin, a water insoluble biopolyester composed mainly of epoxy and hydroxyl fatty acids [1].

As lypolityc enzyme, cutinase shows properties that it can be used to advantage in industrial applications [3]. In vitro, cutinases display hydrolytic activity towards a broad variety of esters incluiding soluble p- nitrofenil esters and triglycerides [4-5]. Synthetic activities of cutinases have also been described for the

triglycerides production, polymers and agrochemicals containing one or more chiral centes [5-3].

The enzymatic kinetics resolution of enantiomers has become a common tool in organic chemistry to prepare enantiomerically pure compounds [6].

It is known that some drugs are produced and sold as it racemic and that the biological activity depends, in many cases, on their absolute configuration. Normally one of isomer (R or S) presents biological activity, while the other is less active or even toxic [7].

The importance of the use of enzymes in biocatalysis has shown increasingly obvious great versatility of catalyzed reactions, soft conditions of reaction, nature regio, quimio and enantioselective are some of their advantages [8].

This work aims to study the ability of esterification enantioselective of different cutinases from *Fusarium oxysporum* in esterification of (R,S)-2-octanol with decanoic acid and the drug (R,S)-Ibuprofen with 1-propanol. In addition non-conventional medium have been explored as system for the esterification reaction which could improve the cutinase activity.

2. Materials and Methods

2.1 Cutinase production

Cutinase from *Fusarium oxysporum* [9] was produced in solid state fermentation using the optimized media (data not shown) wheat bran, soy rind and rice bran. An aliquot of 1 mL containing 2.65 x 10^7 spores was added to medium correspondent with growth conditions optimized (data not shown) composed by medium and distilled water at 1:1 (w/w) in 250 unbaffled Erlenmeyer flasks and cultivated in greenhouse at 28ºC. After 72 hours, 100 mL of water was added to the solid medium and homogenization in a rotary shaker under 100 rpm for 1 hour. After simple filtration, the supernatants were treated with ammonium sulphate (80% saturation). The precipitates were dialyzed in water and lyophilized for use as crude cutinase preparation in powder form. The protein concentration was determined by the method of Bradford [2].

2.2 Enzyme assay

Cutinase activity was measured using spectrophotometrically method following the hydrolysis of p-nitrophenylbutyrate (Pnpb) at 405 nm. An aliquot (0.070 mL) of the culture supernatant was added to 3.43 mL of reaction mixture with the following composition: 1.12 mM Pnpb dissolved in 50 mM phosphate buffer pH at 7.2, also containing 0.2% (N/P) Triton X-100 and 0.43 M tetrahydrofuran. The reaction was monitored for 15 minutes against blank solution [10]. One unit of cutinolytic activity was defined as the amount of cutinase required to release one micromole of p-nitrophenyl in one minute under the specified conditions. All assays were done independently in duplicate.

2.3 Esterification reation of (R,S)-2-octanol

Ester synthesis containing (R,S)-2-octanol (40mM), octanoic acid (40mM), 2 mL of hexane and lyophilized enzymes. The reaction was carried out at 30ºC and 130 rpm. Experiments without enzyme were carried out to evaluate the spontaneous esterification percentage of the system. Samples of 100µL of the solution were withdrawn at different times and diluted in 1.4 mL of hexane. The amount of ester (conversion degree) formed during the reaction and the enantiomeric excess were determined by gas chromatography as described further [7].

2.4 Esterification reation of (R,S)-Ibuprofen

The standard reaction mixture was composed by (R,S)-Ibuprofen (66 mM), 1-propanol (66 mM) and isooctane (10mL) without water addition. The reaction mixture was composed by 0.4g crude cutinase (from wheat bran, soy rind, rice bran SSF and obtained from liquid medium) to the solution and carried out at 30ºC on shaker at 130 rpm. Experiments without enzyme were carried out to evaluate the spontaneous esterification percentage of the system. Samples of 100µL of the reaction mixture were withdrawn at different times and diluted in 1.4 mL of isooctane. The amount of ester (conversion degree) formed during the reaction

and the enantiomeric excess were determined by gas chromatography as described further [11].

2.5 Chromatography analysis of (R,S)-2-octanol

The ester formation was followed using a Chrompack gas cromatograph (Chrompack Co., Holland) equipped with a flame ionization detector and a column (i.d. 0.32mm, length 30m) CP-WAX 52 CB. The temperature of the injector and detector was 220 and 250℃ respectively. The initial temperature of the column reached 50℃ for 2min then the temperature increased at a rate of 10℃/min up to 210℃. Standard curves of substrates and product were obtained to calculate response factors. The conversion was calculated from the amounts of the ester and alcohol peaks. The enantiomeric excess (ee) of the remaining alcohol was determined using the same gas chromatograph equipped with a fused silica capillary quiral column BETA DEX™ 120 (0.25 mm, 60m, Supelco). The temperature of the injector was 220℃; the detector temperature, 250℃. The initial temperature of the column was 95℃ for 30min and the temperature increased at a rate of 5℃/min up to 220℃ [7].

2.6 Chromatography analysis of (R,S)-Ibuprofen

Gas chromatography was performed using a CHROMPACK CP 9001 gas chromatography equipped with a flame ionization detector (FID) and a CP-Sil 5 CB column (10 m x 0.25 mm x 0.12 µm). Injector temperature was 300ºC and the detector was 350ºC; the oven temperature was maintained at 180ºC. Carrier gas was hydrogen with flow of 12 mL/min. An external standard method was employed to quantify the formed ester and the remaining acid. The enantiomers of the unreacted ibuprofen were separated using a chiral column (Chiralcel OD, Daicel Chemical Industries, Ltd., Japan) [11].

2.7 Enantioselectivity-value measurements

The value of enantioselectivity (E) was calculated from enantiomeric excess (ee) and conversion (c) according to the method described by Chen *et al*. [12].

3. Results and discussion

3.1 Cutinase production

The cutinase activity by *Fusarium oxysporum* was determined using three media solid in solid state fermentation after 72 hours and cutinase production optimized by Pio & Macedo [13]. The solid media composed by wheat bran presented the best total activity (20.99 U/mL) and the less specific activity (0.08). The substrate soy rind presented the best specific activity (0.18). The substrate rice bran presented total activity of 7.55 U/mL and good specific activity (0.15). These results showed the differences of substrates on enzyme activity.

The cutinase activity produced by liquid medium, was greater (22.68 U/mL) when compared with cutinase produced by solid medium. This system contains salt solution and flaxssed oil as inductor after 48 hours, 30ºC and 100 rpm.

Table 1: Cutinase activity production in different fermentation media.

Cutinase Substrate	***Total activity (U/mL)***	***Total protein (mg)***	***Specific activity (U/mg of protein)***
Rice Bran	7.55	0.86	0.15
Soy Rind	15.61	2.76	0.18
Wheat Bran	20.99	1.76	0.08
Liquid Medium*	22.68	2.43	0.11

* Cutinase produced by liquid mineral medium optimized by Pio & Macedo [13].

Fernandes *et al*. [14] produced recombinant cutinase with fatty acid as substrate, in the absence of butyric acid the activity reached to 20 U/mL; the specific activity was 192 using 1500 mol of PEG.

Calado *et al*. [10] used two system medium to cutinase production obtained 113 U/mL and 14.9 U/mg of activity specific in medium containing glucose and 25 U/mL of cutinase activity and 25 U/mg of activity specific using galactose as carbon source after 70 hours of fermentation.

The cutinase activity was produced using various industrial lipases, the results were 76.9 U/mL (5.0 U/mg of specific activity) to *Pseudomonas sp*; 61.7 U/mL to *Candida rugosa* (1.6 U/mg of specific activity) and 5.8 U/mL (5.5 U/mg of specific activity) to *Geotrichum candidum* [15].

3.2 Enantioselective esterification for optical resolution of 2-octanol racemic

Biotransformation is an useful tool in organic synthesis, and it is closely related to enantioselective process. We employed four cutinases to catalyse enantioselectivity esterification of (R,S)-2-octanol with octanoic acid as acyl donors. All cutinases demonstrate enantiopreference for the (R)-enantiomer and the results can see in Table 2.

The retention time of (R)-2-octanol was 31,243 min and (S)-2-octanol was 32,893 min. The formation of (R,S)-ester octanoic was observed in all times of fermentation in different media. The reaction catalysed by cutinase had the best yields with substrate of fermentation was wheat bran, followed by soy rind, liquid medium and rice bran, such were show in Figure 1.

The maximum data was obtained after 200 hours of reaction observing the ester formation using the enzyme from substrate rice bran (27.2%), liquid medium (47.7%), soy rind (71.9%) and wheat bran (76.4%).

An increase curve of conversion can be observed in all cutinases produced in solid substrate from time 0 to 100 hours. The best time to enzyme activity in terms of productivity was 100 hours. After that, the esterification rate remained constant for all enzymes.

The cutinase produced in rice bran was increase around ten times to each reaction time reached up 19.4% of esterification after 100 hours. Cutinase produced in wheat bran and soy rind presented the better results reached up 73.1% and 64.1% of conversion respectively after 100 hours of reaction.

The cutinase produced in the liquid medium reached esterification rate of 48% after 15 hours, followed 52% after 50 hours and remained constant after 200 hours with about 48% of conversion.

Table 2 showed the total conversion (c), enantiomeric excess of (S)-octanol (ee_s and ee_p) and enantiomeric ratio (E) on the best reaction times. The cutinases produced in different substrates showed significant differences in catalysis properties.

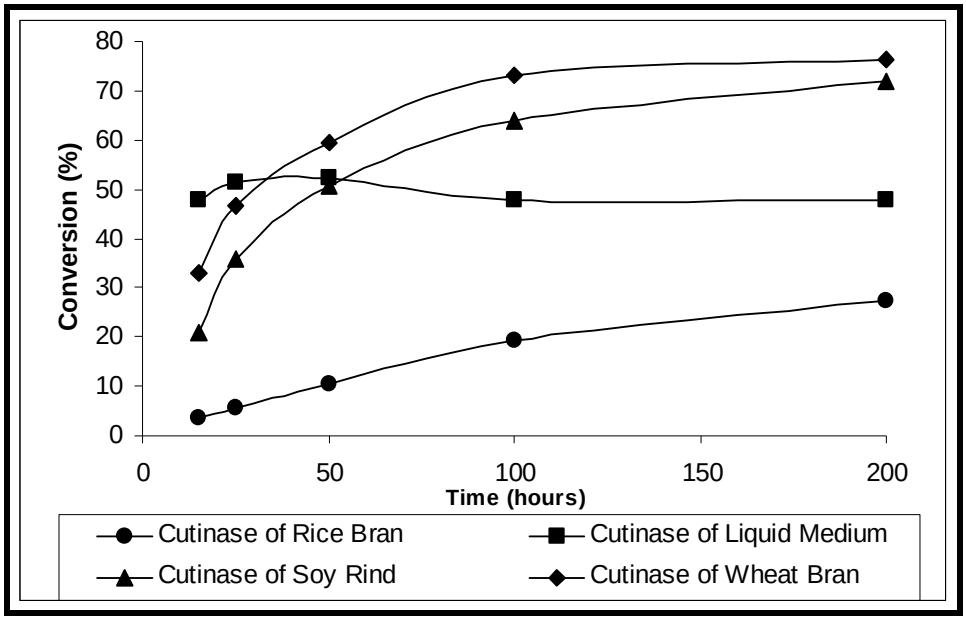

Figure 1: Esterification reaction of 2-propanol racemic with octanoic acid catalyzed by cutinases produced with different media

The maximum E-value was observed with cutinase from soy rind after 100 hours with 64.1% of conversion, 57.4% of ee_s and E= 3.3, the good result occur when analyzed after 200 hours with 71.9% of conversion, 57.7% of ee_s and E= 2.6. The good value of E shows efficiency in racemate resolution that ensures not only an enantiomeric excess high, but also highs yields proportionately [16].

The cutinase of wheat bran showed good ability to esterification, preferentially for the (R)-enantiomer resulting in 34.8% ee_s but the E-value was lower showing that the reaction occurred indiscriminately to ester formation: (R)- and (S)- ester, consequently low value of acid enantiomeric excess (34.8%) and E (1.7) after 100 hours of reaction.

The cutinase produced in the liquid medium reached good values of esterification but low enantioselective values of ee_s, ee_p and E.

Table 2: Esterification of (R,S)-2-octanol with octanoic acid catalyzed by cutinase in hexan at 30ºC.

	100 hours				200 hours			
Cutinase Sustrate	**c(%)**	**ee_s(%)**	**ee_p(%)**	**E**	**c(%)**	**ee_s(%)**	**ee_p(%)**	**E**
Wheat Bran	73.1	34.8	13	1.7	76.4	52	16	2.1
Soy Rind	64.1	57.4	33	3.3	71.9	57.7	23	2.6
Rice Bran	19.4	40	23	2.2	27.2	10	4	1.3
Liquid medium	47.8	4	4	1.1	47.7	2	2	1.1

Mannesse *et al*. [17] synthesized triglyceride analogues of primary acyl ester in different sizes with enantiomers (R)- and (S)- to study the influence of the chain length at the 1-, 2- and 3- position on cutinase activity and stereopreference. The enzyme preferentially hydrolyzed the (R)-enantiomers, but that preference was strongly dependent on the acil chain length distributions, with (R) or (S) activity ratios varying from about ratio 30:1. The enantioselectivity was found in three different systems.

The good yields of esterification of cutinase for chiral secondary alcohols was described of Tyagi & Pleiss [18]. They said that general rule to predict stereopreference from the substrate structure is based on the discrimination between the large and the medium-sized substituent at the chiral center. Discrimination by size explains the experimental observation that enantioselectivity of cutinase is increased by increasing the size of the large substituent.

3.2 Enantioselective esterification for optical resolution of Ibuprofen racemic

In this work, the first stage was evaluated the potential enantioselective of cutinases presented by various substrates in simple molecules of primary alcohols. In second stage the same cutinases were evaluated on enantioselective into more complex molecules using a drug from the profens family, Ibuprofen.

In this test all enzymes were submitted to reactions between (R,S)-Ibuprofen with 1-propanol in isooctane. All reactions were less than 6% of esterification as shown in Figure 2. The cutinases produced by liquid medium and wheat bran were lower significant increase until 50 hours and after this the esterification rate were constant keeping about 2.5% of esterification for medium liquid and 0.5% for wheat bran after 100 hours. However the cutinase produced in soy rind was not able to esterification this drug under the conditions studied.

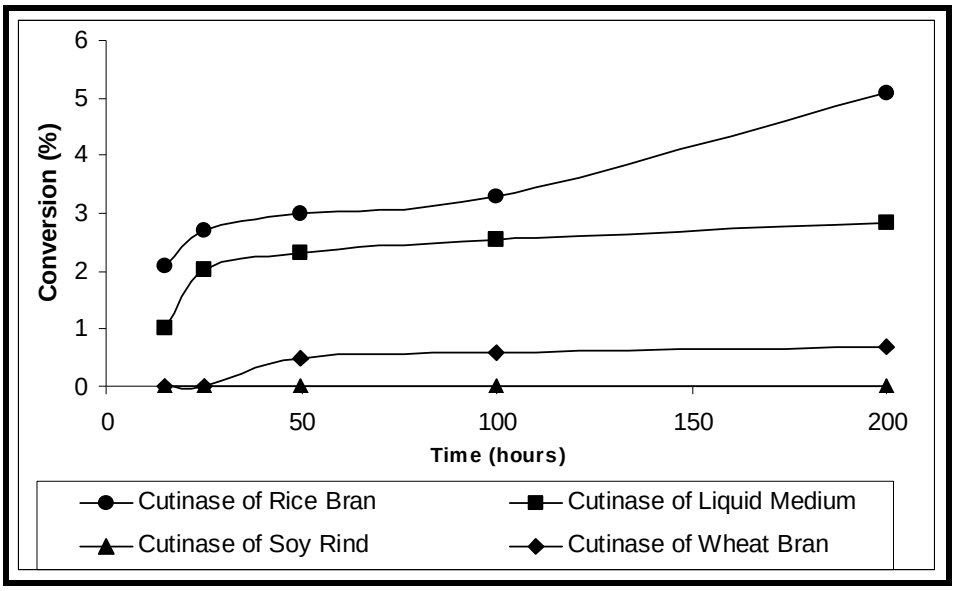

Figure 2: Esterification reaction of Ibuprofen racemic with 1-propanol catalyzed by cutinases produced with different media.

The best result was obtained with cutinase from fermentation of rice bran, presenting 5.1% of ester formed after 200 hours as shown in Table 3. The reaction was slowly increase until 50 hours with 3% of (R,S)-ester propilic of ibuprofen and after this increase around 41.2% in 200 hours with 5.1% of conversion. The E-value had maximum after 100 hours (E= 3.8) and had a decrease when analyzed after 200 hours (E= 1.6). This fact showed that enantiopreference to (S)-Ibuprofen keeping stable until 100 hours and after this did not catalyses preferentially the esterification of (S)-Ibuprofen, but catalyzing esterification of both enantiomers. The same results were found by [18], they reported that cutinase enantioselectivity

towards the alcohol substrate was low (E values among 1 and 2) under all the reaction conditions tested.

Table 3: Esterification of (R,S)-Ibuprofen with 1-propanol catalyzed by cutinase in isooctane at 30ºC.

Time (hours)	c(%)	ee_s(%)	ee_p (%)	E	Enantioprefererence
15	2.1	2	8.8	1.2	S
25	2.7	8	21.1	1.6	S
50	3.0	15	36	2.5	S
100	3.3	23	46.5	3.8	S
200	5.1	16	16.3	1.6	S

All cutinases demonstrate enantiopreference for the (S)-enantiomer and retention time of (S)-Ibuprofen was 7.835 min and (R)-Ibuprofen was 7.061 min, Figure 3 shows an analysis by the gas chromatography.

In all reactions controls, performed to verify the spontaneous esterification system has been observed that the percentage of esterification was significant lower (less than 0.01% of ester formed) under the conditions tested.

Studies were performed with lipases trading as its enantioselective capabilities of recemic ibuprofen with esterificant agent 1-propanol in isooctane, demonstrating that the lipase produced by filamentous fungi *Fusarium oxysporum* had high income during the first 150-200 hours of reaction, enabling to obtain the S-acid, which did not react. In this reaction the largest yield of the enzymes used ester was respectively 70.4% in the period of 336 hours and 84.2% in the period of 264 hours [20].

Borreguero *et al*. [21] tested the regio and enantioselectivity of recombinant cutinase of *Fusarium solani pisi* on three racemic and one prochiral phenylalkanediols. Only the smaller molecules were recognized by the enzyme and there was no correlation with the incubation time.

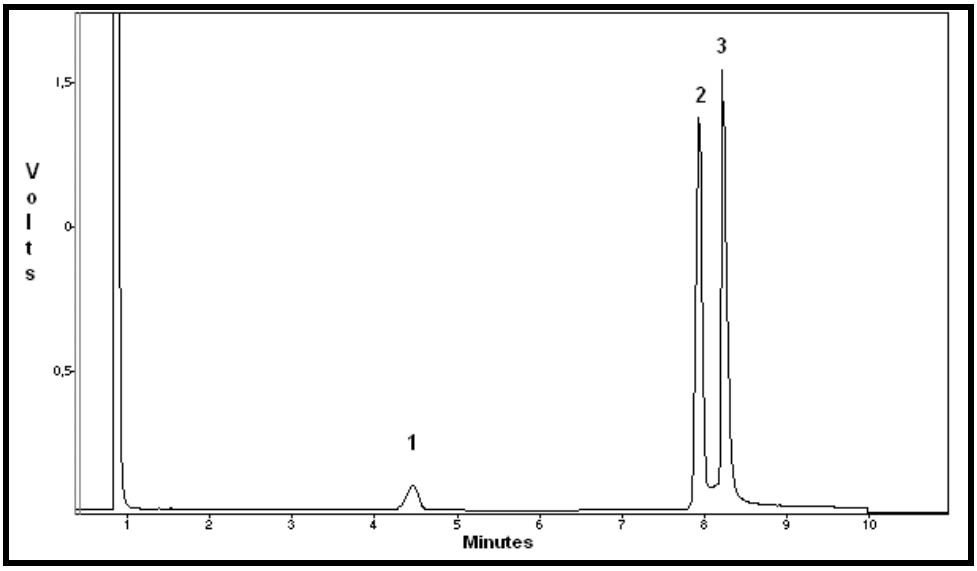

Figure 3: Gas chromatography from enantioselective esterification of racemic Ibuprofen catalysed by cutinase from *Fusarium oxysporum* using rice bran after 200 hours of reaction. Peak 1: Ibuprofen ester propilic; peak 2: (S)-Ibuprofen and peak 3: (R)-Ibuprofen.

It has been observed that even the enzymes which low values of E have been used successfully in the racemic resolutions substrates, considering that different conditions of reaction interfere significantly to the value of E [22,23].

Considering the few reports of attempted use of cutinases in resolution of drugs, the increase in the supply of new cutinases can contribute to the enantiomeric resolution of racemates where the pharmacological property is attributed to only one of enantiomer.

This work contributed also to prove that enzymes produced in different media do not present the same biochemical properties. This statement is very important and must be observed.

Conclusions

During the biocatalysis process any chiralidity is recognized by the enzyme, causing a preference for two possible directions with respect to stereochemical reaction.

The versatility of cutinase relative to a variety of possible substrates makes in an attractive biocatalyst for numerous applications. Non-aqueous enzymology has been one such area, where cutinase, in similarity with lipases, has proved to be an efficient biocatalyst for organic synthesis [23].

The cutinases from *Fusarium oxysporum* showed features high hydrolytic activity and good ability to esterification, we can conclude that the use of cutinases in the racemic resolution alcohols and drugs is an extremely promising possibility, which is still in the phase of studies. Studies involving modifications of the reaction conditions, as activity of water, temperature of the reaction, types of solvents and different concentrations of the enzyme have been made in an attempt to overcome the low values desired.

Another important aspect that could be seen was the large difference between the behavior of cutinases produced by different media, showing that production media of the enzyme influences in the production of isoenzymes with different characteristics when submitted the same reactions with same conditions.

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CAPÍTULO VI – CONCLUSÕES

A partir da análise de todos os resultados obtidos ao longo deste trabalho foi possível chegar as seguintes conclusões:

Quanto à seleção dos meios produtores de cutinase dentre os dez substratos sólidos testados para produção de cutinase por *Fusarium oxysporum*: farelo de trigo (27,5 U/mL), farelo de arroz (18,8 U/mL) e casca de soja (11,9 U/mL) após 120 horas de fermentação foram os substratos que apresentaram os melhores resultados. Através da técnica de planejamento fatorial foi possível avaliar o efeito da adição de fontes de carbono e minerais nos meios sólidos; os suplementos testados nas condições estudadas mostraram ser inibidores da enzima, confirmando que a cutinase é um metabólito secundário. Nesta etapa farelo de trigo, casca de soja e farelo de arroz foram selecionados como melhores produtores de cutinase utilizando 150 % de água para farelo de trigo e casca de soja e 50% de água para farelo de arroz.

No processo de otimização das condições de cultivo: foi utilizado a técnica do delineamento central composto rotacional (DCCR) para o estudo das condições de produção de cutinase avaliando temperatura (ºC) e quantidade de água dos meios (% de água). O melhor rendimento ocorreu após 72 horas de fermentação nas condições: 28ºC de temperatura e 100% de água. Neste estudo também foi observado uma relação inversa entre a concentração do inóculo e a atividade de cutinase. Foi possível aumentar a atividade cutinase em torno de 22% diminuindo a concentração de inóculo para todos os substratos sólidos testados.

No estudo do potencial enantiomérico das cutinases foram produzidas quatro cutinases em meios diferentes (farelo de trigo, casca de soja, farelo de arroz e produzida por meio líquido) e testadas quanto ao seu poder de separação de misturas racêmicas. As cutinases de *Fusarium oxysporum* mostraram ter boas características hidrolíticas e boa capacidade de esterificação podendo-se concluir

que a utilização de cutinases na resolução de álcoois e fármacos racêmicos é uma possibilidade extremamente promissora, que ainda está em fase de estudos.

Outro aspecto importante que pode ser observado é a grande diferença entre o comportamento de cutinases produzidas por diferentes meios, mostrando que o meio de produção da enzima influência na produção de isoenzimas com características diferentes, quando submetidos à mesma reação nas mesmas condições.

SUGESTÕES PARA TRABALHOS FUTUROS:

- Estudo da caracterização bioquímica das cutinases produzidas por diferentes meios sólidos e comparação entre elas;
- Estudos dos fatores como tempo de reação, atividade de água e quantidade de enzima utilizada no processo a fim de otimizar a separação enantiomérica de 2-octanol e do fármaco Ibuprofeno.

CAPÍTULO VII – RERERÊNCIAS BIBLIOGRÁFICAS

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