UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS
DEPARTAMENTO DE CIÊNCIA DE ALIMENTOS

PRODUÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO E APLICAÇÃO DA TANASE DE *Paecilomyces variotii*

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Campinas – SP
Maio/2007

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FEA – UNICAMP

Battestin Vania
B322e Produção, purificação, caracterização e aplicação da tanase de *Paecilomyces variotii* / Vania Battestin. -- Campinas, SP: [s.n.], 2007.

Orientador: Gabriela Alves Macedo
Tese (doutorado) – Universidade Estadual de Campinas.Faculdade de Engenharia de Alimentos

1. Tanase. 2. *Paecilomyces variotii.* 3. Fermentação. 4. Purificação. 5. Caracterização. I. Macedo, Gabriela Alves. II. Universidade Estadual de Campinas.Faculdade de Engenharia de Alimentos. III. Título.

(cars/fea)

Título em inglês: Production, purification, characterization and application of *Paecilomyces variotii* tannase
Palavras-chave em inglês (Keywords): Tannase, *Paecilomyces variotii,* Fermentation, Purification, Characterization
Titulação: Doutor em Ciência de Alimentos
Banca examinadora: Gabriela Alves Macedo
José Roberto Delalibera Finzer
Ângela Maria Moraes
Francisco Maugeri Filho
Lucia Regina Durrant
Hélia Harumi Sato
Data da defesa: 22/05/2007
Programa de Pós Graduação: Programa em Ciências de Alimentos

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Aos meus queridos pais Pacífico e Iracema,
Minhas irmãs e ao Victor W.,
Com muito amor e carinho,

Dedico

AGRADECIMENTOS

À Professora Gabriela Alves Macedo pela oportunidade, amizade e dedicação. Ainda, pela alegria e motivação que demonstrou durante o período do doutoramento.

Aos Professores da banca examinadora pela contribuição ao meu trabalho.

Aos Professores Victor A. P. de Freitas e Nuno Mateus do Centro de Investigação em Química, pela oportunidade de realizar parte dos experimentos na Universidade do Porto-Portugal.

As Professoras Hélia Sato pelo carinho e ajuda e Gláucia Pastore pela disponibilidade do uso do FPLC.

Aos alunos de Iniciação Científica, Luis Katsumi Matsuda, Rafael Goshima e Marcela Matos pela ajuda com os experimentos.

A todas as pessoas com quem tive o prazer de conviver no Departamento de Ciência de Alimentos, incluindo alunos, técnicos do laboratório e funcionários, especialmente Haroldo, Luciana F., Gaby, Priscila H. e Bia.

A minha família e ao Victor Wiendl pelo apoio e carinho.

A Leda pelo carinho e ajuda em todos os momentos.

Aos Professores Alice T. Valduga e José R. Finzer pelos ensinamentos e ajuda no decorrer dos anos.

A Kerry Bioscience do Brasil pelo fornecimento de amostras de tanase comercial, e ao Srº. Leonino Alcantara da Costa pelo fornecimento da palha de café.

Á CAPES pela consessão da bolsa.

Á todos aqueles que, apesar de não serem citados, contribuiram de alguma forma para a realização deste trabalho.

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LISTA DE ABREVIATURAS E SÍMBOLOS

API-	Atmospheric pressure ionization
BSA-	Bovine serum albumin solution (albumina de soro bovino)
DAD-	Diodo array detector (detector de barra de diodos)
DEAE-	Diethylaminoethyl
DPPH-	2,2-diphenyl-1-picrylhydrazyl (2,2-difenil-1-picrilhidrazilo)
EC-	Epicatechin (epicatequina)
ECG-	Epicatechin gallate (epicatequina galato)
EDTA-	Ethylenediaminetetraacetic acid (ácido etilenodiaminotetracético)
EGC-	Epigallocatechin (epigalocatequina)
EGCG-	Epigallocatechin gallate (epigalocatequina galato)
ESI-	ElectroSpray Ionization
FPLC-	Fast protein liquid chromatrography
FRAP-	Ferric Reducing Ability Power
GA-	Gallic acid (ácido gálico)
HPLC-	High liquid performance chromatography (cromatografia líquida de alta eficiência)
LC-MS-	Liquid chromatografy-mass spectrometry (cromatografia líquida-espectrometria de massa)
m/z-	Relação massa/carga
PDA-	Potato Dextrose Agar
SDS-PAGE-	Sodium dodecyl sulphate (polyacrylamide gel electrophoresis)
TAH-	Tanino acil hidrolase
TC-	Taninos condensados
TPTZ-	2,4,6-tris (2-pyridyl)-s-triazine (2,4,6-tris(2-piridil)-s-triazina)

RESUMO GERAL

A tanino acil hidrolase conhecida como tanase (E.C: 3.1.1.20) é uma enzima produzida por fungos filamentosos, leveduras e bactérias. São caracterizadas por sua atividade em complexos polifenólicos, sendo capazes de hidrolisar ligações éster (entre o grupo anel aromático e o resíduo de glicose) e ligações depsídicas (ligação éster entre os anéis aromáticos) em substratos como ácido tânico, epicatequina galato, epigalocatequina galato entre outros. O objetivo deste trabalho foi selecionar linhagens de fungos produtores de tanase e estudar a produção, purificação, caracterização e aplicação desta enzima. A produção da tanase pelas linhagens de fungos selecionados foi testada em meios de cultura contendo resíduos agroindustriais de café e uva com 0,5% e 1,5% de ácido tânico. A linhagem LAB 153G, identificada como *Paecilomyces variotii*, se destacou produzindo 0,275 U/mL de tanase em meio contendo resíduo de café. No estudo de otimização da produção de tanase pela linhagem *P. variotii* utilizando metodologia de superfície de resposta, foi obtido um aumento de 9 vezes na produção da enzima, utilizando-se as seguintes condições: faixa de temperatura de 29-34ºC, meio de cultivo contendo resíduo de café.farelo de trigo, 50% : 50% (p/p), ácido tânico 8-14% e tempo de incubação de 5 dias. A preparação de tanase bruta apresentou atividade ótima em pH 6,5 e 70ºC e estabilidade na faixa de pH 4,0-7,5 após 24 h de incubação a 30ºC. A enzima bruta mostrou-se estável após 30 min de tratamento a 70ºC em pH 6,0. A tanase purificada mostrou atividade ótima em pH 5,5 e 50ºC; e estabilidade máxima na faixa de pH 4,5 – 6,5 após 12 h de incubação a 30ºC. A enzima purificada mostrou-se estável após 30 min de tratamento a 50ºC. A tanase extracelular foi também purificada cerca de 19,3 vezes, utilizando precipitação com sulfato de amônio seguida de purificação com cromatografia de troca iônica (DEAE-Sepharose). A tanase de *P. variotii* apresentou valor de *Km* igual a 0,61 µM e V*máx* igual a 0,55 U/mL, para o substrato ácido tânico. No estudo de aplicação da tanase em substratos naturais como epigalocatequina galato extraído de chá verde, foi verificado que os produtos obtidos da hidrólise apresentaram maior atividade antioxidante quando comparado com epigalocatequina galato.

Palavras chave: tanase, *Paecilomyces variotii,* fermentação, purificação, caracterização

SUMMARY

Tannin acyl hydrolases, commonly referred to as tannases (E.C. 3.1.1.20), are inducible enzymes produced by fungi, yeast and bacteria. Tannases have been mostly characterized by their activity on polyphenolic complexes, are able to hydrolyse the "ester" bond (galloyl ester of an alcohol moiety) and the "depside" bond (galloyl ester of gallic acid) in substrates such as tannic acid, epicatechin gallate, epigallocatechin gallate, chlorogenic acid. The objective of this work was to select tannase producing fungi and to study the production and characterization of this enzyme. These fungi lineages were tested in vegetable residues as coffee and grape, adding 0.5 and 1.5% of tannic acid in the fermentation media. The best result was obtained using LA153G and coffee residues with activity 0.275 U/mL; the best tannase producing fungus was identified as *Paecilomyces variotii*. This strain was used for the optimization of enzyme production by experimental design method. After the optimization process, the tannase activity increased by 9 times. According to the optimization process, the best conditions for tannase production by the lineage of *P. variotii* were: temperature 29 to 34 ºC; % residue, coffee husk:wheat bran - 50:50 (p/p), tannic acid 8 to15 % and 5 days of fermentation. The biochemical properties of tannase were determined. Temperature 70 ºC and pH values from 6.5 were optimum for crude tannase activity. Tannase showed stability at pH 4.0-7.5 after 24 h the incubation at 30ºC. The tannase was stable in a temperature 70 ºC after 30 min in pH 6.0. The purified tannase showed optima activity in pH 5.5 and 50ºC; stability at pH 4.5-6.5 after 12 h the incubation at 30ºC. The tannase was stable in a temperature 50ºC after 30 min. An extracellular tannase was partially purified using ammonium sulphate precipitation followed by DEAE-Sepharose ion exchange chromatography. DEAE-Sepharose column chromatography led to an overall purification of 19.3 fold. The tanase showed values for K_m of 0.61 µM and V_{max} of 0.55 $U.mL^{-1}$. The activity of crude tannase on the epigallocatechin gallate extract of green tea was investigated. This work establish a relationship between the antioxidants effects of epigallocatechin gallate compared to the obtained enzymatic reaction products (epigalocatechin and gallic acid).

Key-words: tannase, *Paecilomyces variotii,* fermentation, purification, characterization

INTRODUÇÃO GERAL

Tanases são caracterizadas por sua atividade hidrolítica em complexos polifenólicos, sendo capazes de hidrolisar ligações éster e ligações depsídicas em substratos como ácido tânico, epicatequina galato, epigalocatequina galato, ácido clorogênico entre outros. A tanase é produzida na presença de indutor, a utilização do ácido tânico como indutor ou única fonte de carbono é fundamental. Mesmo na presença de outras fontes de carbono, a concentração de ácido tânico constitui o fator predominante na produção de tanase através do processo de fermentação. 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. O processo de fermentação sólida oferece aplicações promissoras para produção de compostos bioativos, enzimas e ácidos orgânicos que podem ser gerados por diferentes subprodutos agrícolas como resíduos de café, uva e farelo de trigo. Além deste aspecto, os volumes do fermentador e de efluentes resultantes podem ser menores. O interesse da fermentação sólida para a produção de compostos de importância comercial é conseqüência do interesse por produtos com menor custo de produção. A exploração adequada da fermentação sólida pode significar a redução do custo de produção de tanase. O uso de resíduos agroindustriais pode ajudar não somente a reduzir a poluição ambiental, mas também agregar valor às indústrias processadoras. Estima-se que a produção de enzimas comerciais por fermentação sólida represente somente 5% do mercado de vendas de enzimas. Embora existam muitas aplicações das tanases em potencial, poucas são efetivamente empregadas nas indústrias devido essencialmente ao custo de produção da enzima, que ainda é elevado. A enzima tem vasta aplicação na indústria de alimentos: sucos, cerveja; em cosméticos; fármacos e indústria química.

Este trabalho teve como principal objetivo estudar a produção, purificação, caracterização e aplicação de *Paecilomyces variotii*. Através da fermentação sólida, utilizando resíduo agroindustrial de palha de café e farelo de trigo, os estudos de otimização da composição do meio de cultivo e as condições de fermentação foram conduzidas. O emprego de resíduos agroindustriais representa a busca por novas alternativas de produção de enzimas, a fim de baixar o custo de produção, além de dar destino mais nobre aos

resíduos abundantes em nosso país. Este trabalho traz uma significativa contribuição ao estudo de produção de enzimas induzíveis, a partir de resíduos agroindustriais empregando uma nova linhagem de *P. variotii.* A tanase apesar de apresentar grande potencial de aplicação industrial, ainda tem alto custo e poucos produtores no mundo. O trabalho a seguir será apresentado na forma de artigos científicos, distribuidos na seguinte ordem:

O capítulo 1 trata de uma revisão bibliográfica sobre a tanase e seus substratos, os taninos que estão presentes nos vegetais.

O capítulo 2 descreve a seleção dos microrganismos produtores de tanase e utilização de resíduos agroindustriais no processo de fermentação. Neste estudo foram testadas 500 linhagens fúngicas com o interesse principal de selecionar uma nova linhagem produtora da tanase. Atualmente os principais fungos estudados para a produção desta enzima são linhagens de *Aspergillus* e *Penicillium*. Dada a grande diversidade da flora microbiana e da carência de dados e pesquisas nessa área, a introdução de uma nova linhagem produtora da tanase foi objetivo principal nesse capítulo, bem como, utilizar um resíduo agroindustrial para a produção da enzima através da fermentação sólida.

No capítulo 3 são abordados aspectos da produção da tanase em fermentação sólida utilizando palha de café e a nova linhagem fúngica (*P. variotii*.). O estudo da produção da enzima foi realizado empregando a metodologia de superfície de resposta, avaliando os principais parâmetros de fermentação dessa produção em escala de laboratório.

O capítulo 4 descreve a caracterização bioquímica da enzima bruta. Este capítulo serviu para avaliar aspectos da enzima relacionados a sua atividade catalítica, como por exempo: temperatura ótima e estabilidade, efeito do pH ótimo e estabilidade da enzima, estudo de alguns ativadores e inibidores da atividade da tanase.

No capítulo 5 foram abordados aspectos da purificação da tanase, que foi purificada cerca de 19,3 vezes, utilizando precipitação com sulfato de amônio seguida de purificação com cromatografia de troca iônica (DEAE-Sepharose). Normalmente os protocolos de purificação de enzimas microbianas extracelulares são separados em duas etapas: pré purificação e purificação por métodos cromatográficos. A pré purificação consiste em uma

técnica de concentração de proteínas, geralmente por precipitação, para separação das mesmas dos outros componentes do meio. A metodologia mais utilizada é a precipitação por sulfato de amônio. Na purificação por métodos cromatográficos, como regra geral, são utilizadas colunas com grande capacidade de troca iônica e baixo custo. Estão incluídas nesse item as resinas de interação hidrofóbica e troca iônica. Estas têm sido aplicadas com excelentes resultados na purificação de tanases microbianas.

O último capítulo trata da aplicação da enzima tanase na produção de compostos antioxidantes do chá verde. Esse trabalho foi desenvolvido na Universidade do Porto-Portugal, no Centro de Investigação em Química. O objetivo principal desse estudo foi verificar a ação da enzima na hidrólise de substratos naturais extraídos de chá verde (epigalocatequina galato) para a formação de ácido gálico e epigalocatequina. Foi verificado que os produtos obtidos da hidrólise apresentaram maior atividade antioxidante quando comparado com epigalocatequina galato.

CAPÍTULO 1: FONTES E APLICAÇÕES DE TANINOS E TANASES EM ALIMENTOS

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Publicado em: Alimentos e Nutrição, Araraquara, (15): 63-72, 2004.

RESUMO

Os taninos ocorrem em uma ampla variedade de vegetais, são classificados em hidrolisáveis e condensados. O ácido tânico é um tanino hidrolisável, que é hidrolisado por enzimas. São utilizados para estabilização da cerveja, curtimento de pele e produção de resinas. Atualmente, são empregados em processos biotecnológicos para produção de enzimas como a tanase, que hidrolisa ésteres e ligações laterais de taninos hidrolisáveis produzindo ácido gálico e glicose. A maior aplicação desta enzima tem sido para produção de ácido gálico, que é utilizado na produção de trimetropim e sínteses de ésteres como propil galato, usado como antioxidante na indústria de alimentos. A enzima também é aplicada no processamento de cerveja e clarificação de sucos, processamento de chás instantâneos e tratamento de efluentes contaminados com compostos fenólicos.

Palavras-chave: taninos, tanase, biotecnologia, alimentos

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INTRODUÇÃO

Os taninos ocorrem em uma ampla variedade de vegetais, podendo ser encontrados nas raízes, folhas, frutos, sementes e cascas de plantas. São classificados em dois grupos: taninos hidrolisáveis e condensados. Tanase é uma enzima extracelular, induzível, produzida na presença de ácido tânico por fungos, bactérias e leveduras (Aguilar *et al*., 1999). Embora existam muitas aplicações industriais da tanase em potencial, poucas são efetivamente empregadas devido essencialmente ao custo de produção da enzima, que ainda é elevado. A enzima tem vasta aplicação na indústria de alimentos, sucos, cervejaria, cosméticos, farmacêutica e indústria química (Lekha e Lonsane, 1994).

TANINOS

Os taninos pertencem a um grupo de compostos fenólicos provenientes do metabolismo secundário das plantas e são definidos como polímeros fenólicos solúveis em água que precipitam proteínas (Haslam, 1966). O teor e a espécie de tanino variam, não só de um vegetal para outro como também de uma parte para outro do mesmo vegetal conforme Tabela 1 (Santos, 2000).

Tabela 1: Teor de taninos totais em algumas espécies vegetais

Produtos	Teor de tanino	Referência
Abacaxi		
Folha	0,81 %	Santos, 2001
Caule	0,61 %	Santos, 2001
Sorgo	0,60 – 2,61 %	Rodrigues, 1991
Mandioca	0,62 – 1,11 %	Carvalho *et al*., 1993
Caju	0,35 – 0,72 %	Embrapa 1992
Café (casca)	1,31 – 2,97 %	Filho *et al*., 2000

ESTRUTURA E FONTES DOS TANINOS

As principais características dessa classe de compostos são: solubilidade em água, exceto os de elevado peso molecular; possuem a habilidade de ligar-se a proteínas, combinar-se com a celulose e a pectina para formar complexos insolúveis (Pinto, 2003). Os taninos são classificados em dois grupos: taninos hidrolisáveis e taninos condensados (Salunkhe, 1990; Carneiro, 2001).

a) Taninos hidrolisáveis: Os taninos hidrolisáveis estão presentes em folhas, galhos, cascas e madeiras de várias árvores como: *Terminalia, Phyllantus e Caesalpina*, dentre outros gêneros. Os taninos hidrolisáveis são unidos por ligações esterásicas(ligação éster entre o grupo anel aromático e o resíduo de glicose) e depsidásica (ligação éster entre os anéis aromáticos), sendo prontamente hidrolisados por ácidos, bases e enzimas (ex. tanase) em suas unidades formadoras (Fig 1) (Hagerman e Butler, 1981). A unidade básica estrutural desse tipo de tanino é um poliol, usualmente glicose, com seus grupos hidroxilas esterificados pelo ácido gálico (galotaninos) ou pelo hexahidroxidifênico (elagitaninos).

A Figura 2 mostra a estrutura química dos galotaninos e os grupos fenólicos que esterificam o núcleo glicosídico são constituídos por ácido gálico ou digálico**.** Há grande abundância de ésteres de glicose mono ou di-galoil na natureza que são considerados taninos. É necessário que pelo menos três grupos hidroxil da molécula de glicose estejam esterificados para exibir a capacidade de se ligar e precipitar proteínas para serem considerados taninos, o mais comum dos galotaninos é o ácido tânico (Figura 1) (Pinto, 2003). A Figura 3 mostra a estrutura molecular de elagitaninos. Nesta classe de taninos, os grupos fenólicos utilizados são moléculas de ácido hexahidroxidifênico.

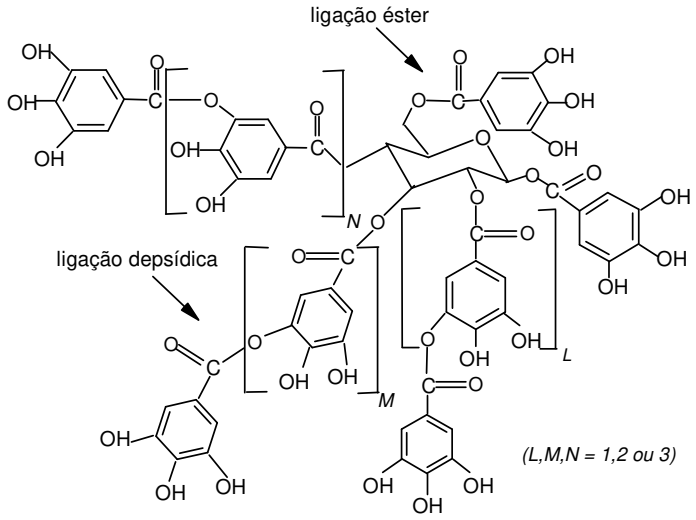

Figura 1: Estrutura química de tanino hidrolisável (Nakamura, 2003).

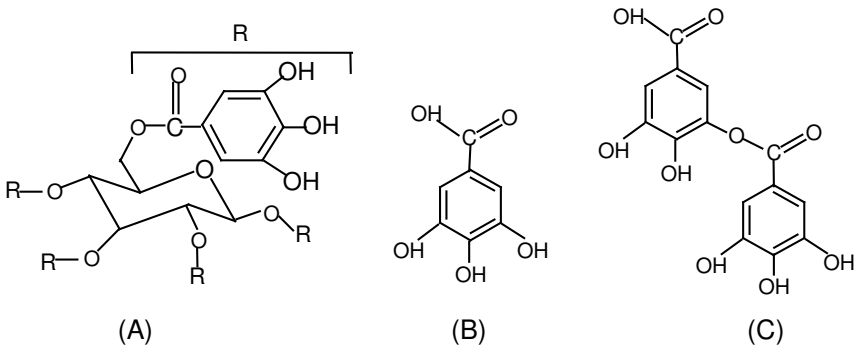

Figura 2: Estrutura de galotaninos (A), ácido gálico-galoil (B) e digálico - digaloil (C) (Bhat, 1998).

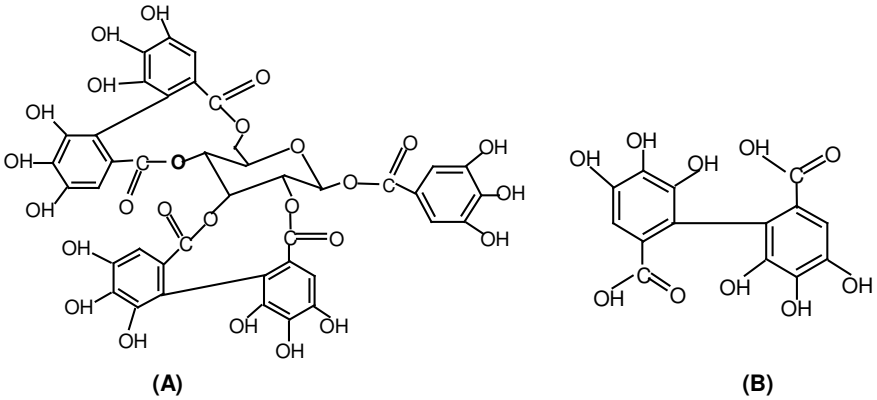

Figura 3: Estrutura de elagitaninos (A) e do ácido hexahidroxidifênico (B) (Bhat, 1998).

b) Taninos condensados

Os taninos condensados (TC) ou proantocianidinas são mais vastamente distribuídos no reino vegetal que os taninos hidrolisáveis. Ainda são chamados taninos condensados devido a sua estrutura química compacta (Fig 4).

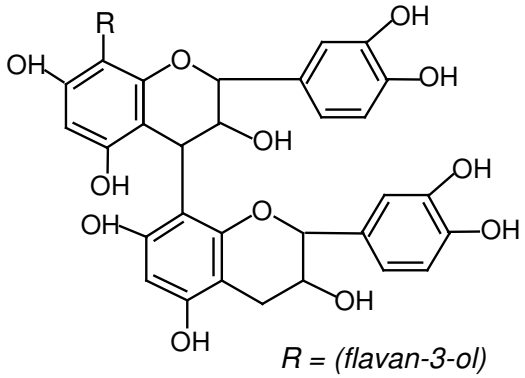

Figura 4: Estrutura de tanino condensado (Lekha e Lonsane, 1997).

Eles estão presentes em grande quantidade nos alimentos normalmente consumidos (Desphande *et al*., 1986; Salunkhe *et al*., 1990). Os TC podem conter duas a cinqüenta unidades flavanóides. Os flavonóides são a maior classe de polifenóis, são constituídos por dois anéis aromáticos ligados por um anel heterocíclico. Podem ser divididos em subclasses como: flavonóis, flavonas, flavanonas (Faria, 2005). Os taninos condensados possuem estruturação complexa; são resistentes à hidrólise, mas podem ser solúveis em solventes orgânicos aquosos, dependendo de sua estrutura. Em razão de suas estruturas químicas, sem a presença de ligações éster e depsídica, as proantocianidinas não são susceptíveis a hidrólise pela tanase (Pinto, 2003).

TANASE

Tanino acil hidrolase (TAH) conhecida como tanase (E.C: 3.1.1.20) é uma enzima que hidrolisa ésteres e ligações laterais de taninos hidrolisáveis produzindo glicose e ácido gálico (Banerjee *et al*., 2001). Tanase é uma enzima extracelular, induzível, produzida na presença de ácido tânico por fungos, bactérias e leveduras. Mesmo na presença de outras

fontes de carbono, a concentração de ácido tânico constitui o fator predominante na produção de tanase (Aguilar *et al*., 1999). A Figura 5 mostra a reação de hidrólise do ácido tânico.

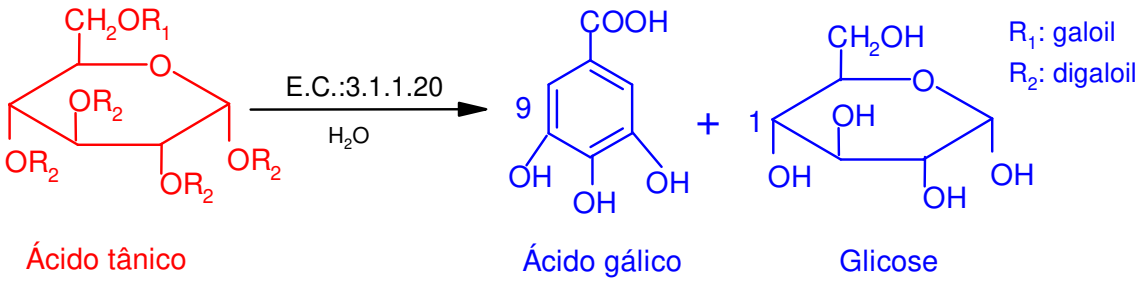

Figura 5: Hidrólise do ácido tânico (Aguilar e Gutiérrez-Sanchez, 2001).

PROPRIEDADES DA TANASE

A decomposição de taninos hidrolisáveis é medida por duas enzimas (Figura 1). Uma com atividade esterásica sobre a ligação éster (entre o grupo anel aromático e o resíduo de glicose) e a outra depsidásica (sobre a ligação éster entre os anéis aromáticos). Em estudo realizado por Beverini e Metche (1990), foram isoladas duas isoformas de tanase fúngica. As tanases denominadas I e II apresentaram as duas atividades, sendo que a tanase I apresentou maior característica esterásica e tanase II atividade depsídica mais pronunciada. De acordo com Haslam e Stragroom (1966) a proporção entre essas duas atividades pode variar de acordo com as condições de cultivo, bem como, ambas podem ser responsáveis pelas duas atividades. A tanase apresenta pH estável na faixa de 3,5-8,0; com pH ótimo entre 5,5-6,5; temperatura de estabilidade entre 30ºC e 70ºC, ótima na faixa de 30-50ºC e massa molecular entre 70kDa e 300kDa. Estas propriedades dependem fortemente das condições de cultura e também da linhagem utilizada. Tanino acil hidrolase é inibido por Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} e Mg^{2+} e é inativada pela ação de EDTA, 2-mercaptoetanol, sulfatos e cloretos de magnésio e cálcio (Aguilar e Gutiérrez-Sanchez, 2001).

APLICAÇÕES INDUSTRIAIS DA TANASE

Embora existam muitas aplicações industriais da tanase em potencial, poucas são efetivamente empregadas devido essencialmente ao custo de produção da enzima, que ainda é elevado. A enzima tem vasta aplicação na indústria de alimentos: sucos, cerveja; em cosméticos, fármacos e indústria química. Ela é principalmente utilizada para produção de ácido gálico, chás instantâneos, na estabilização da cor do vinho, processo de tratamento de couro, detanificação de alimentos, produção de antioxidantes e para tratamento de efluentes na indústria de couros (Banerjee *et al*., 2001).

a) *Chá instantâneo:* Uma das principais aplicações industriais da tanase é o emprego no processamento de chá instantâneo, a fim de eliminar complexos insolúveis indesejados, conhecidos como "creme de chá", resultantes da polimerização de polifenóis (taninos) e da interação de cafeína e proteínas com tais componentes, fenômeno que ocorre quando a bebida é mantida a baixas temperaturas. A utilização de tanase evita o emprego de substâncias químicas para a eliminação do "creme de chá", garantindo um produto final de excelente qualidade, solúvel em água e caracterizado pelo alto conteúdo de componentes aromáticos e coloração apropriada (Sanderson *et al*., 1974).

b) *Fabricação de Vinhos:* Na fabricação de vinhos, a aplicação de tanase nos resíduos do tratamento de uva favorece a remoção de compostos fenólicos, estabilizando e incrementando a qualidade dos vinhos (Lekha e Lonsane, 1997). Cerca de 50% da coloração do vinho se deve à presença de taninos; a oxidação destes componentes em contato com o ar (gerando quinonas) pode causar uma turbidez indesejável e conseqüentemente perda de qualidade do produto final. Essa turbidez pode ser evitada com o emprego da tanase, que impede a reação de oxidação (Lekha e Lonsane, 1997).

c) *Clarificação de sucos:* A tanase também é utilizada como agente clarificador em alguns sucos de frutas e em bebidas geladas a base de café, onde seu uso se concentra na remoção de compostos fenólicos presentes no material vegetal a fim de evitar sua complexação e precipitação (Lekha e Lonsane, 1994).

d) *Tratamento de efluentes:* Adicionalmente, a tanase tem um grande potencial de aplicação no tratamento de efluentes resultantes do processamento de couro e ácido tânico. Estes efluentes contêm altas quantidades de taninos, principalmente polifenóis, que são poluentes perigosos. O uso de tanase, a fim de decompor os taninos presentes, constitui em um tratamento de baixo custo e alta eficácia na remoção destes compostos.

e) *Produção de antioxidantes:* A tanase pode ser utilizada para produção de antioxidantes como epigalocatequina, epicatequina e ácido gálico. Segundo Banerjee *et al*. (2001), o ácido gálico tem várias aplicações na indústria química e farmacêutica. É utilizada para síntese de propil galato, pirogalol, trimetropim e resinas semicondutoras. O propil galato é uma substância amplamente utilizada como aditivo na indústria de alimentos e como antioxidante em óleos e produtos ricos em lipídeos. O pirogalol é utilizado como conservante na indústria de alimentos (Lekha e Lonsane, 1997), o trimetropim é um agente antibacteriana utilizada na indústria farmacêutica (Aguilar e Gutiérrez-Sanchez, 2001).

f) *Ração animal:* As aplicações de enzimas em rações para dietas animais vêm crescendo muito na última década. Os efeitos antinutricionais dos taninos são bem conhecidos: complexam-se com proteínas indisponibilizando-as, reduzindo o valor nutricional da dieta. O sorgo é geralmente utilizado como um complemento de rações animais. No entanto, vários cultivares de sorgo apresentam um alto conteúdo de taninos. A utilização desta enzima pode ser efetuada de duas diferentes formas: por contato direto dos extratos enzimáticos com o material a ser tratado, hidrolisando os polifenóis e evitando uma polimerização indesejada; ou pelo cultivo de linhagens de fungos produtores de tanase em substratos ricos em taninos, os quais são degradados a compostos simples, caracterizados pela propriedade de não se polimerizar ou não formar complexo com as proteínas e/ou cafeína presente nos materiais brutos.

A fim de reduzir custos e viabilizar o uso de tanase, faz se necessária à realização de estudos em diversas áreas de pesquisa a respeito da produção de tanase fúngica em escala industrial. Por esta razão, é necessário que se alcance um completo entendimento a respeito da regulação, capacidade catalítica, especificidade e aspectos de otimização desta produção em escala industrial (Aguilar e Gutiérrez-Sanchez, 2001).

PRODUÇÃO DE TANASE MICROBIANA

Tanino acil hidrolase pode ser obtida a partir de fontes vegetal, animal e microbiana. De fontes animais, TAH pode ser extraído do intestino bovino e das mucosas dos ruminantes. Os microrganismos são a fonte mais importante de obtenção da tanase, uma vez que as enzimas produzidas desta forma são mais estáveis do que aquelas obtidas por outros meios (Banerjee e Kar, 2000).

Aceita-se em geral, que as bactérias são muito sensíveis à presença de ácido tânico, porém bactérias como *Bacillus, Corynebacterium, Klebsiella, Streptococcus bovis e Selemonas ruminantium* são capazes de crescer neste composto e também de degradá-lo. Entretanto, os microrganismos mais estudados são os fungos filamentosos, entre eles *Assochyta, Aspergillus, Chaetomiun, Mucor, Myrothecium, Neurospora, Rhizopus, Trichothecium* e *Penicilium* (Lekha e Lonsane, 1997). Apesar de vários microrganismos produzirem tanase, estas não são igualmente ativas com todos os taninos hidrolisáveis. As tanases de leveduras são efetivas somente na decomposição do ácido tânico, em contrapartida as tanases bacterianas e fúngicas são eficientes na degradação de ácido tânico e outros taninos hidrolisáveis que ocorrem na natureza (Pinto, 2003).

A tanase é produzida na presença de um indutor, assim a utilização do ácido tânico como indutor ou única fonte de carbono, é fundamental. Apesar de outras fontes de carbono poderem estar presentes no meio, a concentração de ácido tânico é o fator predominante na produção da enzima. Diferentes fontes de nitrogênio, inorgânicas e orgânicas, como sulfatos de amônio, nitrato de sódio e peptona, têm sido utilizados para a produção de tanase através de fermentação sólida (Pinto, 2003).

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 (Pinto, 2003). O primeiro relato de produção de tanase por fermentação sólida data de 1917, onde se utilizou a linhagem de *Aspergillus oryzae* crescendo sobre farelo de trigo. Neste trabalho observou-se que a produção de tanase era maior à medida que se aumentava a concentração de tanino no meio. A partir de 20% de tanino, o crescimento do fungo foi inibido (Lekha e Lonsane, 1994). No final da década de 50 vários fungos foram

testados em meio contendo farelo de trigo. Observou-se que a tanase podia ser produzida por *Penicillium spp*, tendo como indutor ácido tânico ou ácido gálico. Segundo Lagemaat e Pyle (2001), a fermentação sólida para a produção de enzimas oferece um grande número de vantagens sobre o método de fermentação submersa e líquida convencional. O meio de produção é simples, utiliza subprodutos agroindustriais ou subprodutos como farelo de trigo, arroz ou aveia, acrescidos de ácido tânico.

O processo de fermentação sólida oferece aplicações promissoras para produção de compostos bioativos, enzimas e ácidos orgânicos que podem ser gerados por diferentes subprodutos agrícolas. Além deste aspecto, os volumes do fermentador e de efluentes resultantes podem ser menores (Lagemaat e Pyle, 2001). Estudos comparativos da produção de tanase por *Aspergillus niger* empregando três métodos de fermentação revelaram que a fermentação sólida produziu 2,5 a 4,8 vezes mais enzima do que as fermentações submersa e líquida, respectivamente, conforme indica a Figura 6 (Lekha e Lonsane, 1994).

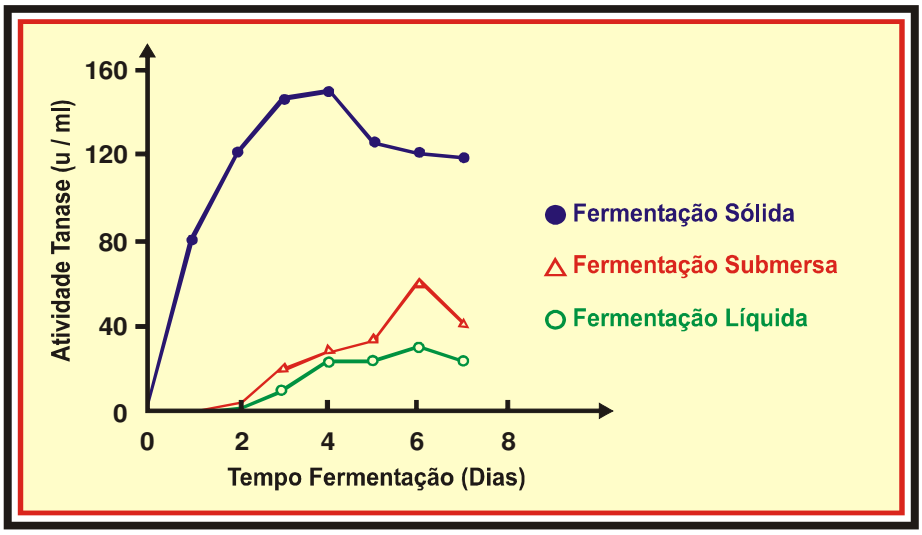

Figura 6: Comparação da produção de tanase por *A. niger PKL 104* em três diferentes tipos de fermentação: sólida, líquida e submersa (Lekha e Lonsane, 1994).

O interesse da fermentação sólida para a produção de compostos de importância comercial é conseqüência do interesse por produtos com menor custo de produção. A exploração adequada da fermentação sólida pode significar a redução do custo de produção de tanase. O uso de resíduos agroindustriais pode ajudar não somente a reduzir a poluição ambiental, mas também agregar valor às indústrias processadoras (Pandey *et al*., 2000). Estima-se que a produção de enzimas comerciais por fermentação sólida represente somente 5% do mercado de vendas de enzimas (Frost, 1986). Em geral, os substratos para a fermentação sólida são resíduos ou subprodutos da agroindústria. Farelos, cascas, bagaços são materiais considerados viáveis para a biotransformação por fermentação sólida. Estas matérias primas são recursos naturais renováveis e geralmente produzidos em grandes quantidades, muitas vezes tornando-se um problema ecológico e sanitário (Pinto, 2003).

CONCLUSÕES

As tanases são enzimas com grande potencial de aplicação industrial, mas com poucos casos comerciais evidenciados destas aplicações. A produção de insumos de alto valor agregado bem como detanificação de resíduos para rações ou resolução de problemas ambientais são alguns exemplos que se tornariam viáveis se o custo desta enzima fosse reduzido. Além deste aspecto, o estudo das características de ação da tanase ainda é pouca elucidada, dado a grande diversidade dos substratos (taninos) e à biodiversidade microbiana que sintetiza a enzima. Estes pontos por si só justificariam maior interesse e atenção ao assunto ora apresentado, de grande importância em nosso país que é essencialmente agrícola.

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CAPÍTULO 2: SELEÇÃO DE FUNGOS PRODUTORES DE TANASE EM RESÍDUOS VEGETAIS

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Publicado em: Ciência e Agrotecnologia, Lavras, (29): 833-838, 2005.

RESUMO

Tanino acil hidrolase conhecida como tanase (E.C: 3.1.1.20) é uma enzima que hidrolisa ésteres e ligações laterais de taninos hidrolisáveis produzindo glicose e ácido gálico. A tanase é uma enzima extracelular, induzível, produzida por fungos, bactérias e leveduras através da fermentação sólida, líquida ou submersa. A fermentação sólida para a produção desta enzima oferece um grande número de vantagens sobre o método de fermentação submersa e líquida convencional. O meio de produção é simples, utiliza resíduos vegetais de uva, caju, café ou subprodutos como farelo de trigo, arroz ou aveia, acrescidos de ácido tânico. A aplicação de resíduos é uma forma de utilizar substratos alternativos e solucionar problemas de poluição que possam causar. O objetivo deste trabalho foi selecionar linhagens fúngicas potencialmente produtoras de tanase em resíduos vegetais ricos em taninos. A primeira etapa da seleção foi realizada utilizando como substrato farelo de trigo suplementado com 0,5% de ácido tânico. Dentre as 500 linhagens testadas 7% produziram tanase. As linhagens que apresentaram as melhores atividades em farelo de trigo foram: LAB345G, LAB53G e LAB153G com atividades de 0,2862; 0,2149; 0,1848 U/mL. Essas linhagens foram testadas nos resíduos agroindustriais de café e uva acrescentando 0,5% e 1,5% de ácido tânico ao meio de fermentação. O melhor resultado foi obtido para o resíduo de café com a linhagem LAB153G apresentando atividade de 0,275 U/mL.

Palavras-chave: fungos, tanase, resíduos, taninos

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ABSTRACT

Tannin acyl hydrolase (E.C: 3.1.1.20) or tannase is an enzyme which hydrolyses ester and depside bonds of hydrolysable tannins releasing gallic acid and glucose. Tannase is an extracellular, inducible enzyme, produced by fungi, bacteria and yeast. The tannase is produced by solid-state, liquid surface and submerged fermentation. The solid-state fermentation offers a number of advantages over submerged and liquid conventional fermentation for the enzymes production. The production is simple, using vegetable residues such as coffee wastes, grape, cashew or by-products as wheat bran, rice or oats, to which tannic acid, is added. The use of residues is an alternative to solve pollution problems that can be caused by an incorrect environmental disposal. Tannase enzyme has several applications on food, juices and pharmaceutical industries. The objective of this work was to select tannase producing fungi and to study the production of the enzyme in vegetable residues rich in tannin. The first stage of the selection was carried out in solid-state fermentation using as substrate wheat bran supplemented with 0.5% of tannic acid. Among the 500 tested lineages, 6.75% of the fungi produced the enzyme. The lineages that showed the best activities were: LAB345G, LAB53G, and LAB153G with 0.3862; 0.2149; 0.1848 U/mL activities values. These lineages were tested in vegetables residues as coffee and grape, adding 0.5 and 1.5% of tannic acid in the fermentation environment. The best result was obtained using LA153G and coffee residues, with an activity of 0.275U/mL being observed.

Key words: screening, tannase, residues, tannin

INTRODUÇÃO

Tanino acil hidrolase, conhecida como tanase (E.C: 3.1.1.20), é uma enzima que hidrolisa ésteres e ligações laterais de taninos hidrolisáveis (Banerjee *et al.*, 2001). O ácido tânico é um típico tanino hidrolisável, que pode ser hidrolisado por tanase em glicose e ácido gálico (Helbig, 2000). A tanase pode ser obtida a partir de fontes vegetal, animal e microbiana (Banerjee *et al.*, 2000). De fontes animais, TAH pode ser extraído do intestino bovino e das mucosas dos ruminantes. Os microrganismos são a fonte mais importante de obtenção da tanase, uma vez que as enzimas produzidas desta forma são mais estáveis do que aquelas obtidas por outros meios. Além disso, microrganismos podem produzir TAH em altas quantidades e de maneira contínua, com grande rendimento (Banerjee *et al.*, 2000). A tanase é uma enzima extracelular, induzível, produzida na presença de ácido tânico por fungos, bactérias e leveduras (Aguilar *et al.*, 1999).

Embora existam muitas aplicações industriais da tanase em potencial, poucas são efetivamente empregadas devido essencialmente ao custo de produção da enzima, que ainda é elevado e, principalmente ao pouco conhecimento sobre seu mecanismo de ação catalítica (Lekha e Lonsane, 1997). No presente estudo, o objetivo foi selecionar linhagens fúngicas produtoras de Tanino Acil Hidrolase, e testar as melhores linhagens em substratos provenientes de resíduos agroindustriais através de fermentação sólida.

MATERIAL E MÉTODOS

Microrganismos

Foram testadas 500 linhagens fúngicas pertencentes ao Laboratório de Bioquímica da Faculdade de Engenharia de Alimentos da UNICAMP, isoladas de diversas regiões do país.

Extração e quantificação dos taninos

Resíduos de café, uva, caju, carambola e acerola na forma em pó, sofreram extração com hexano (remoção de lipídeos e corantes) utilizando 3 mL/g de amostra em cada

extração, através de três lavagens sucessivas, agitados em Shaker a 200 rpm durante 15 min. Em seguida, a amostra foi lavada três vezes com etanol (extração dos taninos) (3 mL/g de amostra). O extrato obtido foi centrifugado a 10,070x*g* por 15 minutos a temperatura de 4ºC e no sobrenadante foram quantificados os taninos. A um volume de 1,0 mL do extrato etanólico obtido foram adicionados 2,0 mL de solução padrão de proteínas (para precipitação dos taninos) composta por tripsina, ovoalbumina e albumina de soro bovino (1,0 mg/mL) em tubos de 15 mL (Hagerman e Butler, 1978). A mistura foi homogeneizada a temperatura ambiente e após 15 minutos foi centrifugada a 10,070x*g* por 15 minutos a temperatura de 4ºC. O sobrenadante foi descartado e o precipitado ressuspenso em 4 mL de SDS-Trietanolamina (detergente com função de romper o complexo formado entre tanino-proteína). A esta solução foi adicionado 1 mL de $FeCl_3$ e após 30 minutos foi medida a absorbância da amostra em 530 nm seguindo a metodologia de Hagerman e Butler (1978).

Preparação do Inóculo

As linhagens fúngicas foram repicadas em meio inclinado PDA (Potato Dextrose Agar) com suplemento de 0,2% (p/v) de ácido tânico ($C_{76}H_{42}O_{56}$) e incubadas em estufa Fanem à 30ºC por 72 horas.

Meio de fermentação

Em frascos Erlenmeyers de 250 mL foram adicionados 10g de farelo de trigo e 10 mL de solução de sais na composição (g/L): KH_2PO_4 (1,0); NH_4NO_3 (2,0); $MgSO_4.7H_2O$ (0,2); $CaCl_2.2H_2O$ (0,02); $MnCl_2.4H_2O$ (0,004); $Na_2MoO_4.2H_2O$ (0,002); $FeSO_4.7H_2O$ (0,0025) e ácido tânico 0,5%. O meio de cultivo foi esterilizado a temperatura de 120ºC por 20 minutos, com umidade relativa 60% e pH 5,7. Após a esterilização os frascos foram inoculados com $5x10^7$ esporos/mL (referente a cada linhagem fúngica testada) e incubados a 30ºC em estufa Fanem por 120 horas (os esporos foram removidos adicionando-se 2,5 mL de água destilada e raspados com auxílio de alças de inoculação, a contagem dos esporos foi feita em câmara de Neubauer). Após a fermentação, foram adicionados 100 mL de solução NaCl 1,5% e os frascos foram agitados a 200 rpm por 2 horas. A solução foi

filtrada e centrifugada a 10,070x*g* por 30 minutos a temperatura de 4ºC; o sobrenadante foi denominado extrato enzimático bruto (Lekha e Lonsane, 1997).

Medida da atividade enzimática da tanase

A solução de substrato foi preparada pela adição de 0,5 % (p/v) de ácido tânico em tampão acetato pH 5,5 - 0,2 M. A reação foi realizada adicionando-se 0,3 mL da solução de substrato com 0,5 mL de extrato enzimático bruto e incubada a temperatura de 60^{o}C por 10 minutos. Após a incubação, a reação foi interrompida pela adição de 3 mL de solução de albumina de soro bovino (BSA) na concentração de 1 mg/mL (essa solução foi preparada em tampão acetato pH 5,0-0,2 M, contendo 0,17 M de cloreto de sódio), sendo em seguida centrifugada a 10,070xg por 15 minutos a temperatura de 4ºC. O precipitado foi ressuspenso em 3 mL de solução SDS-trietanolamina (SDS 1% (p/v) adicionado de 5% (v/v) de trietanolamina em água destilada) acrescido de 1 mL de solução de $FeCl_3$ (0,01M de $FeCl_3$ em 0,01M de ácido clorídrico). A absorbância foi medida após 15 minutos em 530 nm conforme metodologia descrita por Mondal *et al.,* (2001). A atividade enzimática foi calculada pela diferença da leitura de absorbância medida a 530 nm entre amostra e tubo controle (no tubo controle a enzima tanase foi desnaturada utilizando banho em ebulição durante 10 min). Uma unidade de atividade de tanase foi definida como a quantidade de ácido tânico hidrolisado por mL de enzima empregada por minuto de reação: $Abs_{530} = Abs_{controle} - Abs_{teste}$.

As linhagens selecionadas como melhores produtoras potenciais de tanase foram testadas em substratos provenientes de resíduos agroindustriais de indústrias de processamento de polpas e sucos da região de Campinas-SP.

RESULTADOS E DISCUSSÃO

A seleção de linhagens fúngicas produtoras de tanase foi realizada em meio de fermentação sólida utilizando como substrato farelo de trigo suplementado com 0,5% de ácido tânico. Entre as 500 linhagens testadas, 87% dos fungos não sintetizaram a enzima, 6% dos fungos foram inibidos na etapa de pré inoculação quando utilizou-se o meio PDA com 0,2% (p/v) de ácido tânico, comprovando o efeito de inibição que os taninos podem

exercer sobre o crescimento de microrganismos. Em relação às propriedades antimicrobianas dos taninos, muitos fungos, bactérias e leveduras são resistentes aos taninos e podem crescer e se desenvolver na presença destes, como: *Aspergillus, Penicillium, Fusarium, Bacillus cereus, Corynebacterium sp, Candida sp* e *Pichia sp* (Bhat *et a*l., 1998). Os resultados da seleção indicaram que 7% das linhagens testadas são capazes de sintetizar tanino acil hidrolase o que foi comprovado nas condições do teste de atividade da tanase. Com o objetivo de selecionar os melhores fungos produtores da tanase, optou-se pela escolha das linhagens com atividade superior ou igual a 0,12 U/mL (Tabela 1).

Tabela 1: Linhagens pré-selecionadas como produtoras de tanase em farelo de trigo com 0,5% (p/p) de ácido tânico após 120 horas de fermentação

Linhagem	Atividade (U/mL)
LAB345G	0,2862
LAB53G	0,2149
LAB153G	0,1848
LAB306G	0,1681
LAB343G	0,1513
LAB146G	0,1242

As linhagens com maior potencial produtor de tanase foram as de número LAB345G, LAB53G e LAB153G com valores de atividade de 0,2862, 0,2149 e 0,1848 U/mL respectivamente. Essas linhagens foram testadas em meio à base de resíduos agroindustriais de café e uva adicionando-se 0,5% e 1,5% de ácido tânico ao meio de fermentação. Na determinação de taninos totais dos resíduos agroindustriais verificou-se que casca de café e uva tiveram maiores concentrações de taninos dentre os resíduos testados de caju, carambola e acerola, apresentando valores de 0,073% e 0,941% respectivamente. A esses resíduos foram adicionados ácido tânico nas concentrações de 0,5 e 1,5% (p/p) para os testes de fermentação. A Figura 1 mostra os resultados obtidos para a

fermentação sólida em farelo de trigo, resíduos de café e uva com adição de 0,5% e 1,5% (p/p) de ácido tânico.

O farelo de trigo tem sido até hoje o substrato mais estudado para produção de tanase por fermentação sólida. Todas as linhagens testadas neste resíduo foram capazes de produzir a enzima conforme mostra a Figura 1. As linhagens LAB153G, LAB345G, LAB53G apresentaram valores de atividade de 0,522, 0,663 e 0,406 U/mL quando se adicionou 1,5% de ácido tânico, resultando em aumentos de 2,8; 2,3 1,9 vezes se comparados à atividade enzimática com 0,5% de ácido tânico. No resíduo de café, com adição de 1,5% de ácido tânico, todas as linhagens produziram tanase. Para as linhagens LAB153G e LAB345G o aumento da concentração de ácido tânico de 0,5% para 1,5% resultou em acréscimos de 3 e 2,8 vezes na atividade enzimática que passou de 0,089 e 0,038 U/mL para 0,275 e 0,106 U/mL. Para a linhagem LAB53G a produção somente ocorreu com 1,5% de ácido tânico com atividade de 0,216 U/mL. Para o resíduo de uva, as linhagens LAB153G e LAB345G produziram tanase com adição de 1,5% de ácido tânico com atividades de 0,1037 e 0,1021 U/mL. Com adição de 0,5% de ácido tânico somente a linhagem LAB153G produziu a enzima com 0,0498 U/mL.

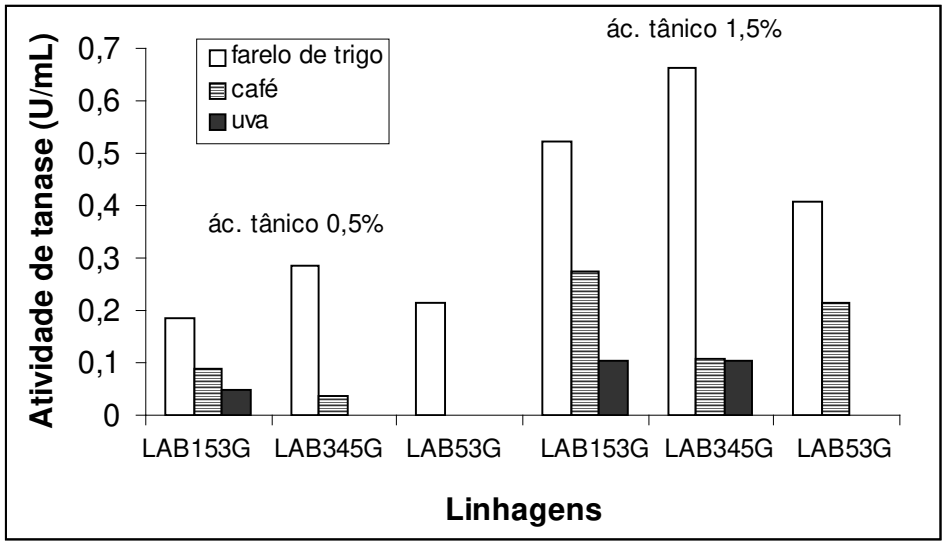

Figura 1: Produção de tanase pelas linhagens LAB153G, LAB345G e LAB53G em farelo de trigo e resíduos de café e uva com 0,5% e 1,5% (p/p) de ácido tânico após 120 horas de fermentação.

O resíduo de casca de café mostrou-se promissor substituto do farelo de trigo, com resultados melhores que os obtidos com os resíduos de uva. O teor de tanino total presente nos resíduos agroindustriais não parece ser assimilável ao metabolismo dos microrganismos sendo necessário suplementação. Apesar de o farelo de trigo ser considerado um bom substrato para a produção da tanase, é necessário que se explore outros resíduos que possam ser eficientes em relação à produção da enzima. Para o resíduo de café, quantidades apreciáveis da tanase foram produzidas, portanto, a utilização deste resíduo consiste em uma alternativa viável e pouco onerosa para produção da tanase em fermentação sólida. Estudos indicam que apenas 6% do conteúdo de uma fruta de café cereja colhida transforma-se em bebida. O restante (polpa, casca e mucilagem) é descartado ao longo do processo, constituindo uma grande proporção de resíduos e subprodutos, requerendo utilização (Pulgarin *et al.*, 1991).

A produção da enzima mostrou estar diretamente relacionada com a concentração de ácido tânico que é adicionada ao meio de fermentação, esta fonte de carbono favorece a produção rápida de tanase que, por sua vez, cliva os taninos fornecendo suprimento contínuo de fonte de carbono. Em estudo realizado por Mondal *et al.,* (2001) quando se utilizou *Bacillus cereus KBR9* para a produção da tanase adicionando 10 g/l de ácido tânico ao meio de fermentação submersa, obteve-se valores para a atividade enzimática de 0,22 U/mL. De acordo com Lekha e Lonsane (1997), Bajpai e Patil (1997) e Pinto (2003), o ácido tânico desempenha o papel de fonte de carbono para o microrganismo, bem como de indutor da síntese. Dessa maneira, a presença de ácido tânico é imprescindível para a síntese de tanase. Em trabalho realizado por Pinto (2003) em experimento preliminar onde não se adicionou ácido tânico ao meio de fermentação, não foi observada atividade de tanase em nenhum tempo de fermentação.

CONCLUSÕES

1. Foram obtidas de 500, 7% de linhagens potencialmente produtoras de tanase através da seleção induzida pela presença de ácido tânico.
2. Mostrou-se que a concentração de ácido tânico no meio de cultura é fator chave na produção da tanase, sendo objeto de mais estudos em andamento.
3. Dentre os resíduos analisados, uva e café foram os melhores resíduos selecionados para a produção de tanase. Nos testes fermentativos, no resíduo de casca de café, foram obtidas as melhores respostas de produção da tanase em relação à uva.
4. O melhor fungo produtor da tanase em resíduo de café e farelo de trigo foi a linhagem LAB 153G. Essa linhagem foi identificada como *Paecilomyces variotii* (ver Capítulo 3 e 4). A identificação da linhagem fúngica foi realizada pela *Fundação André Tosello* (Sao Paulo, Brasil).

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CAPITULO 3: TANNASE PRODUCTION BY *Paecilomyces variotii*

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Publicado em: Bioressource Technology, (98): 1832-1837, 2007

ABSTRACT

Surface response methodology was applied to the optimization of the laboratory scale production of tannase using a lineage of *Paecilomyces variotii*. Preliminary study was used to evaluate the effects of variables, including temperature (ºC), residue (%) (coffee husk:wheat bran), tannic acid (%) and salt solutions (%) on the production of tannase during 3, 5 and 7 days of fermentation. Among these variables, temperature, residues and tannic acid had significant effects on tannase production. The variables were optimized using surface response methodology. The best conditions for tannase production were: temperature (29-34°C); tannic acid (8.5 – 14%); % residue (coffee husk:wheat bran 50:50) and incubation time of 5 days. The supplementation of external nitrogen and carbon sources at 0.4, 0.8 and 1.2% concentration on tannase production were studied in the optimized medium. Three different nitrogen sources included yeast extract, ammonia nitrate and sodium nitrate and carbon source (starch) were studied. Only ammonia nitrate showed a significant effect on tannase production. After the optimization process, the tannase activity increased by 8.6 times.

Key words: tannase, response surface, residues

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INTRODUCTION

Tannin acyl hydrolase, commonly referred to as tannase (E.C: 3.1.1.20), is an enzyme that cleaves ester linkages in hydrolysable tannins (Banerjee *et al*., 2001; Belmares et al., 2004), producing glucose and gallic acid (Banerjee *et al*., 2005). Tannase is an extracellular, inducible enzyme produced in the presence of tannic acid by fungi, bacteria and yeast (Aguilar and Sanchez, 2001). The first step in the development of microbial enzyme production is the lineage selection. Extracellular enzymes are preferred because they are easily extracted and do not require expensive extraction methods (Couri *et al*., 1998). Studies on the production of tannase using solid, liquid and submerse fermentation have been reported (Lekha and Lonsane, 1994; Pinto, 2003). Solid-state fermentation provides several advantages over conventional enzyme production processes and agroindustrial by-products can be used with the addition of tannic acid (Lagemaat and Pyle, 2001).

There are several potential industrial applications for tannase, however, due to the high production costs and limited knowledge of its catalytic action, there are currently only a few applications. Tannase has several applications in the food, juice, beer, cosmetic, pharmaceutical and chemical industries (Lekha and Lonsane, 1997; Batra and Saxena, 2005), but its main uses are in gallic acid production, tea production and the stabilization of wine color and coffee flavor, in leather treatment and others (Banerjee *et al*., 2001). Since only a few fungi have been reported to produce this enzyme, the search is on for organisms that produce elevated amounts of tannase. The studies reported in this paper were conducted to examine the tannase production of *Paecilomyces variotii*, and to evaluate optimal reaction conditions.

METHODS

Microorganism and preparation of the pre-inoculum

Paecilomyces variotii is a new lineage obtained by means of fungal isolation procedures and was used for the production of tannase. Five hundred fungal cultures were obtained from the departmental stock culture collection (of the Food Science Department-

Unicamp), collected from different places in Sao Paulo State (Brazil) and screened for their tannase producing ability. The best tannase producing fungus was identified as *Paecilomyces variotii*, the identification was made by *Fundação André Tosello* (Sao Paulo State, Brazil). The lineage was maintained in potato dextrose agar (PDA) slants, stored at temperature of 7 ºC. The lineage was replicated in PDA containing 0.2% (w/v) of tannic acid and incubated at 30 ºC for 72 h. The pre-inoculum was prepared by adding 2.5 mL of distilled water to remove the spores, obtaining a suspension containing $5.0x10^7$ spores /mL.

Fermentation media

For the fermentation process, a 250 mL conical flask was used containing the following constituents: 10 g of wheat bran, 10 mL of a salts solution containing (g/L): KH_2PO_4, 1.0; NH_4NO_3, 2.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.02; $MnCl_2.4H_2O$, 0.004; $Na_2MoO_4.2H_2O$, 0.002; $FeSO_4.7H_2O$, 0.0025 and 3% tannic acid (w/w). The culture medium, pH 5.7, was sterilized at temperature of 120ºC for 20 minutes at a relative humidity of 60%. After sterilization, the flasks were inoculated with 2.5 mL of the pre-inoculum suspension and incubated at temperature of 30 ºC for 120 h. After fermentation, 80 mL of 20 mM acetate buffer, pH 5.0 were added and shaken at 200 rpm for 1 h. The solution was filtered and centrifuged at 10,070x*g* for 30 minutes at temperature of 4 ºC**.**

Determination of tannase activity

The substrate was a 0.7 % (w/v) solution of tannic acid in 0.2 M acetate buffer at pH 5.5. The reaction was conducted by adding 0.3 mL of substrate solution to 0.5 mL of enzyme extract and incubating at temperature of 60 ºC for 10 min. After incubation, the reaction was stopped by the addition of 3 mL of a 1.0 mg/mL bovine serum albumin solution (BSA) prepared in a 0.17 M sodium chloride solution in 0.2 M acetate buffer, pH 5.0. The solution was then filtered and centrifuged at 10,070x*g* for 15 min at temperature of 4 ºC. The precipitate was dissolved in 3 mL of SDS-triethanolamine (solution contained 1% SDS and 5% (v/v) triethanolamine in distilled water) and 1 mL of $FeCl_3$ reagent (contained 0.01 M of $FeCl_3$ in 0.01 M hydrochloric acid) added and held for 15 min for stabilization of the colour. The absorbance was measured at 530 nm (Mondal *et al*., 2001) and the enzyme

activity calculated from the change in absorbance at 530 nm. One unit of tannase activity was defined as the amount of tannic acid hydrolysed by 1 mL of enzyme per minute of reaction: $Abs_{530} = Abs_{control} - Abs_{test}$.

Experimental design

Response surface methodology is an effective tool for optimizing the process. If the proposed model is adequate, as revealed by the diagnostic checking provide by an analysis of variance (ANOVA) and residual plots, contour plots can be usefully employed to study the response surface and locate the optimum operational conditions (Macedo *et al*., 2003). A fractional (2^{4-1}) experimental design was used, statistical analysis was carried out to identify the variables that had significant effect on the response i.e. tannase production.

The variables tested were: temperature (27-33 ºC), residue % (coffee husk:wheat bran) at (100:0-0:100), tannic acid (3-10%) and salts solution (0-100%*). Tannase production was measured after 3, 5 and 7 days of fermentation (Table 1). After the preliminary analysis, the statistical significant variables were temperature (ºC), residue (%) and tannic acid (%). These variables were used for a complete experimental design. The optimum time for fermentation was 5 days. The complete experimental design was done with 2^3 experiments plus 6 axial points and 3 central points to estimate the experimental error (Table 2). The *F*-test was performed to determine factors having a significant effect ($P \leq 0.10$) (Table 3). The data was analysed using the STATISTICA®, 5.0 software.

Effect of nitrogen and carbon source on tannase production in optimized media

Metabolite concentrations and growth of mold are strongly influenced by medium composition, such as the carbon and nitrogen source. Tests were conducted to determine the effect of various external nitrogen and carbon sources on tannase production after media optimization. Nitrogen sources included yeast extract, ammonia nitrate and sodium nitrate were examined. An added carbon source (starch) was also evaluated at different concentrations (0.4, 0.8 and 1.2%). The enzyme production was monitored after 5 days at 32ºC.

RESULTS AND DISCUSSION

Experimental design for the principal variables in the fermentation process

The first optimization step is to identify the variables having a significant effect on enzyme production. The variables evaluated are listed in Table 1 and shows the tannase activity obtained after 3, 5 and 7 days of fermentation. The statistically significant variables at a 90% confidence level were temperature (ºC), % residue (coffee husk:wheat bran) and tannic acid (%). The maximum tannase production observed was in 5 days of fermentation.

Table 1: Tannase activity according to experimental design

No.	Temperature (ºC)	% Residue (coffee:wheat bran)	Tannic acid (%)	Salts solution (%)	Activity (U/mL)		
					3 days	5 days	7 days
1	(-1) 27	(-1) 100:0	(-1) 3	(-1) 0	0.2824	0.2802	0.1009
2	(+1) 33	(-1) 100:0	(-1) 3	(+1) 100*	0.2759	0.2878	0.1778
3	(-1) 27	(+1) 0:100	(-1) 3	(+1) 100	0.2718	0.4273	0.1199
4	(+1) 33	(+1) 0:100	(-1) 3	(-1) 0	0.4132	0.6623	0.2512
5	(-1) 27	(-1) 100:0	(+1) 10	(+1) 100	0.2873	0.3460	0.1315
6	(+1) 33	(-1) 100:0	(+1) 10	(-1) 0	0.2808	0.2740	0.1420
7	(-1) 27	(+1) 0:100	(+1) 10	(-1) 0	0.5396	0.6923	0.2891
8	(+1) 33	(+1) 0:100	(+1) 10	(+1) 100	0.7492	1.3178	0.3913
9	(0) 30	(0) 50:50	(0) 6.5	(0) 50	0.4600	0.5036	0.2202
10	(0) 30	(0) 50:50	(0) 6.5	(0) 50	0.3371	0.4137	0.1995
11	(0) 30	(0) 50:50	(0) 6.5	(0) 50	0.3623	0.5096	0.2035

*Initial salts solution used as described in the fermentation media item

Following the first factorial design, a second experimental design was used to optimize the production tannase. The most important variables were temperature (27-37 ºC); % residue (coffee husk:wheat bran) between (70:30–30:70) and tannic acid (6-18%) (Table 2). The results for activity tannase are presented in Table 2 and Table 3 shows the analysis of variance (ANOVA).

Table 2: Complete factorial experimental design for tannase production

No.	Temperature (ºC)	% Residue (coffee:wheat bran)	Tannic acid (%)	Response (units tannase)	
				Experimental	Predicted
1	(-1) 29	(-1) 62:38	(-1) 8.43	1.0790	0.9455
2	(+1) 35	(-1) 62:38	(-1) 8.43	0.7832	0.9678
3	(-1) 29	(+1) 38:62	(-1) 8.43	1.2767	1.3569
4	(+1) 35	(+1) 38:62	(-1) 8.43	1.4167	1.3792
5	(-1) 29	(-1) 62:38	(+1) 15.57	0.9643	0.9594
6	(+1) 35	(-1) 62:38	(+1) 15.57	0.5177	0.6450
7	(-1) 29	(+1) 38:62	(+1) 15.57	1.5240	1.3708
8	(+1) 35	(+1) 38:62	(+1) 15.57	1.1415	1.0564
9	(-1.68) 27	(0) 50:50	(0) 12	0.9514	1.0746
10	(+1.68) 37	(0) 50:50	(0) 12	0.9446	0.8292
11	(0) 32	(-1.68) 70:30	(0) 12	1.0382	0.9322
12	(0) 32	(+1.68) 30:70	(0) 12	1.5095	1.6233
13	(0) 32	(0) 50:50	(-1.68) 6	1.2767	1.2182
14	(0) 32	(0) 50:50	(+1.68) 18	0.8925	0.9588
15	(0) 32	(0) 50:50	(0) 12	1.4288	1.4387
16	(0) 32	(0) 50:50	(0) 12	1.5263	1.4387
17	(0) 32	(0) 50:50	(0) 12	1.3824	1.4387

Table 3: Analysis of variance for the response of the dependent variables on tannase production

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F test[a]
Regression	1.1899	7	0.1699	8.7577
Residual	0.1750	9	0.0194	
Lack of fit	0.1671	7	0.0238	
Pure error	0.0078	2	0.0039	
Total	1.3650	16		

$R^2 = 0.87$ [a]F 0.9; 7;9 = 2.51

The pure error was minimal, indicating sufficient reproducibility of the data obtained. The correlation coefficient of 0.87 and the F-test was sufficient to obtain an adequate model (equation 1) to represent the real relationship between the response activity and the variables. The regression analysis from the results of Table 2 can fit a second order polynomial where: tannase activity is (Y), temperature (x_1), % residue (x_2) and tannic acid (x_3). The validation model was done using the one-way analysis of variance (Table 3).

$$Y = 1.44 - 0.073x_1 + 0.206x_2 - 0.077x_3 - 0.172x_1^2 - 0.057x_2^2 - 0.124x_3^2 - 0.084x_1x_3 \quad (1)$$

The equation generated allows one to fit the response surfaces to find the best conditions for tannase production. Fig 1 and 2 indicated the best temperature range as being from 29 ºC to 34 ºC. Kar *et al*., (2002) and Lekha *et al*., (1994) in similar work using response surface methodology for tannase production, obtained similar values for the temperature range (32 ºC and 28 ºC) using *Rhizopus oryzae and Aspergillus niger PKL 104*. Pinto (2003), observed a temperature range of 32 ºC – 40 ºC using *Aspergillus niger* 3T5B8.

The addition of tannic acid (8% to 15%) to the culture medium (Fig 1 and 3) can result in an increase in tannase production, but values greater than 15% resulted in a decrease in tannase production. Molecules known as inducers can influence enzyme production. An increment in the amount of inducer can result in an increase in enzyme synthesis. However, a great increase in inducer concentration will not necessarily cause an equivalent increase in enzyme synthesis. Some effects such as inducer solubility; toxicity level and saturation can determine the ideal concentration (Pinto, 2003). Lekha and Lonsane (1994), observed an increase in tannase synthesis by *Aspergillus niger PKL104* with the addition of tannic acid, the best concentration being 4%. Values above this level can induce a reduction in tannase activity. In addition, in a study using *Aspergillus oryzae*, tannase production was highest with a tannic acid concentration of 20%, although fungal growth was inhibited (Lekha and Lonsane, 1997). According to Pinto (2003), the best tannic acid concentrations to induce high tannase production were between 10 and 15%. With 20% tannic acid, a decrease in tannase synthesis was observed. The initial tannic acid concentration affected the levels of enzyme produced, and depending on the microorganism and the amount of tannase produced, the fermentation took from 1 to 10 days. In the present case, peak tannase production was reached with 12% tannic acid after 5 days of fermentation. The tannase activity from *Paecilomyces variotii* was 1.3-1.6 U/mL. Mondal *et al*. (2001) reported tannase activity of 0.22 U/mL from *Bacillus cereus KBR9* using 10 g/L of tannic acid.

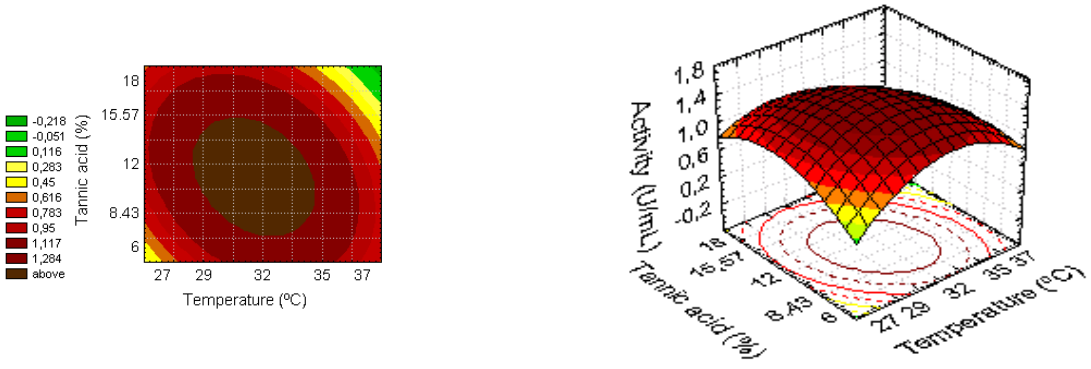

Figure 1. Response surface and contour diagrams for tannase activity considering tannic acid and temperature.

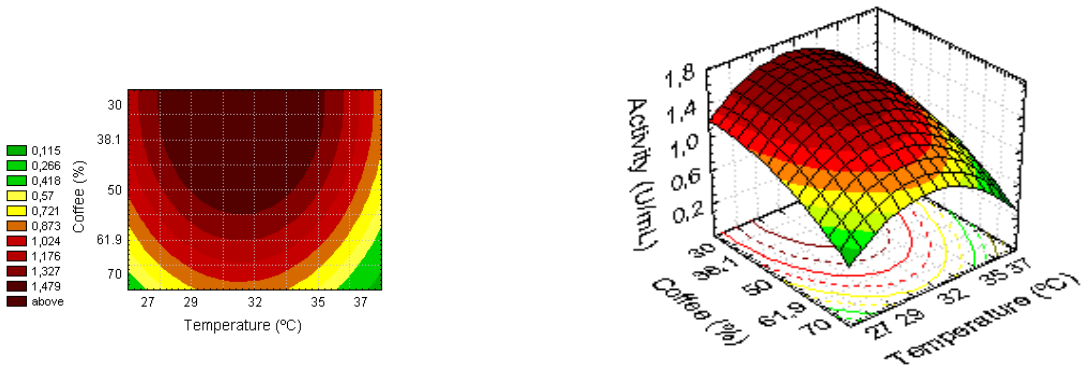

Figure 2. Response surface and contour diagrams for tannase activity considering coffee residue and temperature.

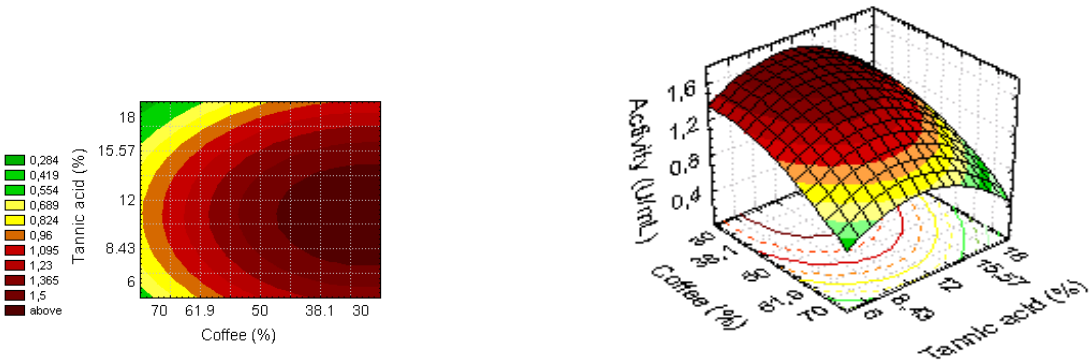

Figure 3. Response surface and contour diagrams for tannase activity considering coffee residue and tannic acid.

In our experiment, tannase activity increased with decrease in coffee residue (Fig 2 and 3). This suggests that wheat bran residue has a more positive effect on tannase production. Wheat bran residue and polyurethane foam are substrates frequently used in solid-state fermentation. Whereas wheat bran is a good substrate for tannase production, coffee husk is an agroindustrial residue economically viable with great disponibility in Brazil. Good results for tannase activity were obtained with 50% coffee residue in a mixture substrate using coffee residue and wheat residue (50:50). The experiments above were conducted using (50:50) coffee husk: wheat bran as substrate.

Effect of supplementation of nitrogen and carbon sources on tannase production in optimized medium

The impact of supplementation of external nitrogen and carbon sources at 0.4-1.2% concentration on tannase activity were studied. Tree different nitrogen sources and one carbon source were examined and the results are shown in Table 4.

Table 4: Effect of nitrogen and carbon source on tannase production

Nitrogen source	Concentration (%)	Relative activity (%)
Ammonium nitrate	Control	100 ± 4.8 [a]
	0.4	172 ± 7.5 [b]
	0.8	170 ± 8.0 [b]
	1.2	186 ± 5.0 [b]
Sodiun nitrate	Control	100 ± 7.5 [a]
	0.4	96 ± 5.5 [a]
	0.8	91 ± 6.3 [a]
	1.2	101 ± 5.0 [a]
Yeast extract	Control	100 ± 2.7 [a]
	0.4	38 ± 5.8 [b]
	0.8	65 ± 5.0 [b]
	1.2	60 ± 5.7 [b]
Carbon source		
Starch	Control	100 ± 4.8 [a]
	0.4	102 ± 7.0 [a]
	0.8	116 ± 5.0 [a]
	1.2	113 ± 5.0 [a]

(Values are means of duplicates and those with different letters are significantly different at $p<0.05$).

Tannase production depends on the availability of both carbon and nitrogen sources in the medium, both have regulatory effects on enzyme synthesis. Among all nitrogen sources tested in our study, ammonium nitrate was the most suitable as it resulted in the highest enzyme activity. By its contribution of ammonium ions, the ammonium nitrate stimulates the synthesis of proteins and is a source of readily utilizable nitrogen (Djekrif-Dakhmouche *et al*., 2006) (Table 4). Other nitrogen source such as yeast extract inhibited enzyme formation by the fungal culture. The yeast extract cause decrease of the tannase activity. These results are probably due to the excessive amount of the yeast extract, which may inhibit the production of enzyme. This decrease may also be caused by the complex nitrogen sources in the media fermentation. On basis of analysing the results, we may conclude that microorganisms necessitate a low level of nitrogen in order to produce enzymes because nitrogen may be a limiting factor (Djekrif-Dakhmouche *et al*., 2006).

Results recorded in Table 4 showed that supplementation of optimized medium with external carbon source had no effect on enzyme production by *Paecilomyces variotii.* Lekha and Lonsane (1997) reported that tannase production by *Aspergillus niger* PKL 104 was not affected by the addition of another readily utilizable carbon source. Similar results were reported by Bradoo *et al*. (1997) when using different additives on tannase production by *Aspergillus japonicus*. Starch addition at each level had no effect on tannase production, which leads us to conclude that residues coffee husk constitute an adequate carbon source for tannase production. These nutrients were probably available for fungal metabolism and an additional source of nutrients was not required**.**

CONCLUSION

According to the optimization process, the best conditions for tannase production by the lineage of *Paecilomyces variotii* were: temperature (29 to 34 ºC); % residue (coffee husk:wheat bran - 50:50); tannic acid (8 to15 %) and ammonium nitrate 1.2%. Using the optimized process, the best set resulted in 9 times more enzyme production than was obtained before optimization.

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CAPÍTULO 4: BIOCHEMICAL CHARACTERIZATION OF TANNASES FROM *Paecilomyces variotii* AND *Aspergillus niger*

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Publicado em: Food Science and Biotechnology, vol. 16, nº 2, pxx. (2007).

ABSTRACT

A biochemical characterization of the tannases from *Paecilomyces variotii* (produced at Unicamp), *Aspergillus niger* (purchased from Industrial Kerry Bio-Science) and *Aspergillus niger* cnpat 001 (purchased from Embrapa Agroindustrial Tropical-Brazil) was carried out. *Paecilomyces variotii* is a new strain obtained from the screening of five hundred fungi that were tested for their production of tannase. The biochemical properties of this new tannase from *Paecilomyces variotii* were determined and compared with those of two other tannase preparations. The tannase produced from *Paecilomyces variotii* showed optimum activity at pH 6.5, whereas *Aspergillus niger* and *Aspergillus niger* cnpat 001 showed pH optima at 4.0 and 5.5. The tannases produced by *Paecilomyces variotii,* by the commercially obtained *Aspergillus niger* and by *Aspergillus niger* cnpat 001 showed 100% stability at pH 6.5, 5.5 and 4.0 respectively. The functional temperature range of the tannases was from 20-70 ºC, with optima at 70 ºC for *Paecilomyces variotii* and at 60 ºC for the commercially obtained tannase, whereas *Aspergillus niger* cnpat 001 showed optimum activity at 40 ºC. The tannase from *Paecilomyces variotii* was shown to be more stable under extreme conditions of pH and temperature. The effects of 1mM preparations of cations and anions, inhibitors, surfactants and chelators on the tannase activity from *Paecilomyces variotii* were also studied.

Key words: Biochemical characterization; Tannase; *Paecilomyces variotii*

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INTRODUCTION

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an inducible enzyme that catalyses the breakdown of ester linkages in hydrolysable tannins such as tannic acid, resulting in glucose and gallic acid (Saxena and Saxena, 2004; Van de Lagemaat and Pyle, 2005). Studies on the production of tannase using solid, liquid and submersed fermentation have been reported (Lekha and Lonsane, 1994). Solid-state fermentation provides several advantages over conventional enzyme production processes (Van de Lagemaat and Pyle, 2001). The fermentation broth can use by-products such as wheat bran, rice bran, sugar beet pulp, fruit pulps, banana waste, cassava waste and coffee residues, adding tannic acid. The use of by products or residues rich in sources of carbon for fermentation purposes is an alternative way of solving pollution problems that can be caused by incorrect disposal in the environment (Battestin and Macedo, 2005). In the present work, a *Paecilomyces variotii* lineage obtained by fungal isolation procedures was used for the production of tannase using coffee husk and wheat bran residues. Tannase is extensively used in wine, beer and coffee-flavoured soft drinks or as an additive in food detanification. Gallic acid is also used in the enzymatic synthesis of propyl gallate, which is mainly used as an antioxidant in fats and oils (Vaquero *et al*., 2004; Belmares *et al*., 2004).

This paper reports on the determinations of pH and temperature optima and stabilities of tannases from the newly isolated strain P*aecilomyces variotii, Aspergillus niger* and *Aspergillus niger* cnpat 001. The effects of cations, anions, inhibitors, chelators and surfactants on the tannase activity of *Paecilomyces variotii* were also determined.

MATERIAL AND METHODS

Screening and Microorganisms

Five hundred fungal cultures were obtained from the departmental stock culture collection of the Food Science Department-Unicamp and were screened for their tannase producing ability. The best tannase producing fungus was identified as *Paecilomyces variotii* (Fig 1). The tannin acyl hydrolases studied were obtained from the three strains, *Paecilomyces variotii* (produced at Unicamp), *Aspergillus niger* (purchased from Industrial

Kerry Bio-Science - this is an enzyme system containing pectinase, tannase and esterase activities), *Aspergillus niger* cnpat 001 (purchased from Embrapa Agroindustrial Tropical-Brazil).

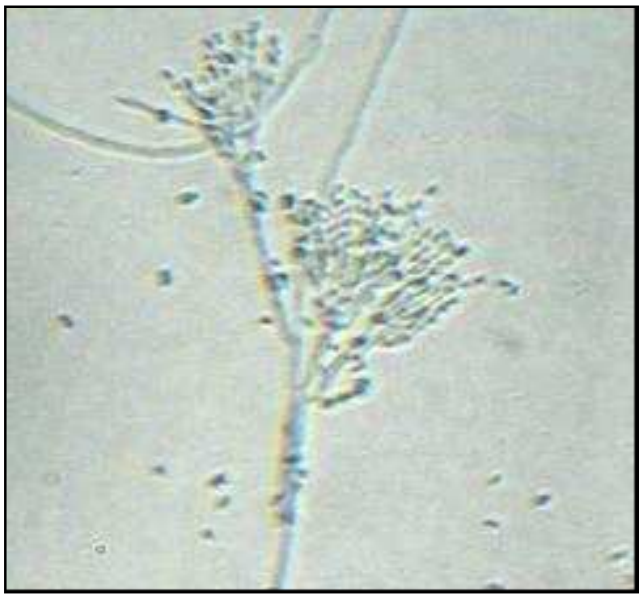

Figure 1: Optical microscopic image of *Paecilomyces variotii* at 1000x.

Chemicals

All the chemicals were of analytical grade. Tannic acid was from Ajinomoto OmniChem Division.

Microorganism preservation and preparation of the pre-inoculum

A *Paecilomyces variotii* strain obtained using fungal isolation procedures was used for the production of tannase. The strain was maintained in PDA (potato dextrose agar) slants, stored at temperature of 7 ºC. The lineage was replicated in PDA containing 0.2% (w/v) of tannic acid and incubated at temperature of 30 ºC for 72 h. The pre-inoculum was prepared by adding 2.5 mL of distilled water to remove the spores, obtaining a suspension containing $5.0x10^7$ spores/mL.

Fermentation media

For the fermentation process, a 250 mL conical flask was used containing the following constituents: 5 g of wheat bran and 5 g of coffee husk, 10 mL of distilled water, 10% of tannic acid (w/w) and ammonium nitrate 1.2% (w/w). The culture medium was sterilized at temperature of 120 ºC for 20 min and the relative humidity of the medium after sterilization remained at 60%. After sterilization, the flasks were inoculated with 2.5 mL of the pre-inoculum suspension and incubated at temperature of 30 ºC for 120 h. After fermentation, 80 mL of 20 mM acetate buffer, pH 5.0 were added and Shaker at 200 rpm for 1 h. The solution was filtered and centrifuged at 10,070x*g* for 30 min at temperature of 4 ºC (Centrifuge Beckman J2-21, Beckman-Coulter, Inc. Fullerton, CA, USA). The supernatant was than treated with solid ammonium sulphate (80% saturation) and allowed to stand overnight at temperature of 7 °C. The precipitate was collected by centrifugation (10,070x*g* -30 min), dissolved in distilled water and dialysed against distilled water. The dialysed preparation was used as crude tannase.

Tannase assay

A colorimetric assay was used to determine tannase activity, based on measuring the residual tannic acid content after the enzymatic reaction (Mondal *et al*., 2001). The reaction mixture consisted of 0.3 mL of the substrate tannic acid (0.7% (w/v) in 0.2 M acetate buffer at pH 5.5) and 0.5 mL of the enzyme extract, incubating at temperature of 60 ºC for 10 min. The enzymatic reaction was paralysed by the addition of 3 mL of a bovine serum albumin solution - BSA (1mg/mL- prepared in a 0.17 M sodium chloride solution in 0.2 M acetate buffer, pH 5.0.), leading to the precipitation of the remaining tannic acid. The tubes were than centrifuged at 9,650x*g* for 15 min at temperature of 4 ºC and the precipitate dissolved in 3 mL of SDS-triethanolamine (solution contained 1% SDS and 5% (v/v) triethanolamine in distilled water), followed by the addition of 1 mL of $FeCl_3$ reagent (contained 0.01 M of $FeCl_3$ in 0.01 M hydrochloric acid) and holding for 15 min for colour stabilization. The absorbance was measured at 530 nm and the enzyme activity calculated from the change in absorbance at 530 nm. One unit of tannase activity was defined as the amount of tannic

acid hydrolysed by 1 mL of enzyme per minute of reaction): $Abs_{530} = Abs_{control} - Abs_{test}$. (Mondal *et al*., 2001).

Biochemical characterization of the tannases

The determinations of the pH and temperature optima and stabilities were used for the biochemical characterisations of the tannase from *Paecilomyces variotii*, the commercially obtained tannase from *Aspergillus niger* and the tannase from *Aspergillus niger* cnpat 001. The effects of cations, anions, inhibitors, chelators and surfactants on the tannase activity of *Paecilomyces variotii* were also determined.

Effect of pH on tannase activity

The optimum and the pH range for tannase activity were determined by testing the enzyme activity in different buffers for a pH range from 3.5 to 9.0. Acetate buffer (0.2 M) was used for the range from 3.5 to 5.5, phosphate buffer (0.2 M) for pH 6.0 to 8.0 and Tris-HCl buffer (0.2 M) for pH 8.5 to 9.0 for 10 min at 60ºC. The stability of the enzyme was examined at different pH values by incubating the enzyme in buffers at different pH values ranging from 3.5 to 9.0 for 24 h at 30 ºC. The residual activity was estimated and expressed as a percentage of the relative tannase activity.

Effect of temperature on tannase activity

The optimum temperature and the temperature range for tannase activity were determined by carrying out the reaction at different temperatures ranging from 20 to 90 °C at pH 6.5 (at regular intervals of 10 °C). The heat stability of the enzymes was examined by incubating the test sample at different temperatures ranging from 20 to 100 °C for 30 min. Residual activity was estimated and expressed as a percentage of the relative tannase activity. All the above tests were carried out in duplicate.

RESULTS AND DISCUSSION

Effect of pH on tannase activity and stability

The tannase produced by *Paecilomyces variotii* showed optimum activity at pH 6.5, whereas *Aspergillus niger* and *Aspergillus niger* cnpat 001 showed pH optima at 4.0 and 5.5 (Fig 2). These results are in agreement with earlier reports in which a pH optimum of 5.0-6.0 was reported for *Aspergillus niger* (Barthomeuf et al., 1994), *Penicilliun chrysogenum* (Rajkumar and Nandy, 1983) and *Aspergillus caespitosum*, *Penicillium crustosum* and *Penicillium variable* (Batra and Saxena, 2005). The tannases produced by *Aspergillus versicolor*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Penicillium restricticum* showed optimal tannase activity at pH 5.0 (Batra and Saxena, 2005).

Tannase from *Paecilomyces variotii* showed 100% stability at pH 6.5 and 88% and 86% stability, respectively, at pH 4.0 and 7.5 after 24 h of incubation at 30 ºC. This enzyme showed a wide range of pH stability, was moderately active at an alkaline pH of 8.0 (retaining 77% activity) and at an acidic pH of 3.5 (retaining 67% activity). In contrast, the tannase produced by the commercially obtained *Aspergillus niger* and that produced by *Aspergillus niger* cnpat 001 showed 100% activity at pH 5.5 and 4.0 respectively. These enzymes were active at an acidic pH 3.5 (retaining 88% and 95% activity) and at an alkaline pH of 8.0 (retaining 16% and 8.0% activity respectively) (Fig 2).

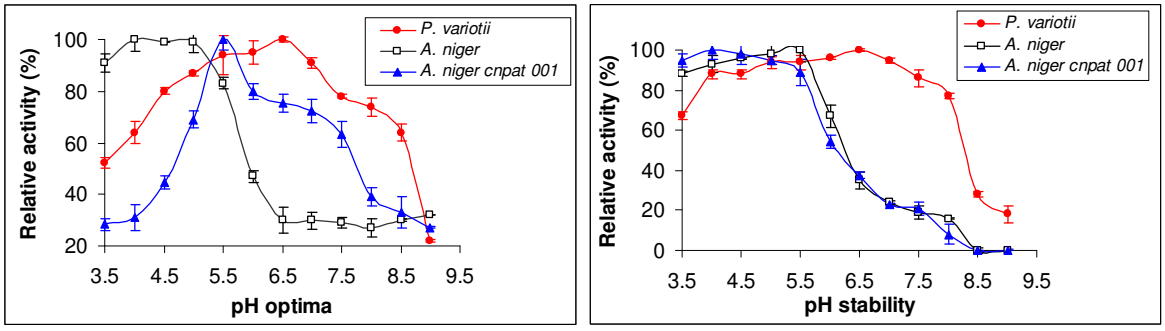

Figure 2. pH optima and pH stability of tannases from *Paecilomyces variotii*, *Aspergillus niger* and *Aspergillus niger* cnpat 001.

Similar results were reported for *Aspergillus niger* LCF 8 (Barthomeuf *et al*., 1994), *Candida sp* (Aoki *et al.*, 1976), and *Penicilliun restricticum* (Batra and Saxena, 2005), where the tannases were found to be stable in the pH range from 3.5 to 8.0 for 16 h and 24h. On the other hand, tannases produced by *Penicillium chrysogenum* (Rajkumar and Nandy, 1983), *Aspergillus flavus* and *Penicillium charlesii* (Batra and Saxena, 2005) were only stable in a narrow pH range of from 5.0-5.5 and a wider pH range of 4.5-6.0, 3.0-6.0 and 4.0-5.0 respectively, for 30-60 min.

Effect of temperature on tannase activity and stability

The functional temperature range of the tannases produced was 20-70 ºC with optima at 70 ºC for *Paecilomyces variotii,* 60 ºC for the tannase obtained commercially (produced from *Aspergillus niger)* and 40 ºC for that from *Aspergillus niger* cnpat 001 (Fig 3). These results are also in agreement with previous reports concerning *Aspergillus niger* van Tieghem (Sharma *et al*., 1999) and *Aspergillus niger* PKL 104 (Lekha and Lonsane, 1994), where the temperature optima were 50, 60 and 70 ºC, respectively.

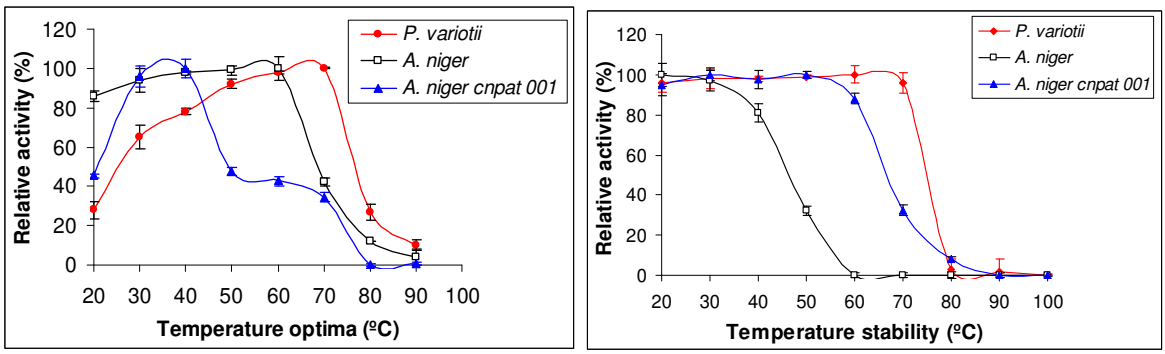

Figure 3. Temperature optima and temperature stability of tannases from *Paecilomyces variotii*, *Aspergillus niger* and *Aspergillus niger* cnpat 001.

However, lower temperature optima of 40 ºC have also been reported for *Aspergillus caespitosum*, *Peniccilium charlesii*, *Penicillium crustosum* and *Penicillium restrictum* (Batra and Saxena, 2005) and of 30 ºC for *Aspergillus oryzae* (Iibuchi *et al*.,

1968), *Aspergillus niger* (Barthomeuf *et al*., 1994) and *Penicillium chrysogenum* (Rajkumar and Nandy, 1983).

The tannase from *Paecilomyces variotii* was stable in a temperature range from 20-70 ºC, with maximum stability at 60 ºC, retaining 100% activity after 30 min. The tannase obtained commercially (produced from *Aspergillus niger)* showed maximum stability at 20 ºC, but 81% and 32% residual activity was observed at 40 and 50 ºC. This tannase was relatively less stable at higher temperatures. The tannase from *Aspergillus niger* cnpat 001 was stable in a temperature range of 20-60 ºC, showed 100% stability at 30 ºC, and 65% and 88% residual activity was observed at 20 and 60 ºC respectively (Fig 2). Similarly, the tannases from *Aspergillus fumigatus* (Batra and Saxena, 2005) have been reported to be stable at 60ºC for 30 and 60 min respectively. Tannases from *Penicillium charlesii* and *Penicillium crustosum* retained 100% activity at 40 and 50 ºC respectively. *Aspergillus caespitosum* tannase retained 100% and 92% residual activity at 40 and 50 ºC, but retained only 46% and 18% residual activity at 60 and 70 ºC, respectively (Batra and Saxena, 2005).

Effect of monovalent and divalent cations on tannase activity

The influence of various compounds, including $BaCl_2$, $CaCl_2$, $MgSO_4$, $ZnSO_4$, $MnSO_4$, K_2SO_4, $AgNO_3$, NaOH, $CaCO_3$ and $Na_2S_2O_3$ on tannase activity produced by *P. variotii* was studied (Fig 4). Samples of 0.12% (v/v) solutions of the enzyme containing 1 mM of the compounds were incubated at 60ºC for 10 min. The tannase assays were then carried out following the procedure described by Mondal *et al*. (2001). Of the monovalent and divalent cations studied Ba^{+2}, Ca^{+2}, Zn^{+2}, Mn^{+2} and Ag^{+} acted as inhibitors of the tannase activity produced by *Paecilomyces variotii.* Kar *et al.,* (2003) and Brado *et al*., (1997) reported similar results, where Ba^{+2}, Ca^{+2}, Zn^{+2}, Mn^{+2} and Ag^{+} inhibited tannase activity at concentrations of 1 mM and 0.2% respectively. In contrast, Mg^{+2} stimulated the maximum tannase activity at concentrations of 1 mM and 5 mM (Koyama, 1998; Kar *et al*., 2003). The inhibitory effect of metal ions is well documented in the literature. It is known that mercury ions react with protein thiol groups (converting them to mercaptides) and also

react with histidine and tryptophan residues. Heavy metal ion (Ag^{+}), react rapidly, and a low concentrations, with sulfhydryl groups of enzyme (Whitaker, 1972).

Effect of monovalent and divalent anions on tannase activity

Figure 4 shows the effect of OH^{-}, CO_3^{-2} and $S_2O_3^{-2}$ on tannase activity, of these anions, only $S_2O_3^{-2}$ induced tannase activity at a concentration of 1 mM. Kar *et al*., (2003) observed similar results, with $S_2O_3^{-2}$ enhancing tannase activity and CO_3^{-2} inhibiting tannase activity at a concentration of 1 mM.

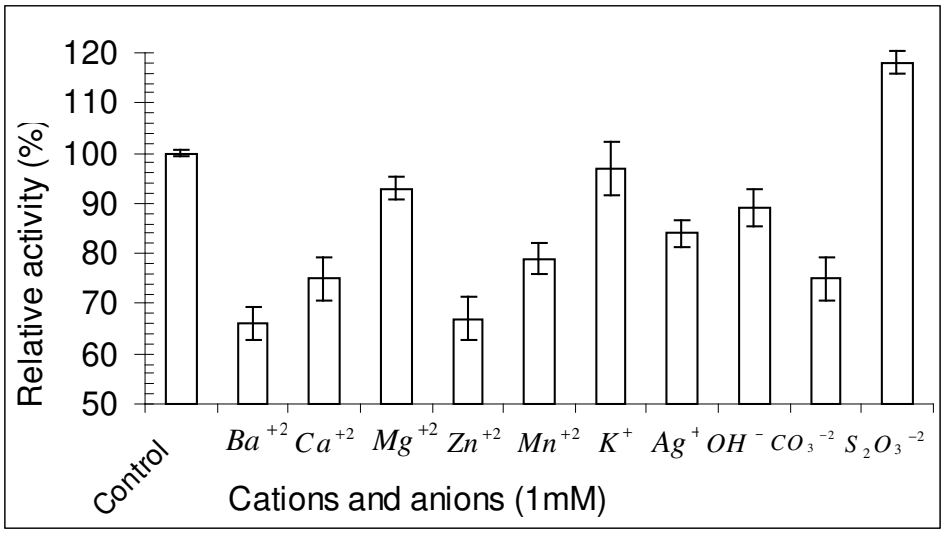

Figure 4. Effect of cations and anions on tannase activity produced by *P. variotii*.

Effect of inhibitors on tannase activity

The inhibitors evaluated for their effects on tannase activity were sodium bisulphite, iodoacetamide, 2-mercaptoethanol, 4-aminobenzoic acid, sodium azide, n-bromosuccinimide and cysteine. Tannase activity was inhibited by sodium bisulphite, 2-mercaptoethanol, 4-aminobenzoic acid, sodium azide, n- bromosuccinimide and cysteine at a concentration of 1 mM (Fig 5). The tannase from *Aspergillus niger* was reported to be inactivated by 2-mercaptoethanol (Barthomeuf et *al*., 1994; Aguilar and Gutiérrez-Sánchez, 2001). When added to the reaction medium, cysteine inhibited the tannase activity of

Paecilomyces variotii. The inhibition of tannase activity by cysteine and 2-mercaptoethanol suggests the present of sulphur containing amino acids at the active site of the enzyme. Inhibition by n- bromosuccinimide indicated that tryptophan residues played an important role in maintaining the active conformation of the enzyme (Saxena *et al*., 2003).

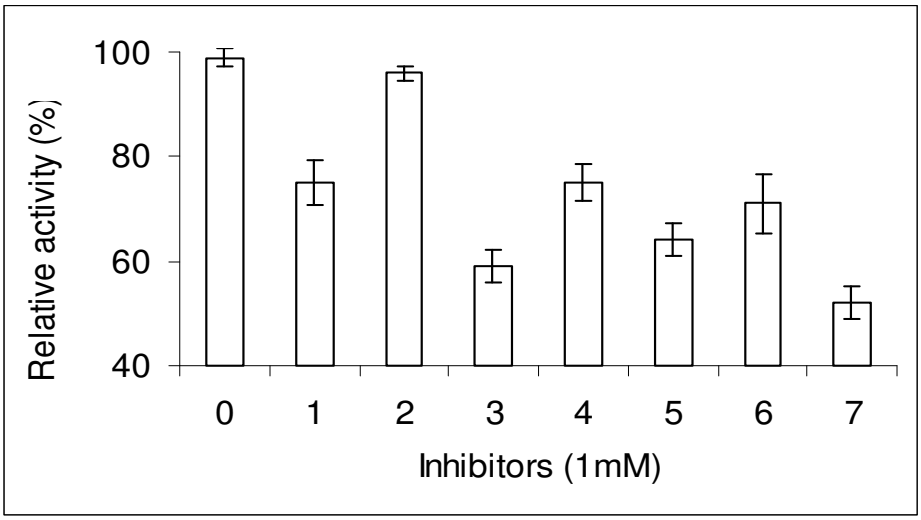

Figure 5. Effect of inhibitors on tannase activity produced by *P. variotii*: 0) control; 1) sodium bisulphite; 2) iodoacetamide; 3) 2-mercaptoethanol; 4) 4-aminobenzoic acid; 5) sodium azide; 6) n-bromosuccinimide; 7) cysteine .

Effect of chelator on tannase activity

The chelator ethylene diamine tetra acetic acid disodium salt (EDTA disodium salt) at a concentration 1 mM, inhibited the tannase from *Paecilomyces variotii* (Table 1). The tannase from *Aspergillus niger* was inactivated by EDTA (Barthomeuf *et al*., 1994; Kar *et al*., 2003) and the tannase from *Aspergillus oryzae* was completely inactivated by EDTA (Iibuchi *et al*., 1968). The decrease detected in the presence of EDTA could be due to its influence on the interfacial area between the substrate and enzyme (Lopes *et al*., 2002), and EDTA forms complexes with divalents cations (Whitaker, 1972).

Table 1: Effect of chelator on tannase activity produced by *P. variotii*

Chelator	Concentration	Relative activity (%)
Control	-	100 ± 2.67
EDTA	1 mM	62 ± 3.59

Effect of surfactants on crude tannase activity

The effects of Tween 80, Tween 20 and Triton X-100 (0.25-1% (v/v)) were studied, using enzyme solutions containing 0.12% (v/v) and the above chemical substances at the concentrations mentioned. Tween 80 and Tween 20 caused a decrease in tannase activity produced by *P. variotii* at concentrations of 0.25, 0.5% and 1% (v/v) (Table 2). This inhibition may be the result of a combined effect of factors such as the reduction in the hydrophobic interactions that play a crucial role in holding together the tertiary protein structure, and a direct interaction with the protein molecule (Kar *et al*., 2003). Triton X-100 caused a decrease in tannase activity at concentrations of 0.5 and 1 % (v/v)). These results are in agreement with those of Kar *et al*., (2003), who used Triton X-100 at concentrations of 0.03-0.5% (v/v) and showed a reduction in tannase activity.

Table 2: Effect of Tween 80, Tween 20 and Triton X-100 on tannase activity produced by *P. variotii*

Additives	*Concentration (%(v/v))*	*Relative activity (%)*
Control	-	100 ± 0.64
Tween 80	0.25	85 ± 1.83
	0.5	77 ± 1.53
	1.0	70 ± 1.84
Tween 20	0.25	81 ± 2.12
	0.5	75 ± 2.44
	1.0	66 ± 0.91
Triton X-100	0.25	96 ± 0.31
	0.5	93 ± 0.61
	1.0	87 ± 0.62

CONCLUSION

In most countries where the economy is largely based on agriculture and where farming practice is very intensive, accumulation of agricultural residues is a serious problem. Solid-state fermentation technology using non-pathogenic microorganisms that can produce hydrolytic enzymes such as tannases would be advantageous for the proper utilization of these residues. Our isolate, identified as *Paecilomyces variotii*, was able to grow in media containing a mixed substrate including coffee husk and wheat bran residues. Wheat bran is a good substrate for tannase production and coffee husk is a highly available, economically viable agro-industrial residue in Brazil.

The biotechnological use of agricultural residues is becoming more and more significant, with the dual goal of waste disposal and value addition. Since microbial activity, especially fungal activity, is the key aspect in this area, there is enormous opportunity for the cost effective production of tannase. So far, few reports are available on tannase production from *Paecilomyces variotii*. This work allowed for a better understanding of the effects of temperature, pH, cations, anions and inhibitors on the tannase activity of *Paecilomyces variotii*, presenting important data from a newly-isolated fungus that produces an interesting tannase. The biochemical properties of this new tannase produced by *Paecilomyces variotii* were determined and compared with those of two other tannase preparations. The tannase from *Paecilomyces variotii* was shown to be more stable under extreme values of pH and temperature. The tannase produced was functional at a wide range of temperature and pH values. Our inhibitory reagent studies suggest the presence of sulphur containing amino acids at the active site and also tryptophan residues. These properties can be further exploited in developing tannase for a wider range of applications in the pharmaceutical, food; feed and leather industries and thus their production at higher levels should be sought for. Therefore, *Paecilomyces variotii* would provide a new source for the efficient production of tannase for industrial applications.

ACKNOWLEDGMENTS

This work was supported by CAPES. The authors are grateful to Embrapa Agroindustrial Tropical and Kerry Bio-Science.

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CAPÍTULO 5: PARTIAL PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF TANNASE PRODUCED BY *Paecilomyces variotii*

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Aceito para publicação em: Food Biotechnology, Issue 3 (2007).

ABSTRACT

An extracellular tannase was isolated from *Paecilomyces variotii* and partially purified using ammonium sulphate precipitation followed by DEAE-Sepharose ion exchange chromatography. *Paecilomyces variotii* is a newly isolated strain obtained in São Paulo, Brazil from the screening of five hundred fungi tested for their production of tannase. The tannase was separated into 2 peaks. SDS-PAGE analysis indicated that the purified enzyme migrated as a single protein band corresponding to molecular masses of 87.3 kDa (major peak) and 71.5 kDa (minor peak). The peaks eluted very close together between 150 and 250 mM NaCl. DEAE-Sepharose column chromatography led to an overall purification of 19.3 fold. The K_m was found to be 0.61 µmol and the V_{max} = 0.55 $U.mL^{-1}$. Temperatures from 40 to 65ºC and pH values from 4.5 to 6.5 were optimum for tannase activity and stability. This tannase could find potential use in the food-processing industry.

Key Words: tannase, purification, characterization, electrophoresis, fermentation

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INTRODUCTION

Tannins are phenolic compounds, which can be grouped as hydrolyzable and non-hydrolyzable tannins. Tannin acyl hydrolase (E.C.3.1.1.20), commonly named tannase, hydrolyses the hydrolyzable tannins and catalyses the hydrolysis of ester and depside bonds in tannic acid releasing glucose and gallic acid (Mahapatra et al., 2005). It has extensive applications in the food, pharmaceutical, beverage, chemical industries, extensively used in wine, beer and coffee-flavoured soft drinks or as an additive in food detanification (Mukherjee and Banerjee, 2006). Gallic acid is also used in the enzymatic synthesis of propyl gallate, which is mainly used as an antioxidant in fats and oils (Vaquero et al. 2004; Belmares et al. 2004).

Tannase production can be done by various methods such as liquid surface, submerged and solid-state fermentation (Mahapatra et al., 2005). Tannase production by solid-state fermentation (SSF) is more advantageous as compared to submerged or liquid surface fermentation (Purohit, 2006). Filamentous fungi are ideally suited for successful SSF, since they grow in nature on solid substrates such as the wood, stems, roots and leaves of plants in the absence of free water (Mukherjee and Banerjee, 2006). The fermentation broth can use by-products such as wheat bran, rice or oats, adding tannic acid. The use of by products or residues rich in carbon sources for fermentation purposes is an alternative to solve pollution problems that can be caused by incorrect environmental disposal (Battestin et al., 2005).

Usually the end products of a fermentation process contain some unwanted components, which have to be eliminated as far as possible by downstream processing (Mukherjee and Banerjee, 2006). The purification and characterization of tannase has been previously attempted owing to its wide applications in various food, leather and pharmaceutical industries. Various media preparations can be used with tannic acid as the sole carbon source for the production of microbial tannase, but the biotransformation of tannin rich agro residues is cost-effective (Mukherjee and Banerjee, 2006).

The extent and technique of this purification is governed by a number of factors, like its intended application and the nature of the starting material. Generally the techniques employed to attain a very high level of purity, such as affinity chromatography, ultra-

filtration, high performance liquid chromatography, electrophoresis, etc., make the purification process very expensive and result in very small volumes of enzyme with low final yields (Mukherjee and Banerjee, 2006). The enzymes thus purified lose their activity and become suitable any for biochemical research studies and structural studies that can be carried out on a denatured enzyme. In contrast, activity studies require the preservation of function, and hence denaturation and proteolysis should be minimized. For commercial applications, extremely high levels of enzyme purity are not required and a quick purification procedure is necessary to keep the process inexpensive. The enzyme is required in a reasonably pure form with high catalytic activity to be successfully applied in industry (Mukherjee and Banerjee, 2006). This paper reports on the results obtained from studies on the partial purification and biochemical characterization of a tannin acyl hydrolase extracted from a newly isolated strain of *Paecilomyces variotii*.

MATERIAL AND METHODS

Microorganism and Screening

Paecilomyces variotii is a new lineage obtained by means of fungal isolation procedures and was used for the production of tannase using coffee husk and wheat bran residues. Five hundred fungal cultures were obtained from the departmental stock culture collection (of the Food Science Department-Unicamp), collected from different places in Sao Paulo State (Brazil) and screened for their tannase producing ability. The best tannase producing fungus was identified as *Paecilomyces variotii*. The identification was made by *Fundação André Tosello* (Sao Paulo State, Brazil).

Microorganism Maintenance and Pre-inoculum Preparation

The strain was maintained in PDA (potato dextrose agar- Acumedia Manufactures Inc. Lansing, Michigan, 48912, USA) slants, stored at temperature of 4ºC. The lineage was replicated in PDA containing 0.2% (w/v) of tannic acid (N.V.Ajinomoto OmniChem S.A.) and incubated at 30ºC for 72 h. The pre-inoculum was prepared by adding 2.5 mL of distilled water to remove the spores obtaining a suspension containing $5.0x10^7$ spores.mL^{-1}.

Fermentation Media

A 250 mL conical flask containing the following constituents: 5g of wheat bran and 5g of coffee husk, 10 mL of distilled water and 10% of tannic acid (w/w), was used for the fermentation process. The culture medium (pH 5.7) was sterilized at temperature of 120ºC for 20 min, the relative humidity of the medium after sterilization being 60%. After sterilization, the flasks were inoculated with 2.5 mL of the pre-inoculum suspension and incubated at temperature of 30ºC for 120 h. After fermentation, 80 mL of 20 mM acetate buffer, pH 5.0 were added and shaken at 200 rpm for 1 h. The solution was filtered and centrifuged at 10,070x*g* for 30 min at temperature of 4ºC (Beckman J2-21 centrifuge, Beckman-Coulter, Inc. Fullerton, CA, USA). The supernatant was than treated with solid ammonium sulphate (80% saturation) and stood overnight at temperature of 7°C. The precipitate was collected by centrifugation (10,070x*g* - 30 min), re-suspended in distilled water and dialysed against distilled water. The dialysed preparation was used as crude freeze-dried tannase.

Tannase assay

A colorimetric assay was used to determine tannase activity, based on measuring the residual tannic acid content after the enzymatic reaction (Mondal et al., 2001). The reaction mixture consisted of 0.3 mL of the substrate tannic acid (0.7% (w/v) in 0.2 M acetate buffer at pH 5.5) and 0.5 mL of the enzyme extract, incubated at temperature of 60ºC for 10 min. The reaction was terminated by the addition of 3 mL of a bovine serum albumin solution - BSA (1mg.mL^{-1} - prepared in a 0.17 M sodium chloride solution in 0.2 M acetate buffer, pH 5.0), leading to the precipitation of the remaining tannic acid. The tubes were than centrifuged at 10,070x*g* for 15 min at temperature of 4ºC and the precipitate dissolved in 3 mL of SDS-triethanolamine (solution contained 1% SDS and 5% (v/v) triethanolamine in distilled water). 1 mL of $FeCl_3$ reagent (contained 0.01 M of $FeCl_3$ in 0.01 M hydrochloric acid) was then added and held for 15 min for colour stabilization. The absorbance was measured at 530 nm and the enzyme activity calculated from the change in absorbance at 530 nm. One unit of tannase activity was defined as the amount of tannic acid hydrolysed by 1 mL of enzyme per minute of reaction): $Abs_{530} = Abs_{control} - Abs_{test}$.

Partial Purification and Characterization of Tannase

Ammonium Sulphate Fractionation and Dialysis

Ammonium sulphate was added to the supernatant to give a final concentration of 80% saturation. The ammonium sulphate was added with constant stirring at 5ºC and the mixture stood overnight at temperature of 7°C. The precipitated proteins were separated by centrifugation at 10,070x*g* at temperature of 5ºC for 30 min. The separated proteins were then re-suspended in a minimum amount of distilled water and the solution dialyzed (using cellulose dialysis tubing - Size 43mm x 27mm Sigma-Aldrich D9527-100ft) for 24 h against distilled water and concentrated by freeze-drying.

Anion-exchange Chromatography (FPLC) on a DEAE Sepharose Column

The partially purified enzyme was dissolved in acetate buffer (20 mM – pH 6.0) and passed through a diethylaminoethyl (DEAE) Sepharose column (0.7 x 2.5 cm) equilibrated with the same buffer. The solution was passed through the column at a flow rate of 1 $mL.min^{-1}$ with acetate buffer (20 mM – pH 6.0), followed by a linear gradient from 0-1M NaCl in the acetate buffer. The eluted fractions were collected in an automated fraction collector (Pharmacia Biotech) and the absorbance of the fractions was measured at 280 nm. The major peak fractions were then assayed for tannase activity, and only the fractions possessing tannase activity were pooled.

Molecular Mass Determination by SDS-PAGE

The properties of the purified tannase were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1971) using a 12.5% separating gel. The protein bands were detected by Coomassie blue staining and then de-stained using a mixture of methanol, glacial acetic acid and distilled water. The molecular weights of the proteins were determined using the standard protein mixture of 94, 67, 43, 30, 20 and 14 kDa.

Optimum pH and Temperature for Tannase Activity

The optimum pH for tannase activity was determined at 60ºC by incubating the enzyme at different pH values (3.5 to 9.0) for 10 min. Acetate buffer (0.2 M) was used for the range from 3.5 to 5.5, phosphate buffer (0.2 M) for pH 6.0 to 8.0 and Tris-HCl buffer (0.2 M) for pH 8.5 to 9.0. The optimum temperature was determined by incubating the reaction mixture for 10 min at different temperatures from 20 to 80ºC at pH 5.5 (at regular 10°C intervals).

Optimum pH and Temperature for Tannase Stability

The stability of the enzyme was examined at different pH values by incubating the enzyme in buffers at different pH values ranging from 3.5 to 9.0 for 12 h at 30ºC (the residual activity was examined at 60ºC). The thermal stability was examined by incubating the test sample at different temperatures ranging from 20 to 90°C for 30 min at pH 5.5. Residual activity was estimated at 50ºC and expressed as a percentage of the respective tannase activity.

Determination of K_m and V_{max}

K_m and V_{max} were determined by plotting the reaction velocity against the substrate – tannic acid - concentration [S]. To calculate the kinetic constants, the data were plotted and fitted directly to the Michaelis-Menten equation. Calculations were also performed using the linear transform method of Lineweaver and Burk (1934) (Annex p. 86).

RESULTS AND DISCUSSION

Purification of Tannase

Tannase was produced extracellularly by a newly isolated strain of *Pecilomyces variotii* using solid-state fermentation on wheat bran and coffee husk residues. A typical purification is shown in Table 1. The substrate tannic acid was used to monitor tannase activity throughout the purification procedure. Fractional precipitation with 80%

ammonium sulphate removed some of the non-enzymatic proteins and about 34% of the total tannase was recovered (Table 1).

Table 1 Purification of tannase isolated from *Paecilomyces variotii*

Purification step	*Total activity*[a] *(U)*	*Total protein*[b] *(mg)*	*Specific activity* *($U.mg^{-1}$)*	*Purification (fold)*	*Yield (%)*
Crude enzyme	90	307.60	0.29	1	100
Ammonium sulphate	30.77	54.74	0.56	1.93	34
DEAE-Sepharose	2.7	0.48	5.6	19.3	3

[a] One unit of tannase activity was defined as the amount of tannic acid hydrolysed by 1 mL of enzyme per minute of reaction.

[b]Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

The elution profile of the tannase extract obtained from the DEAE Sepharose column is shown in Figure 1, showing five protein peaks, tannase activity being found in 2 of the peaks. These results agree with those of Beverini and Metche (1990), where a commercial tannase from *Aspergillus oryzae* was purified by affinity chromatography on Con A-Ultrogel and resulted in the separation of two fractions (tannase I and tannase II). The tannase activity from *Paecilomyces variotii* was found in the third and fourth peaks, the peaks eluting very close to each other, between 150 and 250 mM NaCl. Maximum tannase activity (major peak) was found in the fourth peak between fractions nº 48 and 54 and the minor peak between fractions nº 34 and 38. The active fractions referring to the fourth peak were pooled and used for studying the biochemical properties of the tannase. DEAE Sepharose column chromatography led to an overall purification of 19.3 fold with a yield of 3% (Table 1), results in agreement with those of Sharma et al (1999), who purified a tannase from *Aspergillus niger* van Tieghem. The yield of 3% was lower than the value of 7-19% recovery reported by other authors (Rajakumar and Nandy, 1983; Farias et al., 1994). However, the purification factor was similar to that of the purified tannase obtained

from various different fungi, as reported by other workers (Rajakumar and Nandy, 1983; Sharma et al., 1999).

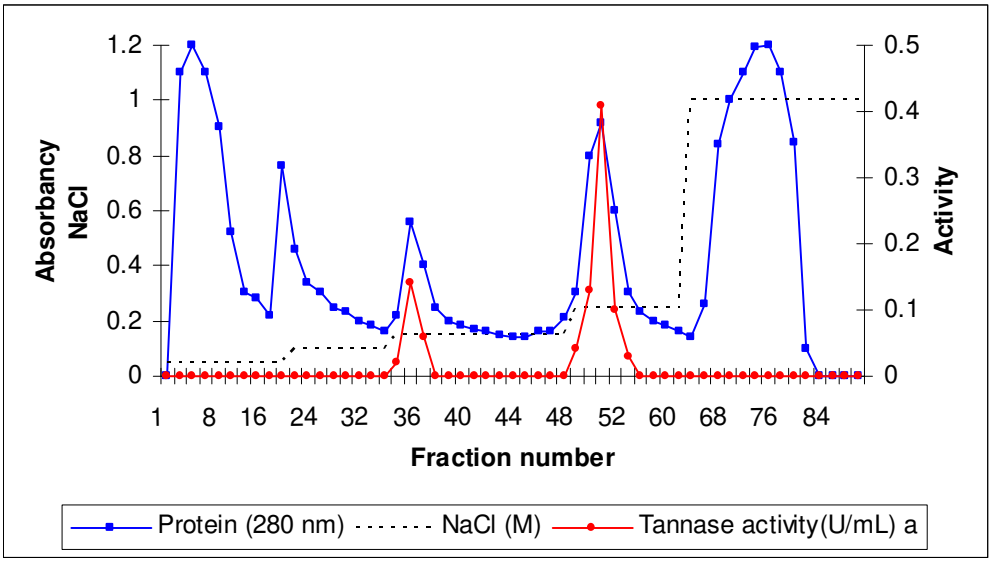

Figure 1: Elution profiles of tannase from *Paecilomyces variotii* using DEAE – Sepharose column chromatography. [a.] One unit of tannase activity was defined as the amount of tannic acid hydrolysed by 1 mL of enzyme per minute of reaction.

Properties of the Tannase

The molecular mass of the purified enzyme was determined by SDS-PAGE (Fig 2). The purified enzyme migrated as a single protein band corresponding to molecular masses of 87.3 kDa (major peak, fourth peak) and 71.5 kDa (minor peak). The tannase from *Paecilomyces variotii* was probably in a monomeric form. Ramìrez-Coronel et al., (2003), reported on multimeric tannases extracted and purified from *Aspergillus flavus* and *Aspergillus oryzae* with molecular masses varying from 186 to over 300 kDa. The purified tannase from the present study had a molecular mass of only 87.3 kDa and the enzyme was also present in a less active form of 71.5 kDa.

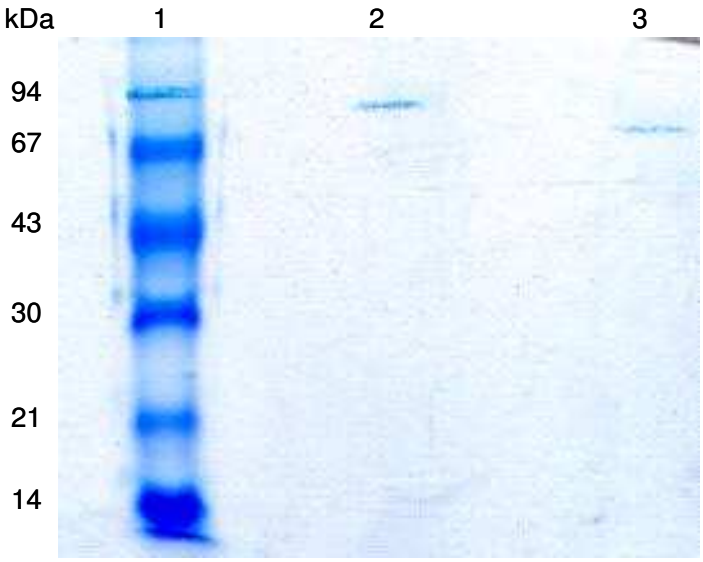

Figure 2: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified tannase from *Paecilomyces variotii*. The lanes contain (1) molecular weight markers, (2) band corresponding to a molecular mass of 87.3 kDa from a purified sample (major peak) and (3) band corresponding to a molecular mass of 71.5 kDa from a purified sample (minor peak).

The variations in enzyme activity and stability with pH and temperature are shown in Figures 3 and 4. With respect to pH (Fig 3), both activity and stability showed similar profiles with an optimum peak around pH 5.5, decreasing on both sides of the peak. However for temperature (Fig 4), the variations in activity and stability showed different profiles. Tannase activity increased moderately in the range from 20 to 50ºC, but decreased rather sharply above 50ºC, whereas stability remained at maximum in the range from 20 to 50ºC and then also decreased above 50ºC.

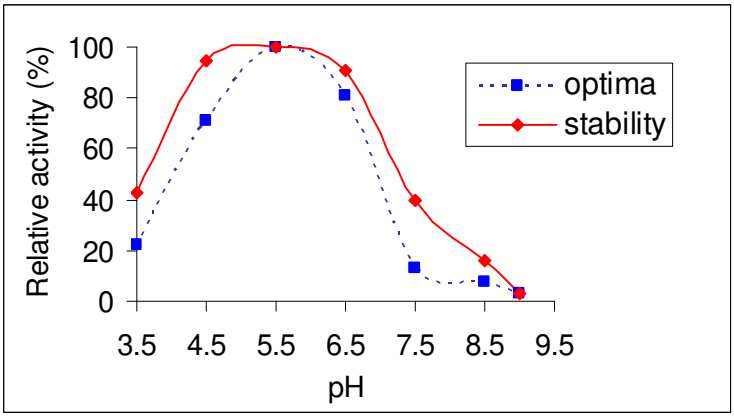

Figure 3: Effect of pH on the activity and stability of purified tannase from *Paecilomyces variotii*.

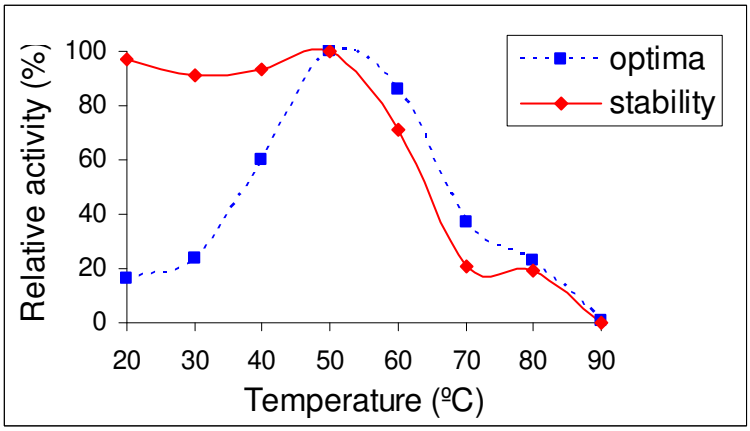

Figure 4: Effect of temperature on the activity and stability of purified tannase from *Paecilomyces variotii*.

The optimum pH range found for tannase activity, from 5.0-6.5, was similar to the values found for the tannases from *Aspergillus niger* (Lekha and Lonsane, 1997) and *Aspergillus niger* ATCC 16620 (Sabu et al., 2005). The enzyme was active at acidic pH values, the activity decreasing as the pH approached the alkaline range. Any change in pH affects the protein structure and the decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability. From the results it was concluded that the tannase from the new isolate needed an acidic environment to be active. In general, fungal tannases are acidic proteins (Mahapatra et al., 2005).

The optimum temperature range for tannase activity (>80%) was from 45 to 60ºC, and stability (>90%) was achieved in the range from 20 to 50ºC. Optimum temperature is defined as that resulting in maximum velocity of the enzymatic reaction, above which the rate of reaction decreases due to thermal inactivation. There was an increase in tannase activity with increase in temperature up to 50ºC, with a subsequent decrease (Fig 4). The temperature for optimum activity of the tannase from *Paecilomyces variotii* was 55ºC, whereas for *Aspergillus sp* and *Penicillium chrysogenum* tannases there are reports of optimal activity between 30 and 40ºC (Rajakumar and Nandy, 1983; Lekha and Lonsane, 1997). Enzymes with high temperature optimum and heat stability are preferred for industrial applications.

To observe the effect of substrate concentration on tannase activity, an assay was performed with various concentrations of tannic acid. The analysis of the graph of substrate concentration against tannase activity yielded values for K_m of 0.61 µM and for V_{max} of 0.55 $U.mL^{-1}$ protein (Fig 5 and Fig 6). The K_m value for the tannase from *Cryphonectria parasitica* using tannic acid as the substrate was found to be 0.94 mM (Farias et al., 1994).

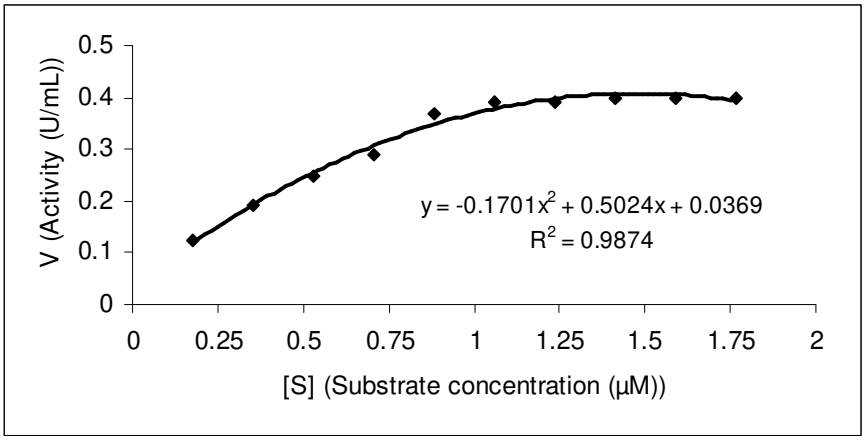

Figure 5: Grapic method of Michaelis & Menten for the calculation of V_{max} and K_m.

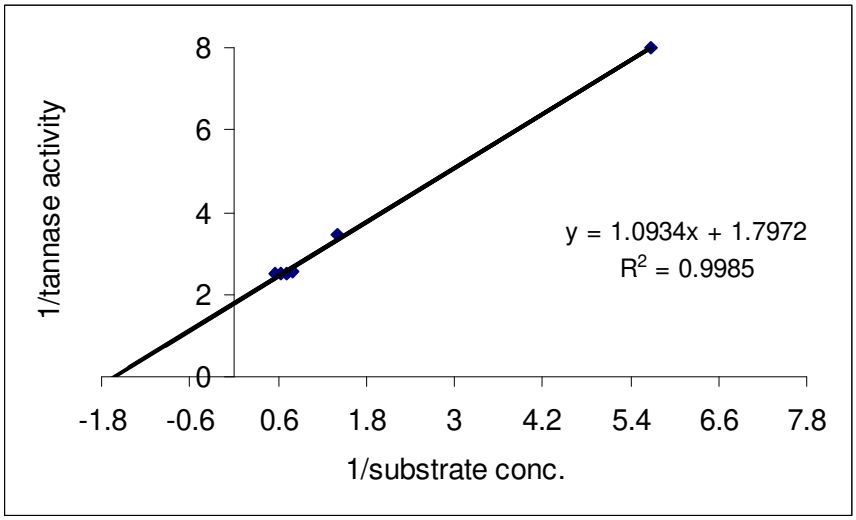

Figure 6: Graphic method of Lineweaver & Burk for calculation of V_{max} and K_m with tannic acid with substrate.

In this study, the overall performance with respect to the pH tolerance and thermal stability of *Paecilomyces variotii* tannase was found to be promising for industrial applications. The purified enzyme is a monomeric protein with a molecular mass of 87.3 kDa and was found to give optimum activity at temperatures between 45 and 60ºC. Similarly the enzyme was active over a wide range of pH values. The purified tannase from *Paecilomyces variotii* is a unique one with a low K_m. All these characteristics were considered favourable for industrial processing, especially in the food-processing industry. Therefore, *P. variotii* could provide a new source for the efficient production of tannase for industrial applications.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from CAPES (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*).

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CAPÍTULO 6: HIDROLYSIS OF EPIGALLOCATECHIN GALLATE BY A TANNASE FROM *Paecilomyces variotii*

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Enviado para publicação (março/2007): Food Chemistry

Trabalho realizado no Centro de Investigação em Química-Universidade do Porto-Portugal, no período de Outubro de 2006 a Fevereiro de 2007.

ABSTRACT

Epigallocatechin (EGC) and gallic acid (GA) were prepared by the degalloylation of an epigallocatechin gallate (EGCG) extract from green tea. EGCG was entirely hydrolyzed using a tannase (from *Paecilomyces* variotii) at pH 6.0, incubating at 40ºC for 30 min. The antiradical properties and the reducing power of these samples were assessed using the DPPH and FRAP assays, respectively. This work established a relationship between the antioxidant effects of epigallocatechin gallate and the enzymatic reaction products (epigalocatechin and gallic acid). The enzymatic reaction products showed higher scavenging activity and antioxidant capacity when compared to epigallocatechin gallate.

Key Words: Tannase; Antioxidant, *Paecilomyces variotii*, Epigallocatechin gallate; Green tea

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INTRODUCTION

Tea is the most widely consumed beverage worldwide and is rich in polyphenolic compounds known as tea flavonoids. Among them, epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), gallic acid (GA) are the most abundant and represent 15–35% of the dry weight (Bunkova *et al*., 2005).

These flavonoids (also named catechins) possess strong antioxidant properties (Majchrzak *et al*., 2004). Catechins have been proven to have antioxidant, antimutagenic, and anticarcinogenic properties and can also prevent cardiovascular diseases. The positive health effects of catechins have attracted much attention by chemists and biochemists during recent years (Cao and Ito, 2004).

The considerable antioxidant potential of tea has long been recognized and is dependent on many factors involved in tea preparation. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelation potential (Atoui *et al*., 2005). Although epigallocatechin (EGC) is an important catechin in tea leaf, it is contained in natural green tea preparations in relatively low amounts compared to its gallate derivative, epigallocatechin gallate (EGCG), which is the most abundant catechin in green tea (Cao and Ito, 2004).

Tannin acyl hydrolases, commonly referred to as tannases (E.C. 3.1.1.20), are inducible enzymes produced by fungi, yeast and bacteria. Tannases have mostly been characterized by their activity on complex polyphenolics, and are able to hydrolyze the "ester" bond (galloyl ester of an alcohol moiety) and the "depside" bond (galloyl ester of gallic acid) of substrates such as tannic acid, epicatechin gallate, epigallocatechin gallate, chlorogenic acid, etc (Garcia-Conesa *et al*., 2001).

In this report, the activity of tannase on the epigallocatechin gallate extract of green tea was investigated. The aim of this work was to study the potential antioxidant properties of epigallocatechin gallate before and after the enzyme reaction using the tannase produced by *Paecilomyces variotii* (Battestin *et al*., 2005). The antiradical properties and the reducing power of these samples were assessed using DPPH and FRAP assays, respectively.

MATERIAL AND METHODS

Reagents

All solvents used in this study were of analytical grade and purchased from Sigma Chemicals. Epigallocatechin gallate (EGCG, 95%), epigallocatechin (EGC, 98%) and gallic acid (GC) were purchased from Sigma –Aldrich, Germany.

Enzyme

The tannase from *Paecilomyces variotii* was obtained according to procedure described in other manuscript (Battestin and Macedo, 2007).

Preparation of epigallocatechin gallate extracts (substrate)

Epigallocatechin gallate was extracted from 1g of green tea (Té verde-Pompadour tee) with 20 ml of ethanol/water/chloroform (1:1:2, v/v) using a blender (Ultra-Turrax) for 5 min according to the procedure described by De Freitas et al (2003). The 50% ethanolic upper aqueous layer was separated from the chloroform layer containing the chlorophylls, lipids and other undesirable compounds. The ethanol was removed using a rotary evaporator and the resulting aqueous solution containing catechins was dissolved in acetate buffer (pH 6.0, 0.2M) for the enzymatic hydrolysis.

Quantification of EGCG, EGC and gallic acid in the green tea extract

The quantitative determination of individual catechins was performed by HPLC (280 nm) by integration of the peak areas at 280 nm, using the equations of the calibration cuves obtained using standards. Standard solutions were prepared by dissolving standard catechins (EGCG, EGC and GA) in trifluoracetic acid at different concentrations (0.1 – 1.5 mg). Linearity (r^2 >0.98) was calculated by plotting the peak area of each concentration against the respective catechin concentration.

Enzymatic hydrolysis

The epigallocatechin gallate obtained from the green tea extract was used as the substrate for the enzymatic hydrolysis by tannase, isolated from *Paecilomyces variotii* (Battestin and Macedo, 2007). The commercial substrate (EGCG Sigma) was also used for the enzymatic reaction. Epigallocatechin gallate (0.1 mL) (0.3 mg/mL final concentration) was prepared in acetate buffer (pH 6.0) and incubated with 0.01 mL of tannase (1-10 mg/mL) at 40ºC for 30 min. The hydrolysis process was paralyzed by placing in an ice bath for 15 min. The released epigalocatechin and gallic acid moieties were identified by analytical HPLC by comparison with authentic standards.

HPLC conditions

The products formed during the enzymatic reaction were analyzed by HPLC (Elite Lachron-Merck Hitachi) using a 150x4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt), and detection at 280 nm with a Knauer K-2800 diode array detector. The solvents were A: 1% trifluoracetic acid ($C_2HF_3O_2$) in H_2O, and B: 20% solvent A in 80% acetonitrile (C_2H_3N). A linear gradient from 7% to 25% B in 40 min was used with a flow rate of 0.5 ml/min.

LC-MS analysis

A Finnigan Surveyor series liquid chromatograph, equipped with a 150 x 4.6 mm i.d., 5µms LicroCART® reversed-phase C18 column maintained at 25 ºC by a thermostat, was used. Mass detection was carried out using a Finnigan LCQ DECA XP MAX (Finnigan Corp., San José, Calif., USA) mass detector with an API (Atmospheric Pressure Ionization) source of ionization and an ESI (ElectroSpray Ionization) interface. The solvents were A: formic acid (COOH-Carlo Erba) in water (1% (v/v)), and B: acetonitrile (CH_3CN-Panreac). The capillary voltage was 4V and the capillary temperature 275 ºC. The spectra were recorded in the positive ion mode between 120 and 1500 m/z. The mass spectrometer was programmed to carry out a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS-MS of the most intense ion using relative collision energy of 30 and 60.

Ferric Reducing/Antioxidant Power (FRAP)

The FRAP assay developed by Benzie and Strain (1996) was performed with some modifications. In short, the FRAP reagent (10 vol of 300 mM acetate buffer, pH 3.6, + 1 vol of 10 mM TPTZ in 40 mM HCl + 1 vol of 20 mM FeCl3) was diluted to one-third its concentration with methanol and pre-warmed at 37 ºC. An aliquot of 297 µL of this reagent were mixed with 3 µL of extract (enzyme reaction products). This mixture was shaken and the absorbance read at 593 nm. The test was performed at 37 ºC and the 0-4 min reaction time window was used. The results were expressed as Trolox equivalents determined using a calibration curve.

Radical DPPH Scavenging Activity

Following the method described by Bondet et al. (1997) with some modifications, the radical activities were determined using DPPH as the free radical. The reaction for scavenging DPPH radicals was performed in polypropylene tubes at room temperature (22-23 ºC). In each tube, an aliquot of 297 µL of extract (enzyme reaction products) was added to 3 µL of DPPH solution (60 µM in methanol). The decrease in absorbance was measured at 520 nm at time = 0 and then every 5 min for 20 min. Methanol was used as the blank solution, and DPPH solution with no sample extract served as the control. The anti-radical activity was calculated from the equation determined from the linear regression after plotting known solutions of Trolox with different concentrations. For the final results, the 0-20 min reaction time window was used. Antiradical activity was expressed as micromolar Trolox equivalents (Faria *et al*., 2005).

Calculations and statistics

Statistical significance of the difference between the groups was analyzed by the Tukey test. Differences were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

Detection and identification of the compounds extracted from green tea

The hydroalcoholic extract of green tea containing polyphenolic compounds was thoroughly analyzed by HPLC/DAD-MS. The use of mass spectrometry, coupled to high performance liquid chromatography, allowed the identification of epigallocatechin gallate and epigallocatechin compounds (Fig 1).

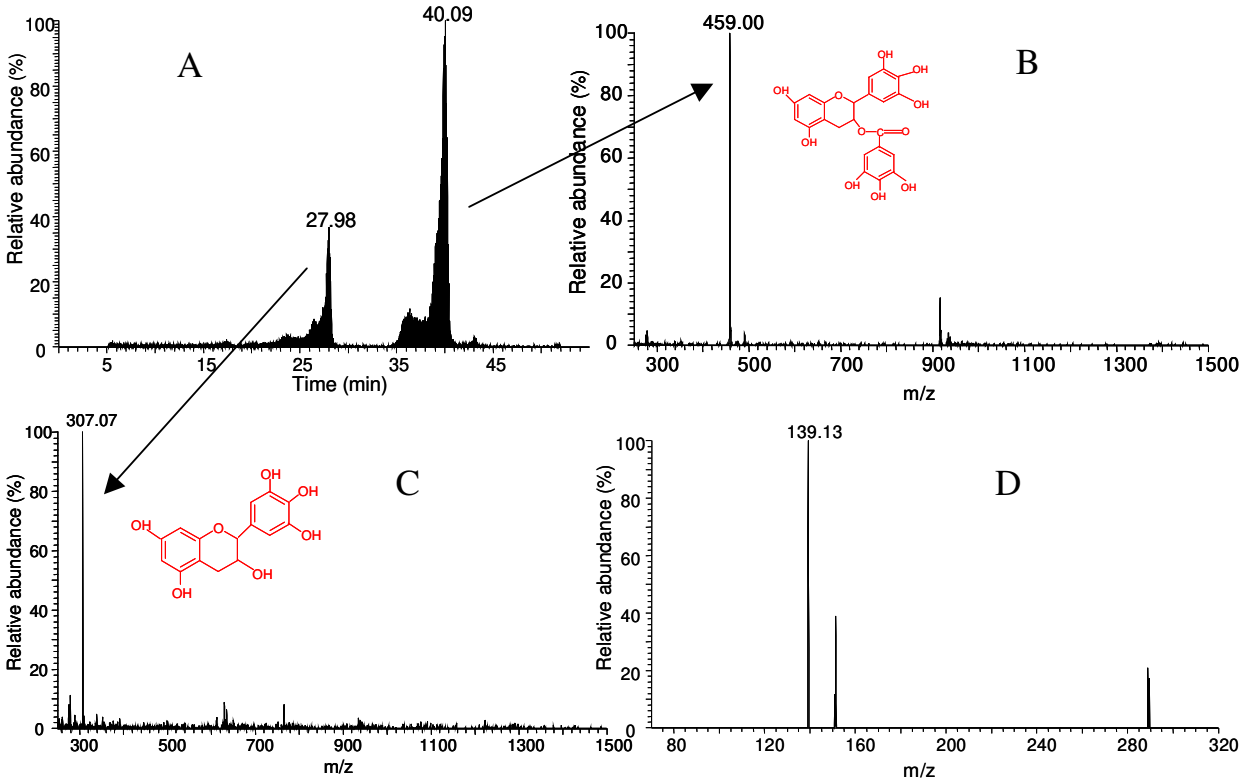

Figure 1. MS analysis of green tea extract (positive-ion mode): (A) total ion chromatogram; (B and C), MS spectrum; (D) MS^2 spectrum of the ion at *m/z* 307.

The mass spectrum showed an ion mass consistent with the structure of epigallocatechin (MW: 306 g/mol). The mass spectrum of this compound revealed a $[M^+]$ molecular ion at *m/z* 307 and a major fragment ion $[M\text{-}168]^+$ at *m/z* 139 which correspond to a retro-Diels-Alder of the catechin moiety (Freitas *et al.*, 2004). The HPLC/DAD-MS

analysis also showed the presence of a very significant peak with the same retention time as the epigallocatechin gallate (retention time of 40 min) in the UV-Vis spectrum. The mass spectrum showed an ion mass [M^+] at *m/z* 459, consistent with the structure of epigallocatechin gallate (Fig 1).

Quantification of EGCG, EGC and gallic acid

The amounts of EGCG, EGC and GA in the green tea extract were determined by HPLC using the calibration curves of the authentic standards. Figure 2 shows the HPLC chromatogram of the green tea extract at 280 nm and Table 1 the catechin contents (mg/mL) of the green tea extract.

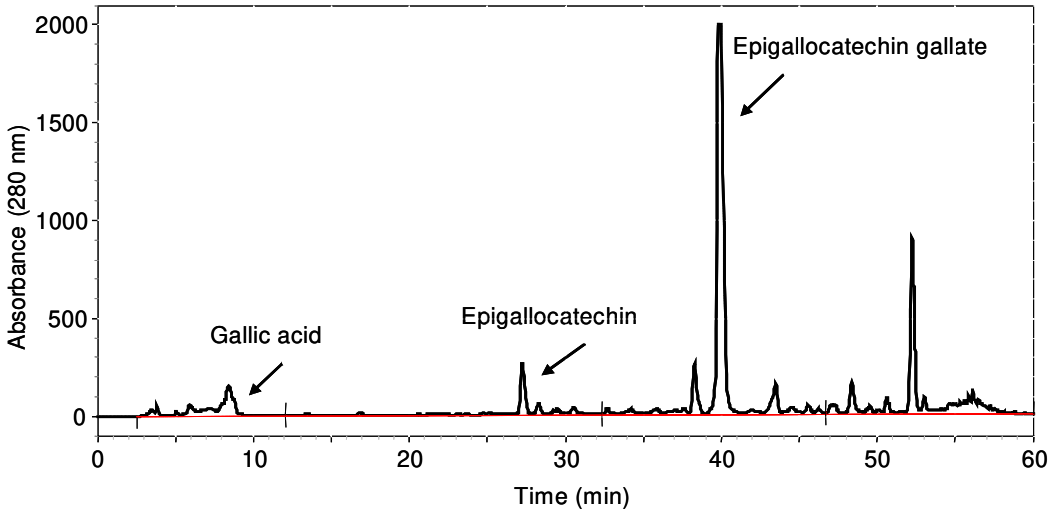

Figure 2: HPLC chromatogram of green tea extract

Table 1: Catechins and gallic acid contents in the green tea extracts and in respective leafs.

Catechins	*Extract content (mg/mL)*	*Extract content (mg/g)*
Epigallocatechin gallate	0. 304	3.8
Epigallocatechin	0.035	0.44
Gallic acid	0.028	0,35

Leaf composition depends on a variety of factors, including climate, season, horticultural practices, and specimen and age from the plant. The usual composition is 10-

15% EGCG and 6-10% EGC, with EGCG being the main constituent (Suganuma *et al.*, 1999).

Enzymatic reaction with commercial EGCG and green tea substrate

The hydrolysis process was carried out using epigallocatechin gallate as the substrate, which was hydrolyzed by the enzyme tannase to form gallic acid and epigallocatechin (Fig 3).

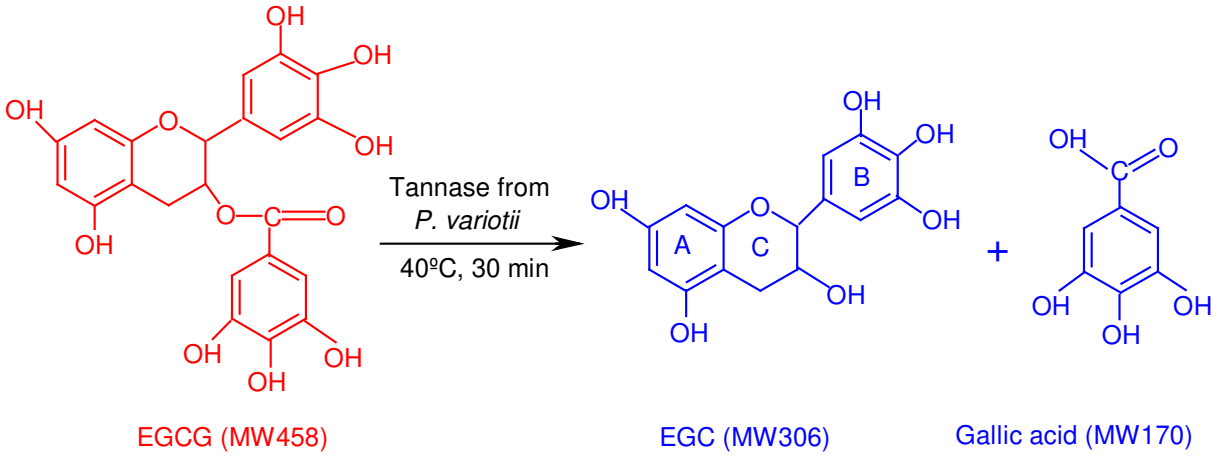

Fig.3: Degalloylation of EGCG by the tannase from *Paeciloyces variotii*

Tannase exhibited high activity on the commercial substrate (EGCG-Sigma) and on the substrate extracted from green tea (Fig 4 and 5). The ability of tannase to hydrolyze the commercial substrate was compared with its ability to hydrolyze that obtained from green tea extract. The results showed that the tannase from *Paecilomyces variotii* was able to hydrolyze the ester bonds from natural substrates. EGC and GA can be formed by the degalloylation of this gallate (EGCG) present in the tea extract. The HPLC analysis of the reaction products (Fig 4 and 5) indicated that commercial EGCG could be completely converted to EGC and gallic acid by adding 0.01 mL (1.2 mg/mL) of tannase containing 0.1 mL (1.0 mg/mL) of EGCG, and that the green tea substrate (EGCG) could be completely converted to EGC and gallic acid by adding 0.01 mL (7 mg/mL) of tannase containing 0.1 mL (0.3 mg/mL) under the conditions mentioned above.

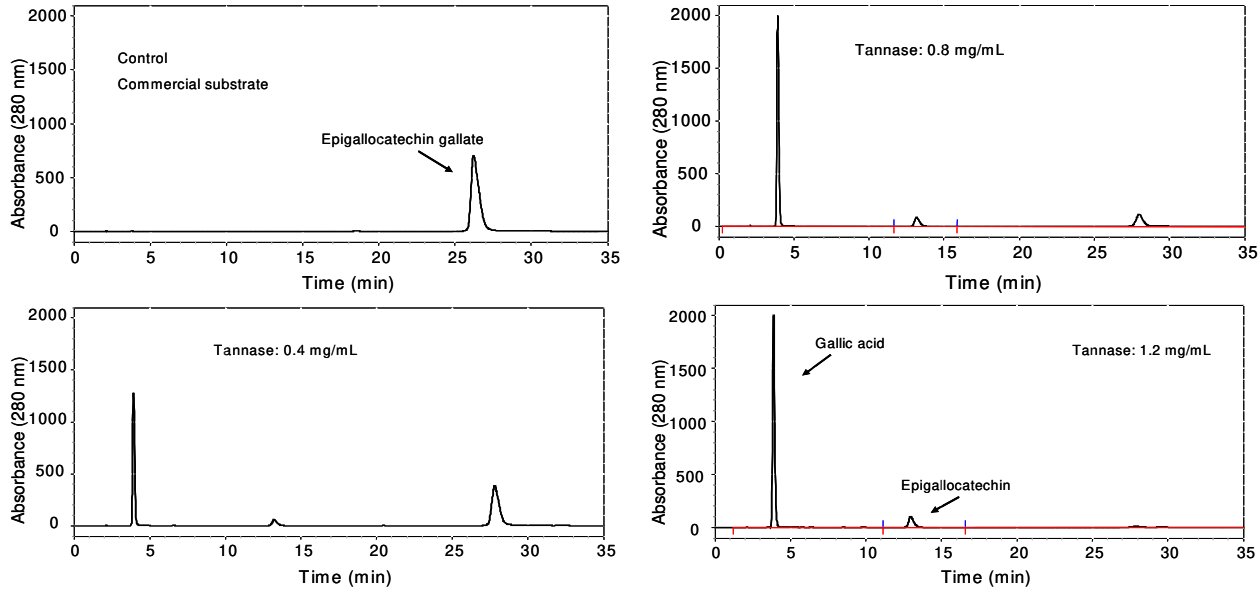

Figure 4: HPLC analysis of the products formed after the enzymatic reaction using EGCG (commercial substrate) at 3 different concentration of tannase (pH 6.0, 40º, 30 min).

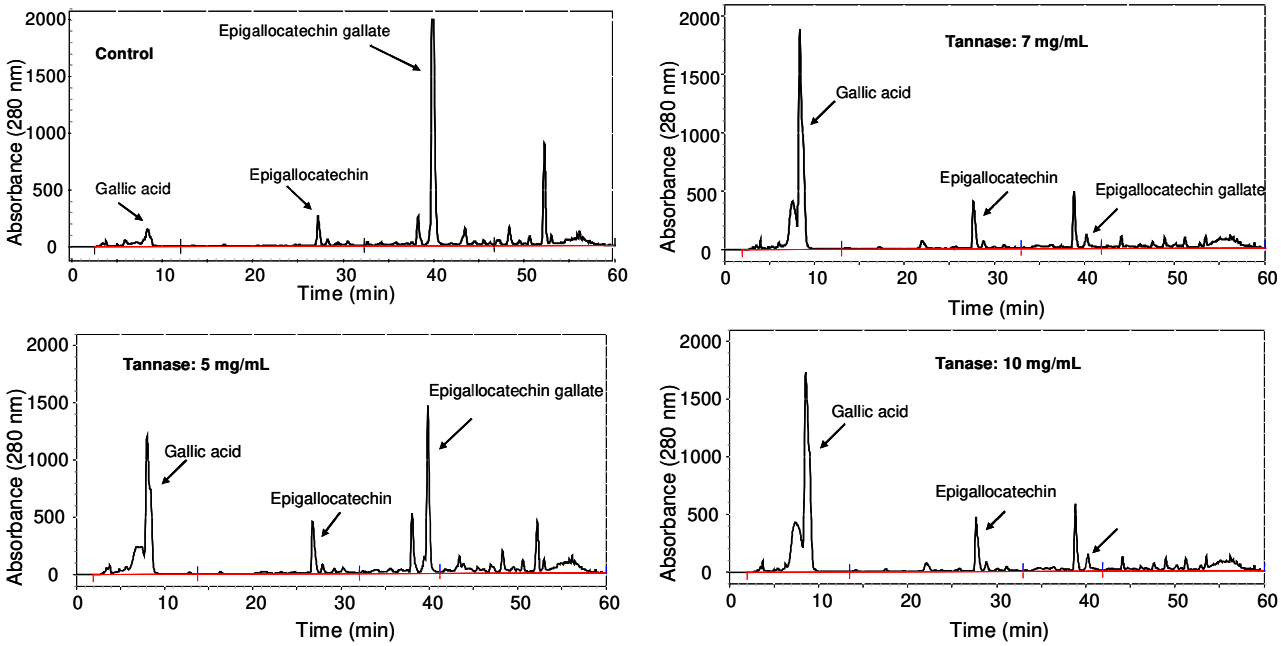

Figure 5: HPLC analysis of the products formed after the enzymatic reaction of the green tea extract (pH 6.0, 40º, 30 min).

Tannases show high specificity for the phenolic site of the substrate (Garcia-Conesa *et al*., 2001). These results are in agreement with those of Cao and Ito (2004), who used tannase for the preparation and purification of epigallocatechin. There is little data about the enzymatic hydrolysis of green tea with the tannase from *Paecilomyces variotii*. All the enzymatic reactions were monitored using denatured enzyme as a control.

Epigallocatechin (EGC) is present in relatively low amounts in natural green tea preparations as compared to its gallate derivative (EGCG) (Cao and Ito, 2004). The EGC concentration of the green tea doubled after treatment with tannase (Figure 6).

Increases in the enzyme concentration (10 mg/mL) reduced the epigallocatechin gallate concentration in the green tea extract from 0.31 mg/mL to 0.0084 mg/mL. In compensation, the gallic acid and epigallocatechin contents increased 16 and 2 times respectively.

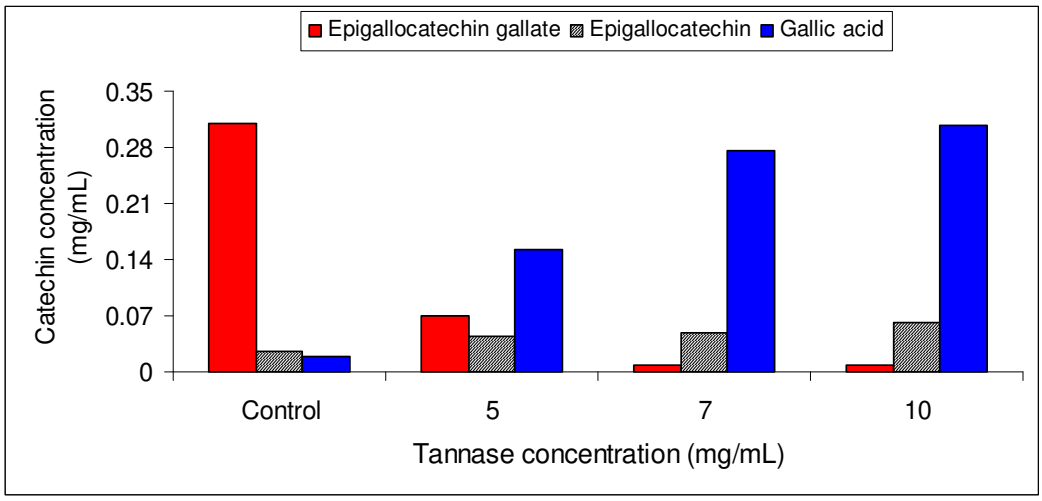

Figure 6: Quantitative determination of epigallocatechin gallate, epigallocatechin and gallic acid obtained using different tannase concentrations.

DPPH and FRAP

The free radical scavenging activity of the extracts was tested using the DPPH method. The substances tested reacted with the DPPH, which is a stable free radical, and induced a decrease in the absorbance measured at 515 nm, which indicates the scavenging

potential of the extracts. It can be seen from the results in Tables 2 that there is a trend for increasing radical scavenging capacity with the increase in the epigallocatechin and gallic acid contents of the green tea extracts. The reducing power of the extracts was subsequently assessed using the FRAP method (Table 3). The products formed after enzyme reaction showed higher scavenging activity and antioxidant capacity.

Table 2: Antiradical activity before and after enzymatic hydrolysis as assessed by the DPPH method

Phenolic compounds	*Commercial substrate (EGCG)*	*Green tea extract*
Control (without tannase)	25.29±0.56 [a]	24.09±0.05 [a]
Enzymatic reaction products (in presence of tannase)	24.60±0,12 [a]	22.78±0.35 [b]

The values are means of duplicates and those with different letters are significantly different at $p < 0.05$.

Table 3: Reducing power before and after enzymatic hydrolysis as assessed by the FRAP method.

Phenolic compounds	*Commercial substrate (EGCG)*	*Green tea extract*
Control (without tannase)	23.95±0.93 [a]	73.4±1.19 [a]
Enzymatic reaction products (in presence of tannase)	39.09±1.33 [b]	85.0±0.59 [b]

The values are means of duplicates and those with different letters are significantly different at $p < 0.05$.

It is well known that a diet rich in fruits, vegetables and tea protects against cardiovascular disease and certain forms of cancer, and perhaps against some other diseases (Block and Langseth, 1994). The polyphenolic components may act as antioxidants or as agents of other mechanisms contributing to anti-carcinogenic or cardio-protective action. The flavonoids constitute a large class of compounds, containing a number of phenolic hydroxyl groups attached to a ring structure, conferring the antioxidant activity (Rice-Evans *et al*., 1996).

Catechins (including epicatechins) with three hydroxyl groups in the B ring are the gallocatechins, and those esterified to gallic acid at the 3-OH group in the C ring are the catechin gallates. The antioxidant activity responds broadly to the tenet that the structures with the most hydroxyl groups exert the greatest antioxidant activity, the catechin-gallate esters reflecting the contribution from gallic acid (Rice-Evans *et al*., 1996). Concerning any structure-activity relationships, the *o*-dihydroxy groups in the B-ring and the hydroxyl group in the C-ring are usually related to the antioxidant properties of the flavonoids (Faria *et al*., 2005).

CONCLUSIONS

In conclusion, this study demonstrates that EGC and gallic acid can be successfully formed from the degalloylation of EGCG by tannase in green tea extracts. This work established a relationship between the antioxidant effects of epigallocatechin gallate and those obtained after the enzymatic reaction (epigalocatechin and gallic acid). The products of the enzyme reaction (epigallocatechin and gallic acid) showed higher scavenging activity and antioxidant capacity than the epigallocatechin gallate. Further studies are needed to explore the mechanism and the potential of this enzyme for future applications in green tea. These results suggest that tannases are able to improve the antioxidant activity of green tea.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from CAPES (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil*) for the scholarship and are grateful to the Department of Chemistry, Faculty of Sciences, University of Porto, Portugal and to Professor Victor A. P. de Freitas for his contribution to this work.

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CONCLUSÕES GERAIS

Entre as 500 linhagens de fungos da coleção de cultura do laboratório de Bioquímica de Alimentos-FEA, 7% de linhagens apresentaram potencial de produção de tanase induzida pela presença de ácido tânico. Dentre os resíduos agroindustriais testados foram obtidos melhores resultados utilizando-se resíduos de uva e café, sendo que nos testes fermentativos foi obtido maior produção de tanase usando-se palha de café. A melhor linhagem produtora de tanase foi identificada como *Paecilomyces variotii*, e esta foi capaz de crescer em meio de fermentação contendo uma mistura de resíduo de café e farelo de trigo.

Utilizando metodologia de superfície de resposta, as melhores condições para a produção da tanase foram: faixa de temperatura 29 - 34ºC; % resíduo café : farelo de trigo (50:50); ácido tânico 8 - 14% e nitrato de amônio 0,4 - 1,2%. Utilizando esta metodologia, foi possível aumentar a produção da tanase em cerca de 9 vezes (0,18U/mL para 1,62U/mL).

A tanase de *P. variotii*, comparada a outras tanases descritas na literatura, apresentou atividade em ampla faixa de temperatura e pH. A preparação de tanase bruta apresentou atividade ótima em pH 6,5 e 70ºC e estabilidade na faixa de 4,0-7,5 após 24 h de incubação a 30ºC. A enzima mostrou-se estável após 30 min de tratamento a 70ºC em pH 6,0. O estudo com os inibidores sugere a presença de íons bivalentes, grupo tiol e resíduos de triptofano no sítio ativo da enzima.

Na etapa de purificação da tanase, esta foi purificada 19,3 vezes através de fracionamento com sulfato de amônio 80% de saturação e cromatografia em coluna de DEAE-Sepharose. A enzima foi separada em 2 picos, apresentando massa molecular de 87,3 kDa e 71,5. Apresentou valores de K_m e $V_{máx}$ 0,61 µM e 0,55 U/mL respectivamente, para o substrato ácido tânico.

Através do estudo de aplicação da enzima, foi possível verificar que a tanase apresenta grande capacidade de hidrolisar substratos de polifenóis, contribuindo para aumentar o poder antioxidante do chá verde.

SUGESTÕES PARA TRABALHOS FUTUROS

1. Conduzir estudos para produzir a enzima utilizando escala piloto e industrial.
2. Com base nos dados da purificação da enzima, estudar características do modo de ação das tanases encontradas, bem como, especificidade em relação a hidrólise do substrato (afinidade de hidrolizar ligações do tipo éster ou depsídica) e o efeito sinérgico entre as duas enzimas.
3. Testar outras aplicações para a tanase, tais como: aplicação para tratamento de efluentes da indústria de couros, indústria de ração animal, e refinar os estudos de aplicação em chás.
4. Testar técnicas de imobilização da enzima.

ANEXO

Determinação de K_m e $V_{máx}$

A cinética é uma metodologia importante para o estudo dos mecanismos enzimáticos. A maioria das enzimas tem muitas propriedades cinéticas em comum. Á medida que a concentração de substrato aumenta, a atividade catalítica de uma concentração fixa de uma enzima aumentará de forma hiperbólica, aproximando-se de uma velocidade máxima, $V_{máx}$, característica na qual toda a enzima está na forma de complexo ES (enzima-substrato). A concentração de substrato que produz a metade da velocidade máxima é a constante de Michaelis-Menten ou K_m, que é característica para cada enzima, agindo sobre um dado substrato. A equação descreve o comportamento cinético da grande maioria das enzimas, e todas essas enzimas que exibem uma dependência hiperbólica de V_0 em relação a [S] são ditas seguir a cinética de Michaelis-Menten.

A equação de Michaelis-Menten relaciona a velocidade inicial de uma reação enzimática com a concentração do substrato e a $V_{máx}$ através da constante K_m. Tanto K_m como $V_{máx}$ podem ser medidas; elas tem significados diferentes para enzima diferentes. A equação e Michaelis-Menten pode ser transformada algebricamente em formas que são mais úteis na determinação prática de K_m e $V_{máx}$. Essa Tranformação é chamada de equação de Lineweaver-Burk. Para enzimas que obedecem a equação de Michelis-Menten, obtemos uma linha reta quando $1/V_0$ x $1/[S]$ são lançados no gráfico de Lineweaver-Burk. A linha reta obtida tem inclinação igual a $K_m/V_{máx}$, o intercepto no eixo $1/V_0$ é igual a $1/V_{máx}$ e o intercepto no eixo $1/[S]$ é igual a $-1/K_m$. O gráfico de Lineweaver-Burk tem a vantagem de permitir uma determinação acurada de $V_{máx}$ e K_m, o que pode ser feito apenas aproximadamente em gráficos de V_0 x [S] (Lehninger *et al*., 1995).

Lehninger, A. L., Nelson, D. L., Cox, M. M. Princípios de Bioquímica. São Paulo: Sarvier, 1995.