UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

KÁTIA KELLE DA SILVA ANDRADE ALBUQUERQUE

EXTRAÇÃO EM SISTEMA DE DUAS FASES AQUOSAS (PEG/CITRATO), CARACTERIZAÇÃO E APLICAÇÃO DA TANASE DE *Aspergillus* sp. SIS 25 EM CHÁ VERDE (*Camellia sinensis*)

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas.

Área de Concentração: Biotecnologia.

Orientadora: Prof.ª Dr.ª Ana Lúcia Figueiredo Porto.

Co-Orientadora: Dr.ª Polyanna Nunes Herculano.

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COMISSÃO EXAMINADORA

MEMBROS TITULARES

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A fé é o firme fundamento das coisas que se esperam, e

a prova das coisas que não se vêem.

Hebreus 11:1

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“Até aqui nos ajudou o Senhor”. I Samuel 7:12

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RESUMO

Enzimas são proteínas com atividade catalítica capazes de integrar diferentes processos biotecnológicos. Dentre as enzimas com aplicação na indústria destaca-se a tanino acil hidrolase (EC 3.1.1.20) ou simplesmente tanase, uma enzima extracelular produzida na presença de ácido tânico por fungos filamentosos, bactérias e leveduras. A tanase (TAH) catalisa a hidrólise de taninos liberando ácido gálico e glicose. TAH pode ser utilizada no tratamento de efluentes, na indústria farmacêutica, de alimentos, bebidas entre outros. Na biotecnologia, o grande desafio na produção de enzimas é extrair a molécula a partir de métodos economicamente viáveis. Assim, o sistema de duas fases aquosas (SDFA) tem sido cada vez mais utilizado para purificar parcialmente diversos produtos biológicos. Neste sentido, o presente trabalho teve como finalidade extrair em SDFA, caracterizar bioquimicamente e aplicar em chá verde a enzima tanase obtida de *Aspergillus* sp. SIS 25 por fermentação em estado sólido, utilizando a fibra do coco como substrato. Um planejamento fatorial 2^3 foi utilizado para avaliar a influência das variáveis principais: massa molar (MMPEG) do PEG (1000, 3350 e 6000 g/mol), concentração (20, 22 e 24% m/m) do PEG (CPEG) e concentração (15, 17,5 e 20% m/m) de citrato de sódio (CCIT), sobre as variáveis resposta: coeficiente de partição (K), recuperação (Rec) e aumento de pureza (AP), em pH 6. A tanase foi preferencialmente particionada para a fase sal do sistema uma vez que em todos os ensaios os valores de K foram menores do que 1. As variáveis MMPEG e a interação entre MMPEG-C_{PEG} apresentaram os resultados mais significativos para o valor de K, sendo ambos os efeitos negativos. Com relação ao aumento de pureza, o melhor resultado (3,2) foi observado no ensaio 8 com 24% de PEG 6000 e 20% de sal. A tanase extraída do sistema apresentou temperatura ótima a 30° C e pH ótimo 5,0. A perda da estabilidade foi observada a 50 °C. A TAH desse estudo foi estimulada na presença de Na^+ e completamente inibida na presença de Zn^{2+}. Os surfactantes não interferiram significativamente em sua atividade, com exceção do Triton X-100 a 2% que diminuiu a atividade relativa em aproximadamente 50%. No processo de hidrólise dos compostos fenólicos do chá verde, a tanase pré-purificada em SDFA apresentou melhor resultado se comparada ao extrato bruto; 0,75 mL da enzima do sistema reduziu 44% dos fenóis do chá. Os resultados demonstram que o modelo estatístico montado para o SDFA além de permitir a extração de uma tanase parcialmente pura tornou conhecido outros modelos que favorecem a otimização das variáveis estudadas, principalmente o aumento de pureza. Com isso, é possível afirmar que a tanase de *Aspergillus* sp. SIS 25 pode ser extraída através de um método de baixo custo, que emprega material reutilizável, biodegradável e que o processo conservou as características biquímicas dessa enzima devido à abundância de água que ocorre no sistema e pela utilização de componentes inertes à maioria das bimoléculas. A criação desse ambiente favorável para separar moléculas biológicas pode explicar o fato da tanase não ter perdido sua atividade durante o estudo, mantendo sua ação catalítica principalmente durante a aplicação em chá verde.

Palavras-chave: sistema de duas fases aquosas, *Aspergillus* sp., purificação, tanase, chá verde.

ABSTRACT

Enzymes are proteins with catalytic activity capable of integrating different biotechnological processes. One of the enzymes with application in industry stands out the tannin acyl hydrolase (EC 3.1.1.20) or simply tannase, an extracellular enzyme produced in the presence of tannic acid by filamentous fungi, bacteria and yeast. The tannase (TAH) catalyzes the hydrolysis of tannins releasing gallic acid and glucose. TAH can be used for effluent treatment, pharmaceutical industry, food, beveragesand others. In biotechnology, the big challenge in the production of enzymes is to extract the molecule from economically viable methods. Thus, the aqueous two-phase system (ATPS) has been increasingly used to partiallypurify biological products. In this sense, the present work had as purpose to extract in ATPS, characterize biochemically and apply in green tea the tannase enzyme obtained from *Aspergillus* sp. SIS 25 by solid state fermentation using coconut fiber like substrate. A factorial design 23 was used to evaluate the influence of major variables: PEG molar mass (1000, 3350 and 6000 g/mol), PEG concentration (CPEG) and sodium citrate concentration (CCIT), on the response variables: partition coefficient (K), recovery (Rec) and purity increase (AP), at pH 6. The tanase was preferentially partitioned to stage the salt system once in all tests the values of K were lower than 1. The variables MMPEG and the interaction between MMPEG-CPEG presented the results more meaningful for the value of K, being both negative effects. With regard to the increase in purity, the best result (3.2) was observed in 8 test with 24% of PEG 6000 and 20% salt. The tannase extracted from system showed optimum temperature at 30 ° C and optimum pH 5.0. The loss of stability was observed at 50° c. TAH this study was stimulated in the presence of Na^{+} and completely inhibited in the presence of Zn^{2+}. Surfactants not significantly interfere in their activity, with the exception of Triton X-100 2% that decreased the relative activity by approximately 50%. In the process of hydrolysis of phenolic compounds from green tea, tannase pre-purified in ATPS showed better results when compared to the crude extract; 0.75 ml of the enzyme from system has reduced 44% of tea phenols. The results show that the statistical model fitted to the ATPS and allow the extraction of a pure and partially known other models that favor the optimization of the studied variables, especially the increase in purity. With this, it is possible to affirm that the tanase of *Aspergillus* sp. SIS 25 can be extracted through a low-cost method, employing reusable, biodegradable material and the process preserved the biquímicas features of this enzyme because of the abundance of water that occurs in the system and by the use of inert components to most bimoléculas. The creation of this favourable environment to separate biological molecules can explain the fact of tannase didn't lose its activity during the study, keeping their catalytic action primarily during application in green tea.

Keywords: aqueous two-phase systems, *Aspergillus*, purification, tannase, green tea.

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LISTA DE ABREVIATURAS E SIGLAS

PEG	Polietileno glicol
K	Coeficiente de partição
SDFA	Sistema de duas fases aquosas
TAH	Tanino acil hidrolase
MMPEG	Massa Molar do Polietileno glicol
CPEG	Concentração de Polietileno glicol
CCIT	Concentração de citrato
Rec	Recuperação
ATPS	Aqueous two phase systems
Da	Daltons
kDa	Quilodaltons
ANOVA	Análise de Variância
pH	Potencial hidrogênionico
RPM	Rotações por minuto
SDS	Dodecil sulfato de sódio
B.O.D.	Biochemical oxygen demand
BCA	Ácido bicinconínico
AP	Aumento de pureza
PAGE	Gel de poliacrilamida

SUMÁRIO

INTRODUÇÃO

Os taninos são os compostos mais abundantemente extraídos da biomassa vegetal, depois da celulose, hemicelulose e lignina. Podem ser encontrados nas folhas, frutos, raízes e sementes dos vegetais superiores. Devido ao sabor adstringente, os taninos atuam como um mecanismo de defesa das plantas contra o ataque de herbívoros (ARBENZ; AVEROUS, 2015). Estes compostos possuem a propriedade de formar complexos insolúveis em água e por isso são considerados fatores antinutricionais em alimentos, pois podem associar-se a proteínas dificultando a digestão. Os taninos podem ser hidrolisados por ácidos, bases e tanases de diferentes fontes microbianas (JIMÉNEZ et al., 2014; SILVA et al., 2010).

A tanase (tanino acil hidrolase - EC 3.1.1.20), é uma enzima extracelular e induzível, que catalisa a hidrólise de taninos produzindo ácido gálico e glicose (MADEIRA-JUNIOR et al., 2015). Tanino acil hidrolase (TAH) pode ser produzida por fungos filamentosos, bactérias, leveduras e, por ser uma enzima de grande interesse comercial, sua aplicação está sendo estudada principalmente na indústria farmacêutica, tratamento de efluentes do couro, alimentos e bebidas como os chás instantâneos (JANA et al., 2014).

O chá é uma bebida muito apreciada em todo o mundo. O chá verde (*Camellia sinensis*), em especial, é vastamente consumido em vários países, inclusive o Brasil, por apresentar efeitos benéficos à saúde, como por exemplo: auxílio na prevenção do câncer e doenças cardiovasculares, combate ao excesso de gordura no corpo, efeito antioxidante de radicais livres, quelantes de metais, inibidores da lipoperoxidação, entre outros (SANTOS et al., 2014; ZHANG et al., 2016). Na indústria de chás, a tanase ajuda a reduzir a formação de precipitados na bebida, além de melhorar a coloração e sabor (CHÁVEZ-GONZÁLEZ et al., 2012). Apesar de melhorar os aspectos sensoriais do chá, a aplicação de tanase na produção de bebidas ainda é limitado devido aos custos para obtenção da enzima, principalmente no que se refere à purificação, que é um processo bastante complexo.

Atualmente, o grande desafio da biotecnologia consiste em aplicar a enzima em diferentes setores da indústria sem que para isso necessite recorrer a métodos de custos elevados. Apesar dos micro-organismos serem fontes inesgotáveis de diversas enzimas, o processo de extração de moléculas de interesse se torna dispendioso em virtude dos processos empregados para separar a molécula alvo de moléculas contaminantes. Os métodos

convencionais para purificar biomoléculas geralmente incluem etapas muito complexas. Uma alternativa viável é extrair a molécula de interesse em sistema bifásico aquoso (LIMA et al., 2013).

Os sistemas de duas fases aquosas (SDFA) são formados pela incompatibilidade de dois polímeros hidrofílicos ou um polímero e um sal. Por apresentar alto teor de água em ambas as fases esses sistemas constituem um meio adequado para extração de biomoléculas, pois preservam a estabilidade molecular das mesmas (ALI et al., 2014). Os componentes do sistema quando se separam favorecem o particionamento do produto biológico para uma das fases e, através de ensaios laboratoriais e estatísticos, é possível definir os parâmetros que levam a uma separação ideal. Com isso, a simplicidade da técnica faz do SDFA um processo atrativo e de fácil reprodução em larga escala para extrair enzimas de interesse comercial (TANG et al., 2014).

Deste modo, o objetivo do presente trabalho é extrair em SDFA e caracterizar bioquimicamente a tanase de *Aspergillus* sp. SIS 25, apresentando uma potencial aplicação desta enzima na redução de compostos fenólicos indesejáveis do chá verde, promovendo a purificação da biomolécula por um método baixo custo.

CAPÍTULO 1

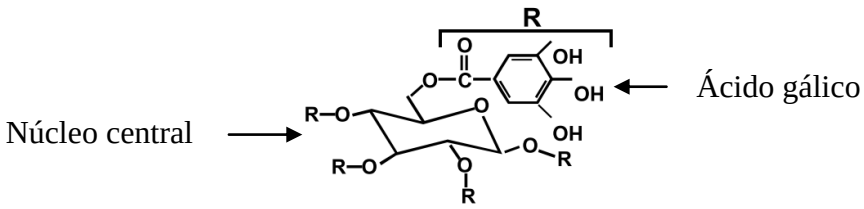
1. REVISÃO DA LITERATURA

1.1 Taninos

Os compostos polifenólicos compreendem uma ampla gama de substâncias que possuem pelo menos um grupo hidroxila (-OH) em um ou mais anéis fenólicos. A maioria dos compostos fenólicos não são encontrados no estado livre na natureza, mas na forma de ésteres ou heterosídeos sendo, portanto, solúveis em água e em solventes orgânicos polares (CARVALHO et al., 2007; CARVALHO et al., 2012). Dentre estes encontram-se os taninos, um subgrupo de compostos fenólicos possivelmente de maior tamanho. O termo tanino foi originalmente utilizado para descrever certas substâncias orgânicas que serviam para curtir peles de animais em um processo conhecido como *tanning.* Atualmente, este termo tem sido amplamente aceito para classificar um grupo bastante heterogêneo de compostos fenólicos de massa molecular relativamente alta (500-20000 Da) e de complexidade elevada - 12-16 hidroxilas em 5-7 anéis aromáticos por cada 1000 Da (OLIVAS-AGUIRRE et al., 2015).

Os taninos são o segundo maior grupo de fenóis abundantes na natureza. São considerados produtos do metabolismo secundário das plantas e, geralmente podem ser encontrados em maior quantidade nas cascas, raízes, folhas e frutos (LENIN; LOKESWARI; SRI, 2015). Em função da sua estrutura química os taninos são classificados em dois grupos: taninos hidrolisáveis (galotaninos e elagitaninos) e taninos condensados (proantocianidinas). Os galotaninos em especial, são formados por várias moléculas de ácidos orgânicos esterificados parcial ou totalmente a uma molécula de glucose. Esta associação é facilmente hidrolisada em meio ácido, alcalino, através da água quente ou por meio de ação enzimática (JANA et al., 2014).

Figura 1. Estrutura molecular de um galotanino (BHAT; SINGH; SHARMA, 1998).

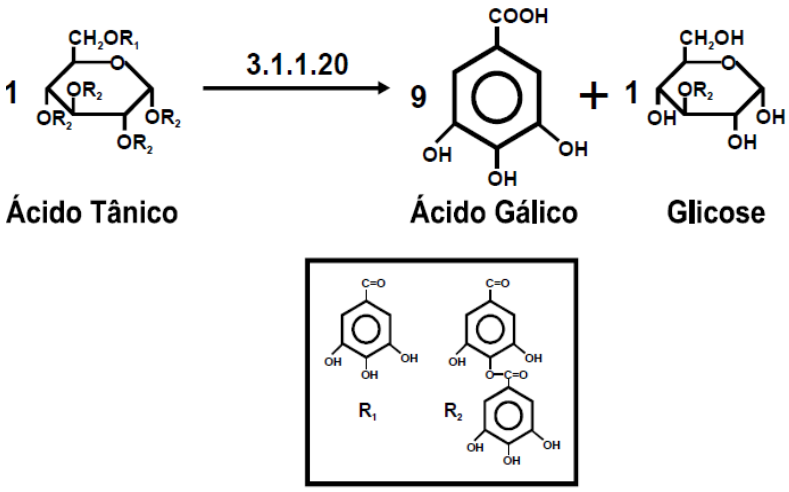


Taninos são capazes de formar ligações estáveis com proteínas e outros polímeros, tais como os polissacarídeos. Por serem fenólicos são muito reativos quimicamente. Formam pontes de hidrogênio intra e intermoleculares e um mol de tanino pode ligar-se a doze moles de proteínas. Estes compostos são facilmente oxidáveis através de enzimas vegetais específicas ou por influência de metais como cloreto férrico, que ocasiona o escurecimento de suas soluções (MELLO et al., 2001; DUARTE et al., 2014).

1.2 Tanase

Tanino acil hidrolase (EC 3.1.1.20) ou simplesmente tanase (TAH) é uma enzima extracelular envolvida na hidrólise de taninos. A TAH hidrolisa ésteres de taninos hidrolisáveis (Fig. 2), produzindo moléculas de glicose e ácido gálico. A tanase pode ser produzida na presença de ácido tânico por diversos micro-organismos como fungos filamentosos, bactérias e leveduras (MADEIRA-JUNIOR et al., 2015).

Figura 2. Reação de hidrólise do ácido tânico, R_1 (galoil) e R_2 (digaloil) pela tanase (AGUILAR; GUTIÉRREZ-SÁNCHEZ, 2001; BATTESTIN; MATSUDA; MACEDO, 2004).



Muitos autores relacionam a presença de taninos como um mecanismo de defesa dos vegetais contra a ação microbiana. Em face disso, a produção de tanase pode ser considerada como um contra-ataque às plantas por parte dos micro-organismos. A TAH atua na invasão da planta hospedeira hidrolisando parte dos compostos fenólicos (taninos hidrolisáveis) presentes nos tecidos vivos ou em decomposição (REDONDO et al., 2014).

A tanase geralmente apresenta pH ótimo entre 4.5-6.5, estabilidade ao pH na faixa de 3.5-8.0; além de temperatura ótima entre 30-50ºC, estabilidade térmica na faixa de 30ºC-70ºC

e, massa molecular entre 50kDa e 320kDa. Diferentes íons metálicos tem sido frequentemente reportados como inibidores de tanase, tais como: íons de Fe^{3+}, Mg^{2+}, Mn^{2+}, Zn^{2+} e Cu^{2+} (PINTO et al., 2005). Além destes, metais pesados como Hg^{2+}, Co^{2+}, Ba^{2+}, Cd^{2+}, Ag^{+}, Pb^{+}, Sn^{2+} também são considerados potentes inibidores de tanase (YAO et al., 2014). O comportamento da TAH em relação ao pH, temperatura e inibição por íons metálicos depende basicamente das condições de cultivo e do micro-organismo utilizado na produção da enzima (BENIWAL; CHHOKAR, 2010; CHÁVEZ-GONZÁLEZ et al., 2012). Dentre os fungos filamentosos produtores de tanase, os gêneros *Aspergillus* e *Penicillium* são considerados os melhores produtores, seja por fermentação em estado sólido ou fermentação submersa (AGUILLAR et al., 2007; RENOVATO et al., 2011; CHÁVEZ-GONZÁLEZ et al., 2012).

1.3 Fontes de obtenção da tanase

A tanase foi primeiramente descrita por Knudson (1913), que descobriu a degradação do ácido tânico por uma cepa de *Aspergillus niger*. Esta enzima pode ser produzida por fermentação submersa e sólida, utilizando resíduos agroindustriais como fonte de carbono (DE LIMA, et al., 2014; GONÇALVES et al., 2011; SOUZA et al., 2015). Poucos trabalhos tratam da produção de tanase por fermentação submersa pois, esse tipo de fermentação é menos viável em virtude da baixa produtividade da enzima tanase (PINTO et al., 2005). A fermentação em estado sólido (FES) apresenta muitas vantagens em comparação com a fermentação submersa, tais como: natureza extracelular da enzima, maior produtividade e maior estabilidade às mudanças de pH e temperatura. Além disso, a FES oferece benefícios econômicos e ambientais por utilizar resíduos agroindustriais como substrato (CHÁVEZ-GONZÁLEZ et al., 2012).

A TAH não é igualmente ativa contra todos os taninos hidrolisáveis. As obtidas de levedura são efetivas somente na decomposição do ácido tânico (galotanino). Já as TAH bacterianas e de fungos filamentosos são eficientes na degradação de ácido tânico e outros taninos hidrolisáveis que ocorrem na natureza (BHAT; SINGH; SHARMA, 1998). O aumento da produção de tanase está intimamente relacionado com a disponibilidade de tanino no meio, contudo, em altas concentrações de tanino o crescimento do fungo é inibido.

A produção de tanase pode ser realizada utilizando uma grande variedade de substratos. Em folhas de chá verde como fonte de carbono, a produção da enzima foi maior (3.6 U/g de substrato) do que utilizando folhas de *Anacardium occidentale* (1,59 U/g de substrato) e 36 vezes maior do que a produção em folhas de *Mangifera indica* (VALERA;

JORGE; GUIMARÃES, 2015). Pesquisa realizada com resíduos de dendê e sementes de tamarindo em pó para produção de tanase utilizando *Aspergillus niger* ATCC 16620, mostra que os rendimentos máximos da enzima (13,03 UI/g) ocorreu após 96 horas de incubação e, após o período de 120h, o rendimento da enzima reduziu para 6,44 Ul/g (SABU et al., 2005). Kumar, Sharma e Singh (2007) produziram tanase através de *Aspergillus ruber* utilizando como substrato folhas de jamelão (*Syzygium cumini*). Após 96 horas de fermentação os autores observaram que a produção atingiu o valor de 69 U/g de enzima. Beniwal et. al (2013), utilizaram serragem de *Dalbergia sissoo* para produção TAH através do *Aspergillus heteromorphus* MTCC 8818. A produção máxima de 1,62 U/g de tanase ocorreu após 96h.

1.4 Gênero *Aspergillus*

As espécies de *Aspergillus* produzem um grande número de enzimas extracelulares e, muitas delas são aplicadas na biotecnologia para degradar produtos e compostos. Dentre as espécies produtoras de enzimas comercialmente importantes encontram-se: *Aspergillus flavus, A. niger, A. oryzae, A. nidulans, A. fumigatus, A. clavatus, A. glaucus, A. ustus* e o *A. versicolor* (SOARES et al., 2010; SCHUSTER et al., 2002). O gênero *Aspergillus* sp. compreende mais de 260 espécies de fungos filamentosos. Podem apresentar colônias de coloração branca, amarela, amarelo-esverdeada, amarronzada, preta ou verde. A cor da colônia é a principal característica macroscópica utilizada para classificar as espécies deste gênero. A forma, tamanho e ornamentação dos conídios também podem auxiliar na identificação de isolados, no entanto, técnicas moleculares e bioquímicas são mais precisas. *Aspergillus* são fungos ubíquos e anemófilos, classificados como os micro-organismos mais abundantes, além de mundialmente distribuídos, podendo ser isolados do solo, ar, água, alimentos, plantas, material em decomposição e superfícies (SAMSON; VARGA, 2009; SIDRIM; ROCHA, 2004; WARD et al., 2006).

Os fungos constituem um grupo de microrganismos eucarióticos, uni ou multicelulares, em geral multinucleados, com parede celular. Podem ser filamentosos, constituídos por filamentos longos e ramificados denominados hifas ou leveduriformes, constituídos por células individuais que se reproduzem por brotamento ou fissão binária (STUART; PIMENTEL; MARCON, 2010). Nesse importante grupo de micro-organismos mais de 77.000 espécies são conhecidas, sendo a maioria terrestre. Entre todos os grupos fúngicos existentes, o filo Ascomycota tem sido cada vez mais estudado principalmente devido ao seu potencial na produção de enzimas. Os ascomicetos são fungos filamentosos que

atuam na decomposição da matéria orgânica através da hidrólise das macromoléculas pelas exozimas que secretam. Dentre estas enzimas encontram-se as amilases, pectinases, xilanases, celulases, proteases e tanases que, apesar de serem importantes em vários seguimentos da indústria, não são largamente explorada devido aos custos de seu processamento, principalmente no que diz respeito à purificação (PUTZKE; PUTZKE, 2004).

1.5 Sistema de duas fases aquosas

O sistema de duas fases aquosa (SDFA) é uma tecnologia atraente para purificação de biomoléculas por oferecer vantagens como: simples e rápida separação, clarificação do extrato, baixa desnaturação devido ao alto teor de água em ambas as fases, rápida transferência de massa, partição seletiva e baixo custo. Portanto, ele tem sido utilizado em vários domínios da biotecnologia para separar as moléculas de interesse das moléculas contaminantes (YUZUGULLU; DUMAN, 2015).

O primeiro sistema PEG/sal a ser utilizado pela indústria foi o sistema composto por PEG/fosfato. Além de fosfato, outros sais como sulfatos e citratos, podem ser empregados em sistema de duas fases aquosas (SILVA et al., 1999). O SDFA é formado pela mistura de dois polímeros hidrófilos ou um polímero e um sal, em determinadas concentrações (ASENJO; ANDREWS, 2011; ROSA et al., 2010). Após a homogeneização, cada componente do sistema é concentrado em uma das fases, favorecendo, deste modo, a partição de biomoléculas, tais como proteínas, células, fragmentos celulares ou ácidos nucléicos. A estratégia básica de separação em SDFA baseia-se na predominante partição da molécula de interesse para uma das fases do sistema e as contaminantes para a fase oposta (OLIVEIRA et al., 2001). Neste sistema, as proteínas são divididas entre as duas fases com um coeficiente de partição que pode ser modificado se as condições experimentais do meio como pH, sais, força iônica e outros, forem alterados (BASSANI et al., 2010; SPELZINI et al., 2008).

O polietileno glicol (PEG) é um polímero vastamente utilizado em SDFA por ser uma molécula inerte e de carga neutra que dificilmente desnatura proteínas (PEREIRA et al., 2012). O sal citrato de sódio também é um composto desejável para formar o sistema de duas fases pois ele é atóxico para humanos e biodegradável quando presente em rios, lagos e solo. No SDFA, geralmente quase todas as biomoléculas menores tendem migrar para a fase inferior (fase sal) que é mais polar. Considerando que as proteínas permanecem na fase superior (fase PEG) menos polar, esta não é a ideal para a recuperação de proteínas. A fase

polimérica requer etapas adicionais como ultrafiltração e cromatografia que aumentaria o custo do processo. (YUZUGULLU; DUMAN, 2015).

Os mecanismos que regem a partição de biomoléculas em um determinado SDFA ainda não são totalmente compreendidos. Em geral, o particionamento de proteína é impulsionado por Van der Waals, ligação de hidrogênio, hidrofóbicas e iônicas, interações entre as biomoléculas e a fase circundante. Portanto, várias condições podem influenciar a partição das macromoléculas, tais como: tamanho, carga, hidrofobicidade da molécula; concentração e massa molar do polímero; tipo e concentração do sal utilizado; e, por fim, o pH (NAGARAJA; IYYASWAMI, 2014).

Embora existam vários métodos disponíveis para a purificação de biomoléculas, estes são por vezes considerados onerosos e de etapas complexas que resultam em perdas na recuperação dos produtos biológicos. Por consequência, o interesse em técnicas mais economicamente viáveis, como SDFA, e com menor número de etapas tem se tornado cada vez mais atraente no tratamento de biomoléculas com aplicação industrial.

1.6 Aplicações da tanase

A biodegradação por determinados micro-organismos e enzimas é uma das maneiras mais eficientes de degradar grandes moléculas de tanino em pequenas moléculas com elevado valor. Os taninos apresentam efeitos antinutricionais bem conhecidos. Dependendo da quantidade ingerida e do estado fisiológico do animal, os taninos podem causar diminuição na disponibilidade de nutrientes e na produtividade animal, podendo levar à morte em alguns casos (REDONDO et al., 2014). O uso de tanase em rações ricas em taninos pode trazer efeitos benéficos na remoção desses compostos indesejáveis, favorecendo assim a digestibilidade e aumentando a capacidade de absorção pelos animais (BATTESTIN; MATSUDA; MACEDO, 2004).

Efluentes de curtumes contêm altas quantidades de polifenóis, o que representa um potencial risco ao meio ambiente. A utilização de tanase pode constituir um tratamento efetivo para esse tipo de efluente (AGUILAR; GUTIÉRREZ- SÁNCHEZ, 2001).

A hidrólise do ácido tânico pela tanase libera o ácido gálico, uma importante substância que pode ser aplicada na indústria farmacêutica para a síntese de trimetoprima, uma substância antibacteriana (SHETE; CHITANAND, 2015). Este ácido também pode ser utilizado para síntese de propilgalato, um composto largamente utilizado como aditivo na

indústria de alimentos e como antioxidante em óleos e produtos ricos em lipídeos (KAR; BANERJEE, 2000).

Na indústria de bebidas, a utilização de TAH dispensa o emprego de substâncias químicas para eliminação de complexos insolúveis indesejados em chá instantâneo, garantindo um produto final de excelente qualidade, solúvel em água e caracterizado pelo alto conteúdo de componentes aromáticos e coloração desejada. A hidrólise dos polifenóis do malte pela tanase faz com que não ocorra a descoloração e desenvolvimento de turbidez na cerveja durante a estocagem (PINTO et al., 2005). Em média, 50% da coloração do vinho se deve à presença de taninos. A oxidação destes componentes em contato com o ar pode causar uma turbidez indesejável reduzindo a qualidade do produto final. Essa turbidez pode ser evitada com o uso da tanase, que atua impedindo a oxidação (AGUILAR; GUTIÉRREZ-SÁNCHEZ, 2001). A tanase também é utilizada como agente clarificador em alguns sucos de frutas e em bebidas à base de café (SHARMA; CHATURVEDI; SHARMA, 2015). Sua aplicação em chá verde tem sido recentemente estudada.

1.7 Chá verde (*Camellia sinensis*)

O princípio ativo das plantas medicinais é frequentemente relacionado a seus compostos polifenólicos. No passado, os extratos de planta (chás) ricos em taninos eram utilizados na medicina tradicional da China e Japão, para tratar diarréia, inflamações, hemorragias, intoxicação por metais pesados e câncer (JANA et al., 2014).

Camellia sinensis é um arbusto da família *Theaceae* conhecida popularmente por: chá verde, chá-da-Índia, banchá ou “green tea”. Os principais compostos químicos terapêuticos do material vegetal *C. sinensis*, são polifenóis, que são potentes antioxidantes de radicais livres, quelantes de metais e inibidores da lipoperoxidação, anti-inflamatórios, antimicrobianos, inibidores da enzima conversora de angiotensina, auxiliam na prevenção da osteoporose e podem contribuir na prevenção de câncer (SANTOS et al., 2014).

Para obtenção do chá, são utilizados as folhas secas e os brotos da planta. A composição química dos chás pode variar quanto à espécie, idade das folhas, estação, clima (umidade, temperatura, latitude) e condições de cultivo (solo, água, minerais, fertilizantes, entre outros). Essas diferenças na matéria-prima refletem no sabor, cor e, possivelmente, nos teores de flavonóides, que são utilizados para definir a qualidade da matéria prima vegetal (PERON et. al. 2008; JAYASEKERA et al. 2011; FIMINO; MIRANDA, 2015). As

propriedades funcionais do chá estão relacionadas com o seu conteúdo polifenólico. Uma bebida típica preparada como infusão (em água quente por 3 minutos) de 1 g de erva para 100 ml de água, contém geralmente entre 250-350mg de sólidos solúveis do chá, sendo 30-42% do peso em catequinas e 3-6% em cafeína. No Brasil, o chá verde é comercializado principalmente acondicionado em saquinhos de papel de filtro (sachê). Os estudos do chá verde brasileiro (var. *assamica*) ainda são escassos quando comparados aos realizados com chás verdes produzidos em outros países. Para que os benefícios do consumo da bebida sejam máximos, são necessários estudos que assegurem as melhores formas de preparo, garantindo maior extração e estabilidade de seus compostos bioativos (NISHIYAMA et al., 2010).

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CAPÍTULO 2

EXTRACTION IN AQUEOUS TWO-PHASE SYSTEM (PEG/CITRATE), CHARACTERIZATION AND APPLICATION OF TANNASE FROM *Aspergillus* sp. SIS 25 IN GREEN TEA (*Camellia sinensis*)

Artigo a ser submetido à revista Fluid Phase Equilibria.

EXTRACTION IN AQUEOUS TWO-PHASE SYSTEM (PEG/CITRATE), CHARACTERIZATION AND APPLICATION OF TANNASE FROM *Aspergillus* sp. SIS 25 IN GREEN TEA (*Camellia sinensis*)

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Highlights

- Purification using ATPS and application of tannase from *Aspergillus* in green tea.
- Liquid-liquid extraction of tanase using PEG/citrate ATPS.
- ATPS is an interesting method for purify the tannase.

Abstract

Tannase (EC 3.1.1.20) is an extracellular enzyme that hydrolyzes tannins producing gallic acid and glucose. This enzyme is commercially important because presents applications in the pharmaceutical industry, leather processing, animal feed, food and drinks. To purify enzymes of commercial value, the aqueous two-phase system (ATPS) has been a cost-effective alternative. In this work, tannase (TAH) from *Aspergillus* sp. SIS 25 has been studied in aqueous two-phase system composed of polyethylene glycol (PEG) and sodium citrate and a statistical design 2^3 was used to study the influence of molar mass of PEG (1000, 3350 and 6000), PEG concentration (CPEG) and sodium citrate concentration (CCIT), at pH 6.0. The purified enzyme system was applied in green tea (*Camellia sinensis*) to assess their potential to reduce undesirable compounds to drink. The tannase was preferentially partitioned to salt phase of system once all the tests presented values of K less than 1. The effect of PEG molar mass (MMPEG) and MMPEG-CPEG interaction were significant, both negative. The best increase in purity (3.2) was observed in 8 test with 24% PEG 6000 and 20% salt. The statistical model studied allowed partially purify tannase and also find other models that promote improvements in the studied variables. The tannase obtained presented optimum temperature at 30 °C and optimum pH 5.0; already the stability was lost at 50 °C. In all Na^+ concentrations the enzyme was stimulated, however, in the presence of Zn^{2+} TAH was completely inhibited. Surfactants no have significant influence on enzymatic activity, with exception of Triton X-100 2% which decreased the relative of tannase activity by approximately 50%. The tannase purified in ATPS on concentration of 0.75 mL hydrolysed 44% of phenolic compounds from green tea. Superior performance if compared with the crude extract. Thus, this work provides subsidies for new studies that promote the purification of tannase using ATPS, stressing the great biotechnological potential of this enzyme, especially in green tea industry.

Keywords: aqueous two-phase systems, *Aspergillus*, purification, tannase, green tea**.**

1. INTRODUCTION

Tannin acyl hydrolase (EC 3.1.1.20) or tannase is an extracellular enzyme with great biotechnological potential produced in the presence of tannic acid by various microorganisms, especially filamentous fungi. Tannase (TAH) catalyzes the hydrolysis of tannins converting them into gallic acid and glucose. Its application is well known in the food, pharmaceutical and beverage, like teas [1,2].

Tea is one of the most popular and widely consumed beverages in the world and its composition is rich in polyphenols which have antioxidant activity. *Camellia sinensis* (green tea) is native to Southeast Asia and it is grown in more than thirty countries. Its extracts are commonly used because of low cost and beneficial health effects, for example, in the prevention of cancer, cardiovascular disease, and treating neurological disorders long term. Green tea has also been used in diets in the form of teas and extracts or incorporated into creams, gels, lotions and other pharmaceutical means [3,4].

As regards its use in tea, tannase reduces the adverse effects (bitter and astringent taste) of the tannin in the beverage, enhancing sensory acceptance [5]. In the instant tea production, TAH is used to remove the insoluble precipitates formed when the beverage is cooled at temperatures below 4°C. These precipitates come from the interaction between polymerized phenolic compounds and the caffeine. By degrading tannin, tannase prevents the formation of those polymerized compounds, improving the quality of the tea (once the enzymatic treatment, differently from the chemical treatment, preserves the desirable aromatic compounds) [6].

In this process of applying enzymes for commercial purposes, the main restrictions on the production of molecules with high levels of purity are the various steps required to purification. These procedures, in general, are technically difficult and may lead to enzyme denaturation, besides to require a high energy consumption and greater amount of chemicals [7]. Thus, efforts should be concentrated on the development of new technologies to purify enzymes with lower costs, in a sustainable way and preserving the conformational compatibility of the biomolecules.

Aqueous two-phase system (ATPS) is a biocompatible method that allows the partition and partial purification of biomolecules. The ATPS is usually created by the solution of two immiscible hydrophilic polymers (natural or synthetic) or by the combination of a polymer and a salt [8].

These systems compose an appropriate mean for the biomolecule extraction, once the high water content in both phases (between 70 e 90%) provides an appropriate environment to the activity of biologically active compounds, preserving their molecular stability and allowing their processing. Currently, the process of separation and purification of bioproducts is a very important segment for industries, representing 80% to 90% of the cost of production [9]. Therefore, ATPS is an attractive method to the enzyme extraction.

Studies that allow the improvement of the extraction and purification of TAH by aqueous two-phase system have received increased importance and impacted the cost-benefit of the processes. The aim of this study is, therefore, to extract the tannase obtained from *Aspergillus* sp.SIS 25 in ATPS PEG-citrate, to characterize the enzyme and applying it in green tea (*Camellia sinensis*) from solid state fermentation, using coconut fiber substrate.

2. MATERIAL AND METHODS

2.1 Microorganisms

In this study, was used the filamentous fungi *Aspergillus* sp. SIS 25 isolated from Caatinga soil, Serra Talhada, PE-Brazil.

2.2 Solid state fermentation and crude extract

To produce the tannase from *Aspergillus* sp. SIS 25, solid-state fermentation (SSF) was performed using the coir as a substrate. The strain was maintained on Czapeck Dox Agar and kept at 30 °C for 7 days. The inoculum was prepared by suspending spores from the Czapeck Dox Agar plates in sterile 0.01% Tween-80 solution. The number of spores was determined in a Neubauer counting chamber and 1 x 10^7 spores per mL were used to inoculate the erlenmeyer flasks containing 10 g substrate used for SSF. Flasks were incubated at 30 °C for 48 h before harvesting. After fermentation, 18 mL of 10 mM sodium phosphate buffer (pH 5.5) was added to 3 g of the fermented mixture, and the maceration was performed. The extract was clarified by filtration (Whatman no. 1 under vacuum) and centrifugation at 2000 rpm for 10 min. The supernatant was used as the crude enzymatic extract and was subjected to enzymatic analysis and extraction of tannase in the ATPS.

2.3. Protein determination

Determination of total protein content of both the top and bottom phases of the systems was carried out by spectrophotometrically using bicinchoninic acid following the method described by Smith et al. [10], with absorbance measured at 595 nm. To avoid interference from PEG and citrate, all samples were analysed against blanks containing the same phase composition without proteins.

2.4 Tannase activity

The tannase activity was determined spectrophotometrically according to Sharma's et al. method [11] and modified by Ordoñez et al. [12], based on the formation of a chromogen between gallic acid (released by the esterase activity of tannase) and rhodanine (2-thio-4-ketothiazolidine). One unit of the enzyme was defined as micromole of gallic acid formed per minute.

2.5 Preparation of aqueous two-phase systems

The systems were prepared with PEG of different molar mass (1000, 3350 and 6000 g/mol) and sodium citrate salt. Citric acid was added in an appropriate amount to maintain a pH value of 6.0. The desired amounts of PEG and salt were placed in graduated tubes with conical tips (15 mL). The crude extract containing tannase, which represents 20% of the total system, was added to the tubes. Water was added to a final amount of 5 g. After addition of all components of the system (PEG + citrate + water + crude extract) and vortex shaking for 1.0 min, the two phases were separated by settling for 60 min. The phase volumes were measured, and the top and bottom phases were separately withdrawn with pipettes and assayed for protein concentration and tannase activity.

2.5.1 Experimental design and statistical analysis

The influence of variables PEG molar mass (MMPEG), PEG concentration (CPEG) and citrate concentration (CCIT) on variables results, purification factor (PF), activity yield (Y) and partition coefficient (K), was evaluated from the results obtained by a 2^3 factorial design, plus a central point, which was run in quadruplicate to allow estimation of the experimental error [13]. The values selected for these variables (Table 1) were chosen based on binodal diagrams reported in the literature [14]. All statistical and graphic analyses were carried out with the aid of the Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).

Table 1. Variable levels of the 2^3 experimental design selected for tannase extraction by PEG/citrate ATPS.

Variables	Low (-1)	Central (0)	High (+1)
PEG molar mass (g/mol)	1000	3350	6000
PEG concentration (%, w/w)	20.0	22.0	24.0
Citrate concentration (%, w/w)	15.0	17.5	20.0

2.5.2 Partition coefficient

The tannase partition coefficient was determined as the ratio of the enzyme activity in the top phase (A_t) to that in the bottom phase (A_b):

$$K = \frac{A_t}{A_b} \tag{1}$$

2.5.3 Activity yield

The activity yield was defined as the ratio of the total volumetric activity in the top phase to that in initial extract and was expressed as percentage:

$$Y = \left(\frac{AtVt}{AiVi} \right) \times 100 \quad (2)$$

where V_t and V_i are the volumes of the bottom phase and the initial extract, respectively.

2.5.4 Purification factor (PF)

The purification factor was calculated as the ratio of the specific activity in the bottom phase to the specific activity in the crude extract before partition (A_i):

$$PF = \frac{Ab/Cb}{Ai/Ci} \quad (3)$$

where C_b and C_i are total protein concentrations, expressed as mg/mL, in the bottom phase and crude extract, respectively.

2.6 Enzymatic characterization

2.6.1 Effect and stability to temperature and pH influence on tannase activity

To determine the temperature optimum, enzyme assays were incubated at different temperatures between 20 °C and 90 °C, with 10 °C interval. The thermal stability was performed from the incubation of the enzyme at different temperatures (20 to 50 °C, with 10 °C interval) for 3 hours, aliquots withdrawn for analysis at 30 minute intervals.

The optimum pH for activity tannase purified in aqueous two-phase systems were determined using different buffer solutions: sodium acetate (pH 3.0 to 5.0), Tris-HCl (pH 6.0 to 8.0) and glycine-NaOH (pH 9.0 and 10.0).

2.6.2 Effect of metal ions on tannase activity

The enzyme purification in ATPS was exposed to the following ionic solutions (5, 10 and 20 mM): calcium chloride [CaCl2], potassium chloride [KCl], sodium chloride [NaCl], zinc chloride [ZnCl2], zinc sulfate [(ZnSO4) .7H2O], magnesium sulfate [MgSO4] and

copper sulfate [CuSO4]. The enzyme was incubated at 30 °C for 30 minutes. The salts were dissolved in Tris–HCl pH 7.75 with 0.15 M NaCl.

2.6.3 Surfactant influence on the activity of tannase

The influence of SDS, Triton X-100, Tween 20 and Tween 80 was studied in the following concentrations: 0.5%; 1.0% and 2.0%. The enzyme purified in ATPS was exposed to these surfactants and incubated at 30 °C for 30 minutes. The surfactants were dissolved in Tris–HCl pH 7.75 with 0.15 M NaCl.

2.7 Hydrolysis of undesirable phenolic compounds in green tea by the action of the enzyme tannase

Green tea was prepared using bulk sheets, following the methodology proposed by Lu and collaborators [15]. For hydrolysis of phenols, 5 mL of tea was placed in test tubes with different rates of pre-purified extract in ATPS (0.250 mL, 0.500 mL, 0.750 mL and 1.0 mL). The tea without enzyme was used as control. The tubes were incubated at 30 °C for 120 minutes. The analyzes were performed every 30 minutes (30, 60, 90 and 120 min), applying the Folin-Ciocalteu micro method to quantitate the levels of phenolic compounds of trials [16]. The absorbance was read at 760 nm and gallic acid was used as standard for the calibration curve. The extracts used contained 46.12 U/mL, 15.05 U/mL tannase in the crude extract and partially purified, respectively.

3. RESULTS AND DISCUSSION

3.1 Tannase extraction in aqueous two-phase system

The strategy employed to achieve the aims of this study was to obtain linear models describing the influence of the main variables: PEG molar mass (MMPEG), PEG concentration (CPEG) and sodium citrate concentration (CCIT) on secondary variables: partition coefficient (K), yield (Y) and purity factor (PF). The main experimental results on extraction of tannase are listed in Table 2.

Table 2. Combinations of the levels of three independent variables (concentration of PEG and sodium citrate and molecular weight of PEG) used in a complete factorial design 2^3 and the values of the relative responses.

Run	CPEG% (p/p)	CCit% (p/p)	MMPEG g/mol	PF	K	Y (%)
1	20	15	1500	1,1	0,2	73,4
2	20	15	6000	0,86	0,1	50,3
3	24	15	1500	1,1	0,2	28,6
4	24	15	6000	1,5	0,2	95,7
5	20	20	1500	2,5	0,1	90,9
6	20	20	6000	1,7	0,2	76,4
7	24	20	1500	2,8	0,1	86,8
8	24	20	6000	3,2	0,1	93,6
9 (C)	22	17,5	3350	2,1	0,1	98,9
10 (C)	22	17,5	3350	1,8	0,1	93,0
11 (C)	22	17,5	3350	1,9	0,1	92,8
12 (C)	22	17,5	3350	2,0	0,1	92,5

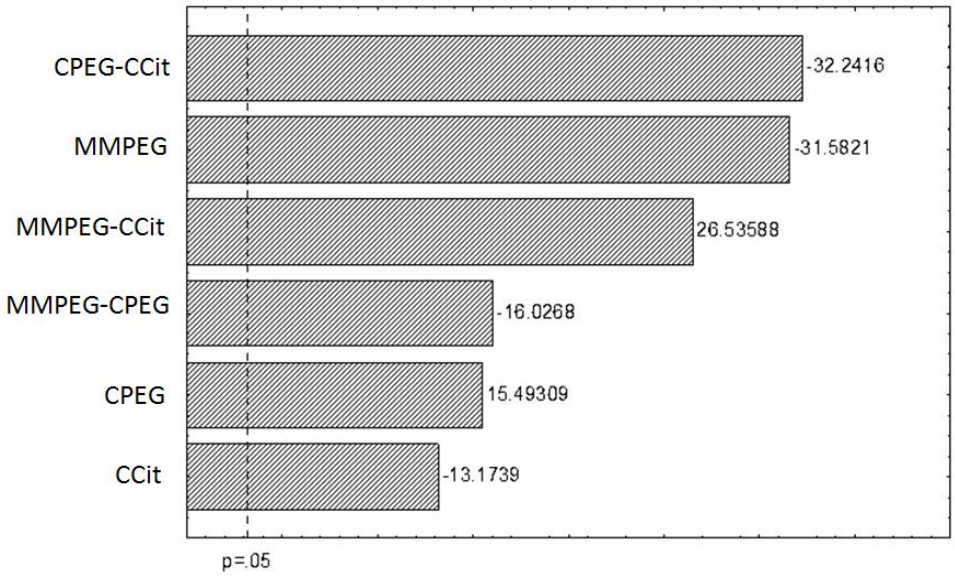
CPEG% and CCIT%= PEG concentration and sodium citrate concentration, respectively; MMPEG = PEG molar mass; PF= purification factor in the botton phase; K= partition coefficient; Y (%) = activity yield in bottom phase.

The studied model showed good results in partial purification of tannase. The 8 test was chosen as the best result for presenting the largest value of PF (3,2). This experiment was comprised of higher concentrations of reactants: 24% PEG 6000 and 20% salt.

In all the tests one can see that the partition coefficient of the activity was lower than 1, indicating that most of the enzyme was extracted from the salt phase. The same was observed by Nascimento and collaborators [17] using PEG/Citrate to purify lectin. Although the partitioning efficiency depends on factors such as an electrical potential between the phases, size and conformation of the molecule; the hydrophobic characteristic is considered the most influential factor [18]. In a PEG/salt system partitioning can occur due to the effect of "*volume exclusion*" in the polymer-rich phase or also cause the "*salting out*" effect in salt-rich phase. The increasing in the salt concentration decreases the solubility of biomolecules in the bottom phase (rich in salt) and propels them to the top phase. On the other hand, the volume occupied by the polymers increases with the polymer concentration and its molecular weight, promoting a decrease in the available space for the molecules in the upper layer, what propels the molecules to the lower phase. [19,20].

The Pareto chart shows, in order of magnitude, the effects of variables and their interactions, which are represented by names and numbers on the vertical axis. The length of each bar is proportional to the standardized effect of the variable and the vertical line can be used to judge the most important effects.

Figure 1. Pareto chart of the main effects and their interactions for the response variable K in the tanase extraction process in ATPS PEG/Citrate using full statistical planning 2^3.

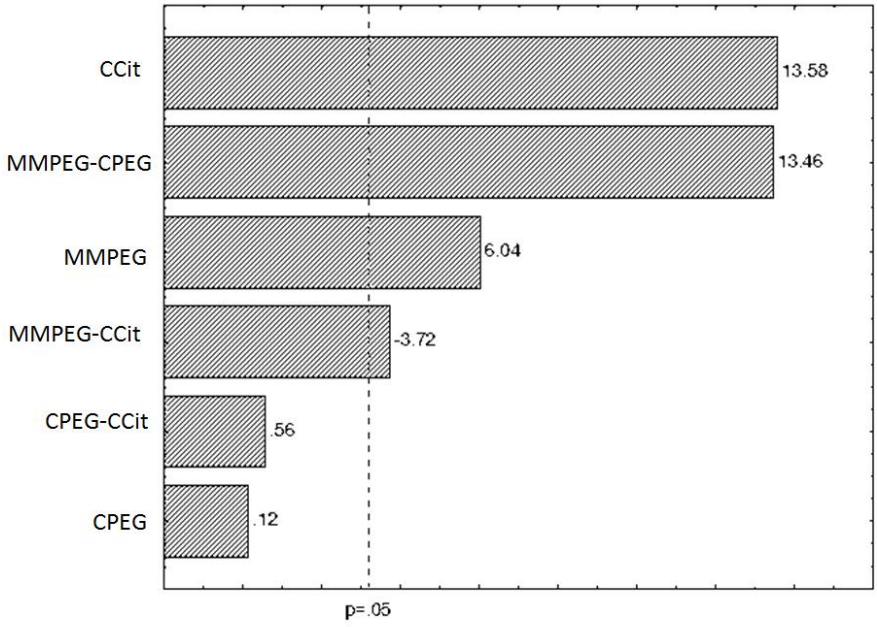


The Figure 1 shows that all terms were significant (at 95% confidence level) for the variable K, for the values of the estimated effects with $p < 0.05$. In order of magnitude, the terms interaction between CPEG-CCIT and MMPEG were the most important effects, both

showing negative algebraic sign. Thus, decreasing CPEG, increasing CCIT and increasing MMPEG, it is possible to obtain a better separation of the salt tannase phase.

Our reports are in agreement with the studies by Rodriguez-Duran et al. [21] which have purified tannase obtained from *Aspergillus niger* by ATPS PEG/phosphate. The authors used PEGs with molecular weights of 400, 600 and 1000, and the results showed that by increasing MMPEG significantly there is a consequent decrease in the enzyme partition coefficient. Also according to those authors, the hydrophobic proteins have high K values of activity. Based on this, the authors concluded that the TAH of *Aspergillus niger* has few hydrophobic areas on its surface since all values of K activity were smaller than 1 (corroborating our results), suggesting that the tannase has a hydrophilic nature. A significant and positive effect on the interaction of MMPEG-CCIT variables was observed. This means that the simultaneous increase of these two variables will displace a better partitioning of the enzyme in bottom phase.

Figure 2. Pareto chart of the main effects and interactions for variable Yeld (%) in the tanase extraction process in ATPS PEG/Citrate using full statistical design 2^3, in bottom phase.



The effect of main variables and their interactions on the response variable Y(%) can be seen in Figure 2. All positive significant terms indicate that increasing the variable promotes better recovery at the salt phase. This occurs with increasing CCIT, simultaneous increase in MMPEG-CPEG and increased MMPEG, as shown in the graph. Contrary to what

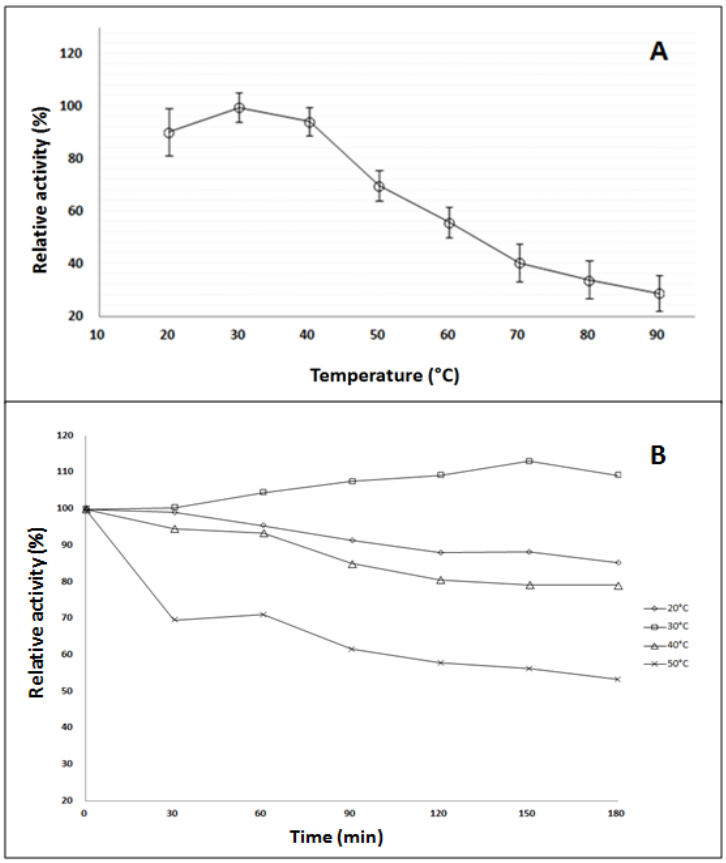
is observed for the variable K, not all effects were significant at the 95% confidence level. CPEG-CCIT and CPEG not presented p <0.05. Although the effect of the main variable CPEG was not significant at the 95% confidence level, the interaction of this term with MMPEG shows that in joint action, this variable may prove significant effect for Y(%).

With ANOVA, the linear model for the yield response variable elucidated 70% (R^2=0,70) of the results obtained in the assays. Some misfit for the model was observed, but with low error of 0.30 at a confidence level of 95%.

3.2 Effect of temperature and pH on tannase activity

The optimum temperature of tannase was observed at 30 °C, in which the enzyme had 100% of its efficiency (Figure 3A).

Figure 3. Influence of different temperatures in the activity of tannase produced by *Aspergillus* sp. 25 SIS (A) and enzyme stability for 3 hours of incubation (B).

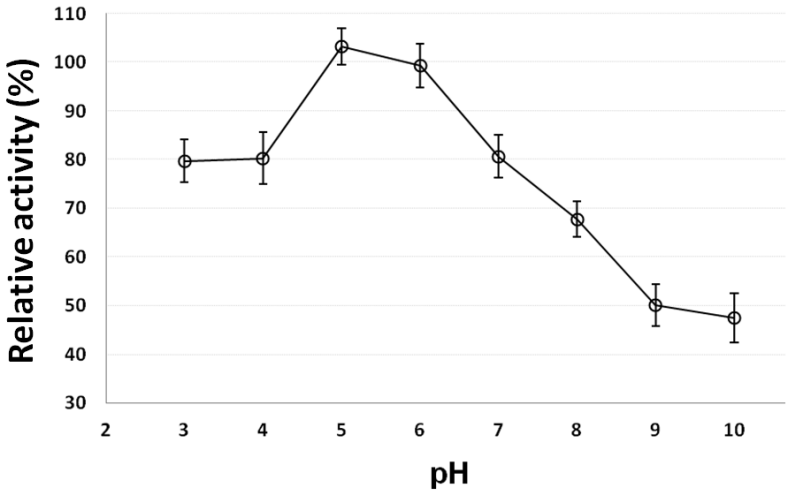


The increase in temperature caused gradual loss in activity of the molecule; at 90 °C, for example, the enzyme lost 71% of its activity. The tanase remained stable at its optimum

temperature for more than 3 hours. At 50 °C the enzyme began to lose stability since its relative activity began to decrease (Figure 3B).

The optimum pH of tannase was observed between 5.0-6.0, in which the enzyme showed 100% and 96% of its activity, respectively (Figure 4), when it was incubated at 30 °C in acetate buffer sodium. Significant activities were also observed at pH's 3.0; 4.0 and 7.0. In an alkaline pH it was verified that the activity of the enzyme decreased significantly, reaching 44% in NaOH-glycine buffer (pH 10).

Figure 4. Influence of different pH in the activity of tannase produced by *Aspergillus* sp. SIS 25 after 30 minutes of incubation.



Microbial tannase generally has the optimum temperature ranges of 20-60 °C and thermostability between 30 and 60 °C [22]. As in this study, Mahapatra et al. [23] They described how great a temperature of 30 °C to tannase different *Aspergillus* species. Similarly, Costa et al [24] also observed an optimum temperature of 30-35 °C to *A. tamarii* tannase, which is stable for more than 2 hours at 40 °C, completely lose their stability at temperatures higher than 45 °C. Likewise, the tannase from *Aspergillus niger* ATCC 6514.07 showed enhanced activity around 35 °C and pH 6.2 [25]. In line with most of the work described herein, Jana et al. [26] observed an immobilized tannase of *Bacillus subtilis* PAB2 that having optimum activity at 40 °C, at pH 5.0, similar to its free form, being stable over a wide pH range (3.0-8.0).

Enzymes are very sensitive to changes in pH and work best on a very limited range [27]. As the tannase from *Aspergillus* SIS 25, the tannase *Penicillium variable* was reported to be stable in a pH range of 4.0-6.0 therefore showed that almost 100% activity for 24 hours

[28]. Tannase of *A. tamarii* studied by Costa et al. [24] also showed pH characteristics relatively similar to the enzyme of the present work. The same presented optimum pH 5.0 with substantial activity at pH 4.0 to 8.0, and is stable over a wide pH range (3.0 to 8.0). Many other acidophilic nature of tannase with optimum pH similar to that observed in this study are reported in the literature, such as *A. awamori* tannase and P. variable with pH 5.0 [23,28] and pH 6.0 tannase obtainable from *A. niger* [29].

The optimum pH acid suggests applicability in the food industry mainly from fruit, where the acidity favors the enzyme activity [30]. The same principle can also be practiced in processing beverages such as green tea, which has a pH of about 6.0, which favors the catalysis of undesirable compounds such as tannins.

3.3 Influence of surfactants on the enzyme activity

In the presence of Tween 20, the enzyme activity remained unchanged showing that the molecules of the surfactant concentrated at 0.5% and 1% did not interfere with the accessibility of the substrate to the active site of the enzyme (Table 3).

Table 3. Influence of various surfactants on tannase enzyme activity.

Surfactant	Concentration (%)	Relative activity (%)
Tween 20 (% v/v)	0,5	105,37
	1	99,29
	2	85,89
Tween 80 (% v/v)	0,5	79,28
	1	62,27
	2	55,96
Triton X-100 (% v/v)	0,5	64,81
	1	61,86
	2	46,69
SDS (mM)	0,5	65,76
	1	70,54
	2	66,76

In the presence of SDS and Tween 80 surfactan, the enzyme lost its activity especially in higher concentrations. Although, Triton X-100 2% had greater interference with the binding of substrate, decreasing the relative activity to 46.69%.

Surfactants are substances that can denature proteins and therefore play an important role in the catalytic activity of enzymes. Their effects on the catalytic ability of tanase vary considerably [22]. Unlike the present study, a significant loss of activity in the presence of

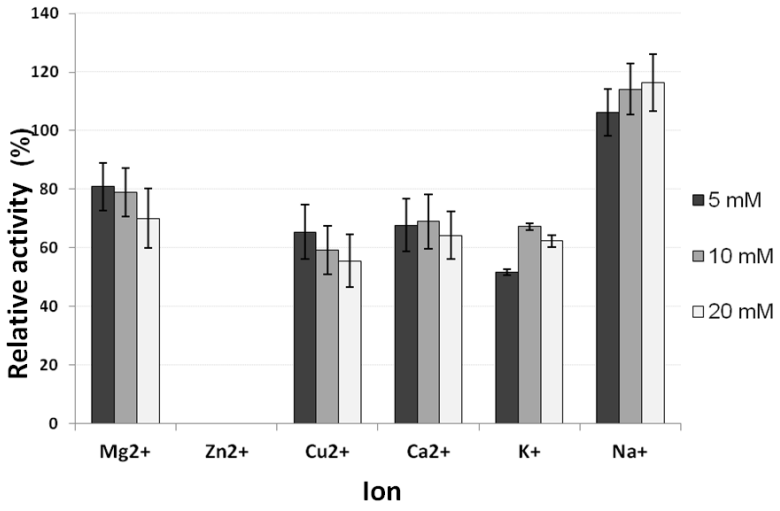
Tween 20 was reported to tannase *Verticillium* sp. P9 [31], while small positive effect was observed for tannase from *Aspergillus niger* GH1 [32]. Tannase activity of *Penicillium variable* and *Aspergillus foetidus* were completely inhibited by SDS and Tween 80 [28,33], different this work, where there was observed complete inhibition by any of the surfactants. With this, it is clear that the tannase from *Aspergillus* SIS 25 has a certain resistance to most surfactants, seen here as tannase denaturing agents.

3.4 Effect of metal ions on the enzyme activity

More than 75% of the enzymes require metal ions to express its maximum catalytic ability. At low concentrations, some metals may act as cofactors which enhance enzymatic activity but at high concentrations, what happens is an inhibition [32].

They tested different concentrations of metal ions that in the literature as inhibitors or stimulators of the tannase activity, as can be seen in Figure 5. Tannase from *Aspergillus* sp. SIS 25 was resistant to most of the studied ions. The enzyme showed maximum activity in the presence of Na^{+} and Mg^{2+}, lost about 40% activity in the presence of Cu^{2+} but was Zn^{2+} that completely inhibited the tannase action.

Figure 5. Influence of various metal ions concentration on tannase activity of *Aspergillus* sp. SIS 25, extracted in aqueous two-phase system.



The inhibitory effects of Fe^{3+}, Cu^{2+} and Zn^{2+} have been frequently reported in the literature. The tannase *A. niger* GH1, for example, was strongly inhibited by Cu^{2+} and Zn^{2+} by slightly inhibited, in accordance with Mata-Gómez et al [32]. However, other strains of *A. niger* has produced tannase strongly inhibited by Mg^{2+} and Mn^{2+}, as the tannase from

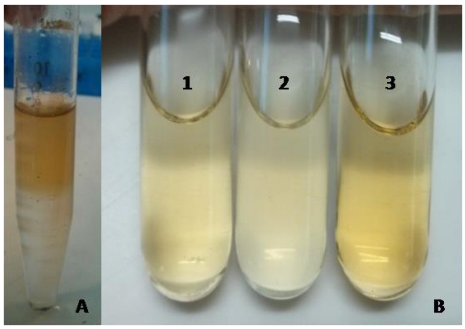
Aspergillus niger ATCC 16620 [34] and *Aspergillus niger* ITCC 6514.07 [25] have shown that the presence of magnesium cations. In contrast, tannase *Verticillium* sp. was inhibited by Mn^{2+}, Zn^{2+} and Cu^{2+} however, activated by Mg^{2+} [31], as well as TAH seen in this work and tannase *Rhizopus oryzae*, *Aspergillus foetidus* [35] and *Aspergillus awamori* MTCC 9299 [36].

Cofactors are usually not required for the tannase activity, but divalent cations, such as magnesium, often stimulate the enzyme activity. This probably happens due to a variety of mechanisms, including activation by metal ions, altering the equilibrium constant of the enzyme reaction, or causing a change in the surface charge of the enzyme. Furthermore, the tannase activity is generally inhibited by heavy metals such as Hg^{2+}, Co^{2+}, Ba^{2+}, Cd^{2+}, Ag^{+}, Pb^{2+}, Sn^{2+} [22].

3.5 Hydrolysis of undesirable phenolic compounds in green tea by the action of the enzyme tannase

The aqueous two-phase system is a method of partial purification which simultaneously clarifies the enzymatic extract (Figure 6.A).

Figure 6. Pigment retention in the PEG phase (top) of the ATPS (A) and qualitative difference in color of green tea (1), green tea treated with partially purified tannase (2) and green tea with crude extract (B).



The retention of undesirable components (such as pigments) in this system facilitates the use of the enzyme in various applications such as green tea, as shown in Figure 6.B.

The action of crude enzymatic extract and purified enzyme in ATPS on hydrolysis of green tea compounds it is represented in the Table 4. The hidrolyzes was tested at different concentrations of the crude extract and of the purified enzyme in ATPS, in different time intervals. The control (tea without enzyme) initially contained 5.58 U /ml of total phenolics. In tea sample treated with crude extract there was an initial increase of phenolic compounds,

in relation to control, due to the presence of contaminants from phenols extract. Then the reduction of phenols was observed over time, possibly due to a greater exposure of tannins the presence of tannase. The initial increase of phenols shows that contaminants from the crude extract are able to interfere with the tannins hydrolysis process, contributing to the emergence of undesirable results in tea as intensifying color and astringent taste.

Table 4. Effect of concentration of 0.75 mL of crude extract and 0.75 mL of purified tannase enzyme in ATPS (PEG /Citrate) on hidrolyzes of 1mL green tea phenolic compounds.

Assay	Time (min)	Total phenols (%)
Green tea treated with 0.75 mL of crude extract	30	137
	60	137
	90	132
	120	130
Green tea treated with 0.75 ml of purified enzyme by ATPS	30	75
	60	71
	90	64
	120	56

Hydrolysis of phenols extracted from the tannase in ATPS showed gradual reduction of the compounds in relation to control, with each increase of enzyme concentration. The best result was observed after 2 hours incubation with 0.75 mL of tannase from ATPS at 30 °C, wherein the content of phenolic compounds in the tea was reduced to 56% (3.14 U / mL). At this same time interval, green tea sample with 0.75 ml of crude extract still had 7.2 U/mL total phenols, equivalent to 130%. In the presence of the highest concentration (1 mL) of purified enzyme, the phenols remained at an average 68% reduction of 32%. The 0.25 mL and 0.50 mL concentrations showed the smaller reduction of phenolic compounds: 16% and 24%, respectively.

Few studies on hydrolysis of phenolic compounds by tanase are reported in the literature. The existing works mainly deals about the action of the enzyme in the process of clarification of fruit juice. Sharma et al (2014) tested the activity of tannase produced by *Aspergillus niger* in the detanification of guava juice, using tanase at 2%. After a period of 60 minutes, the authors verified a decrease of 59.23% in the beverage tannin content. Lima et al. (2014) using tannase from *Penicillium montanense* for clarifying grape juice, was able to

reduce 46% of tannin content after 2 hours of incubation at 37 °C with 1 mL of crude enzyme extract.

The fact that the enzymes possess specific substrates favors the understanding that the phenolic compounds reduced in green tea are actually tannins. This reduction, which can be followed by quantification of total phenols, shows that it is possible to extract in ATPS one tanase with promising use in biotechnology. Tannase from system when applied to green tea showed superior performance compared to the crude enzyme extract, suggesting the importance of further studies to optimize its purification and consequent use in the industry. Thus, this work confirms the potential of the ATPS as an economically viable method for purification of tannase, making than enzyme promising on improvement of sensory aspects of green tea.

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CONSIDERAÇÕES FINAIS

A melhor extração de tanase em sistema de duas fases aquosas ocorreu no ensaio composto por 24% de PEG 6000 e 20% de citrato de sódio, no qual foi obtido um aumento de pureza (AP) de 3,2. A partir dos efeitos das variáveis independentes sobre as variáveis resposta é possível descrever um melhor modelo estatístico para a extração da enzima de interesse. A massa molar do PEG e a interação entre MMPEG-CPEG apresentou influência negativa na partição da molécula entre as fases do sistema. Isso implica dizer que o aumento da MMPEG resultará em valores melhores do coeficiente de partição, para extrair a enzima tanase na fase sal.

A tanase de *Aspergillus* sp. SIS 25 apresentou máxima atividade a 30 °C e em pH 5,0 porém, perdeu estabilidade a 50 °C. Quanto ao efeito de íons metálicos foi observado uma interessante resistência à maioria dos íons. O Na^{+} potencializou a atividade da enzima e o Mg^{2+}, frequentemente descrito como inibidor de tanase, não interferiu em sua atividade. Já o íon Zn^{2+} mostrou-se um potente inibidor de tanase, zerando completamente sua atividade. Não foram observadas reduções significativas na atividade da TAH quando esta foi exposta à maioria dos surfactantes. Em exceção, o Triton X-100 a 2% reduziu pela metade o potencial catalítico da tanase. A TAH desse trabalho também apresentou resultados interessantes quando aplicada em chá verde. A enzima purificada em SDFA foi capaz de reduzir 44% dos compostos fenólicos indesejáveis, apresentando-se mais viável nessa aplicação se comparada à enzima no extrato bruto.

Os resultados aqui apresentados confirmam que o SDFA é um método interessante para purificar parcialmente enzimas de interesse comercial como a tanase e, fortalece o potencial uso dessa molécula na hidrólise de taninos presentes no chá verde, promovendo novos estudos que explorem o uso biotecnológico dessa enzima.

ANEXO I

NORMAS DA RESVISTA

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FLUID PHASE EQUILIBRIA

An International Journal

AUTHOR INFORMATION PACK

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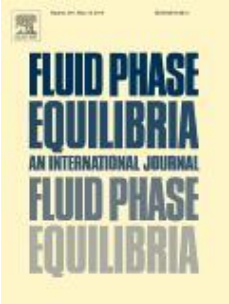

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Fluid Phase Equilibria publishes high quality papers dealing with experimental,theoretical and applied research related to **equilibrium** and **transport properties** of **fluid** and **solid phases**.

The fluid phase properties of interest include:
PVT, enthalpies, heat capacities, Joule-Thomson coefficients, Gibbs and Helmholtz energies, chemical potentials, activity and fugacity coefficients, critical properties, chemical equilibria, multiphase equilibria and interfacial properties, thermal conductivity, viscosity and rheological properties, and diffusion coefficients.
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Unless they are accompanied by contemporary or new theory, papers will be refused if they report experimental data only at pressures and temperatures close to ambient on any of the following liquid or liquid mixture properties: viscosity; density; speed of sound; refractive index; surface tension. Similarly, papers will be refused if they only report phase equilibrium compositions, such as solubilities, at conditions near ambient without theoretical analysis and interpretation.

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All modeling of properties and phenomena based on artificial neural networks, machine learning algorithms, and similar information processing approaches will only be considered when comparisons of accuracy are made with existing physically-based models or if no thermodynamic models are available. Further, the work must describe the procedure well enough that readers may be able to independently reproduce the results.

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Editorial and Introduction

Editorial

New procedures for articles reporting thermophysical properties

Fluid Phase Equilibria, along with other journals in the field, established collaboration with the Thermodynamics Research Center (TRC) of the National Institute of Standards and Technology (NIST) in 2009 for the purpose of ensuring the quality of published experimental data. In a joint statement [1], the editors of the five journals involved set out the rational for the cooperation in terms of helping to ensure that authors and reviewers were made aware of any previously-published literature values for the properties and systems in question. The process involved NIST 'capturing' the new experimental data, comparing it against existing values in the NIST data archive and providing a report that: (a) listed relevant literature sources; and (b) highlighted any obvious discrepancies in the new data.

In order to streamline the process and to further enhance the quality of published articles, we are now introducing one change to the way in which the NIST cooperation is implemented. Effective in February 2013, responsibility for preparing a *Literature Report* will shift from NIST to the submitting authors. Submitting authors will be able to prepare their own *Literature Report* by using *ThermoLit*, a publicly available (http://trc.nist.gov/thermolit/) program. This will eliminate NIST's role in providing this report, and thus speed the review process and provide added benefit to authors who will have literature citation results on hand at a stage when they can do the most good. Please, note that use of *ThermoLit* is designed as an aid to the traditional required literature review and must not be used as a substitute.

NIST will continue to provide a data evaluation at the end of the review process, immediately prior to final acceptance of the article. This data evaluation will compare the reported experimental data with that existing in the NIST Data Archive and highlight any unexpectedly large discrepancies, such as those arising from typographical errors. Though the data evaluation step has not changed, we will use this opportunity for a reminder that experimental results and their uncertainties must be tabulated in the way described in the Guide for Authors. A key feature is that tables be self-contained and include the uncertainties of all reported quantities (variables, constraints, and properties). In addition, we have incorporated new standards relating to the description of chemical samples and we encourage authors to present details of their samples in an easily-readable tabular form. To assist authors, a large number of example tables have been prepared by NIST and are available (http://trc.nist.gov/FPE-Support.html).

The new procedures will provide literature citations to authors before submission of their manuscript and speed the review process. Indeed, authors are encouraged to use *ThermoLit* in advance of experiments to help minimize duplication of effort. In 2012, new IUPAC guidelines for the reporting of phase equilibrium measurements were published (Pure Appl. Chem. 2012, 84(8), 1785-1813), and the requirements of this journal are consistent with these recommendations.

Prior to submission, authors are strongly encouraged to review a checklist based on these recommendations, which is available (http://trc.nist.gov/FPE-Support.html). We are certain that the new Literature Report tool and the procedures described here will further enhance the already high quality of articles published in *Fluid Phase Equilibria*.

Th.W. de Loos, Editor
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References

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Aims and Scope

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Theoretical techniques may be chemical thermodynamics, applied statistical mechanics, molecular physics, molecular simulation, quantum chemistry, applied mathematics. Papers with new models, or modifications of available models, are expected to show comparisons for accuracy and predictive ability with applicable data and contemporary existing models.

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