ALESSANDRA GONÇALVES DE MELO

PURIFICAÇÃO E CARACTERIZAÇÃO DE TANASE DE *Aspergillus* sp. GM4 EM FERMENTAÇÃO SUBMERSA

LAVRAS – MG

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2012

Ficha Catalográfica Elaborada pela Divisão de Processos Técnicos da Biblioteca da UFLA

Melo, Alessandra Gonçalves de.
Purificação e caracterização da tanase de *Aspergillus* sp. GM4 em fermentação submersa / Alessandra Gonçalves de Melo. – Lavras: UFLA, 2012.
66 p.: il.

Dissertação (mestrado) – Universidade Federal de Lavras, 2012.
Orientador: Patrícia Gomes Cardoso.
Bibliografia.

1. Metodologia de superfície de resposta. 2. Delineamento experimental. 3. Enzimas microbianas. 4. Produção enzimática. I. Universidade Federal de Lavras. II. Título.

CDD – 660.634

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APROVADA em 30 de março de 2012.

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2012

Àqueles que me apoiaram e me inspiraram. Em especial, ao meu querido avô Gemiro e a vó Mulata que, com exemplo, me mostraram o valor da integridade e da persistência. À minha mãe Rute, que com um jeito que só ela tem, me ensinou a importância da educação como forma de mudar o nosso futuro. Ao meu pai Elder, irmãos, familiares e amigos que se orgulharam e estiveram ao meu lado em cada uma das minhas conquistas. E ao meu amado José Carlos, que com zelo e amor, me ajudou a me manter centrada e focada nas coisas realmente importantes. A vocês eu dedico!

AGRADECIMENTOS

À Universidade Federal de Lavras, ao Departamento de Biologia e ao Programa de Pós Graduação em Microbiologia Agrícola, pela oportunidade de realização do mestrado.

À CAPES pela concessão da bolsa de estudos e à FAPEMIG e ao CNPq pelo apoio financeiro no desenvolvimento do projeto.

À Professora Patrícia Gomes Cardoso pela orientação, amizade e confiança! Por dar-me liberdade para questionar e instigar-me a pensar no desenvolvimento dos trabalhos.

Ao Professor Luis Henrique Souza Guimarães pela co-orientação, pelas contribuições no desenvolvimento deste trabalho e por me receber em seu laboratório na realização de parte dos experimentos.

Aos Professores Disney Ribeiro Dias, Jose Guilherme Lembi Ferreira Alves e Whasley Ferreira Duarte que aceitaram fazer parte da minha banca e pelas contribuições valiosas na finalização do trabalho.

Ao Professor Eustáquio Souza Dias eu agradeço imensamente pela amizade, paciência e bons conselhos. Agradeço ainda pelas contribuições e pelos conhecimentos transmitidos.

Aos Professores do Setor de Microbiologia pelos conhecimentos transmitidos, que contribuíram para o meu crescimento profissional.

Ao Professor Mário Lúcio e aos colegas do Laboratório de Fitopatologia, em especial ao Pedro, pelo apoio.

Aos Professores e funcionários dos Setores de Microbiologia e Cafeicultura pelo convívio. Em especial a Ivani, Cidinha, Rose, Renata, Magda e Michele, pelos esclarecimentos e ajuda constante.

Aos amigos, colegas, professores e funcionários do Laboratório de Biologia Celular e Microbiologia da Faculdade de Filosofia, Ciências e Letras da

USP de Ribeirão Preto pelo convívio harmonioso e pelas muitas experiências trocadas. Em especial, André, Cynthia, Josana, Lili, Loli, Tati, Telma, Vanessa e Zé pela amizade dentro e fora do laboratório e pela disposição em ensinar! Eu amei conviver com vocês!

Às meninas do Laboratório de Prospecção e Genética de Fungos, Ariela, Natália, Patrícia e Rayssa por todo esse tempo de trabalho em conjunto e por dividirem comigo as alegrias e frustrações das experiências vividas no decorrer dos experimentos. Em especial, agradeço a Ariela e Rayssa que trabalharam diretamente comigo, pelas horas dedicadas, pelo esforço e responsabilidade no desenvolvimento dos trabalhos.

Aos amigos da Igreja Cristã Maranata de Lavras e de Ribeirão Preto pelas orações, amizade e por me ajudarem a manter o equilíbrio. Em especial, Aldenora, Ester, Cidinha, Rebecca, Sarah e Eustáquio por serem a minha família em Lavras e por toda amizade, carinho e cuidado que tiveram comigo! Lili, Lucas, Leila, Cris e Gilvan, velhos amigos, com os quais tive a oportunidade de conviver novamente durante o tempo passado em Ribeirão Preto: obrigada por me acolherem.

Ao José Carlos, me faltam palavras para agradecer o seu cuidado. Pelo incentivo, pela amizade e pelo amor! Pelas conversas que me distraíram ou por aquelas que me fizeram voltar ao foco! Amo ter você ao meu lado!

À minha família, tios, primos, avós, eu agradeço por sempre me apoiarem e compreenderem a minha ausência. Em especial, agradeço aos meus pais, Rute e Elder, e meus irmãos, Alexandre e Victória, por tornarem a minha vida mais feliz! Amo vocês!

A Deus, meu grande amigo, eu agradeço por me fortalecer, por nunca me desamparar e nunca me deixar só! Porque d'Ele, por Ele e para Ele são todas as coisas!

RESUMO

Três espécies do gênero *Aspergillus*; *A. japonicus* 246A, *A. tamarii* 3 e *Aspergillus* sp. GM4; foram previamente selecionadas e avaliadas quanto à produção de tanase nos meios Adams, Czapeck, Khanna, M5 e Vogel. *Aspergillus* sp. GM4 e meio Adams foram selecionados para a realização deste estudo. A produção enzimática foi avaliada utilizando diferentes indutores. A indução máxima foi obtida na presença de 2% de ácido tânico e 1% de metil galato. O delineamento de Plackett-Burman com seis variáveis foi realizado, visando encontrar aquelas que tivessem efeito significativo na produção da tanase. Agitação e $MgSO_4$ foram selecionadas para o fatorial completo. No delineamento composto central rotacional houve um aumento de 2,66 vezes na produção de tanase quando comparado com os experimentos iniciais. Tanase de *Aspergillus* sp. GM4 foi purificada em duas etapas: com filtração da amostra utilizando uma membrana de corte de proteínas com massa molecular menor do que 100 kDa e cromatografia de exclusão molecular utilizando Sephacryl S-200. O rendimento da enzima na primeira etapa foi de 29% sendo purificada 29 vezes seguida de recuperação de 3,5% após uma purificação de 33 vezes. A massa molecular da enzima nativa foi estimada por cromatografia de exclusão molecular revelando uma proteína com massa molecular de aproximadamente 162 kDa. Tanase de *Aspergillus* sp. GM4 apresentou atividade ótima em temperatura de 40 °C e pH 6,0. A enzima manteve atividade no intervalo de pH 3,0 a 8,0 e no ensaio de estabilidade térmica apresentou atividade até 80°C, porém foi completamente inativada após 30 minutos. Tanase foi estável na presença de compostos tradicionalmente descritos como inibidores da atividade enzimática, tal como β-mercaptoetanol, EDTA e íons metálicos Pb^{2+} e Ba^{2+}.

Palavras-chave: Tanase. Produção enzimática. Delineamento experimental. Metodologia de superfície de resposta. *Aspergillus* sp.. Purificação. Caracterização.

ABSTRACT

Three *Aspergillus* species; *A. japonicus* 246A, *A. tamarii* 3 and *Aspergillus* sp. GM4, were previously selected and had their ability to produce tannase tested in Adams, Czapeck, Khanna, M5 and Vogel culture medium for enzyme production. *Aspergillus* sp. GM4 and Adams medium were selected to perform this study. Tannase production was tested with different inducers. The highest induction ratio was in the presence of 2% tannic acid and 1% methyl gallate. The Plackett-Burman screening design was used and $MgSO_4$ and agitation rate were selected for the complete factorial. The Central Composite Rotatable Design allows an increase of 2.66-fold in the enzyme production and reduced the costs of the medium production. The purification process of tannase from *Aspergillus* sp. GM4 was performed with filtration using 100 kDa molecular mass cut-off membrane and gel-filtration chromatography using Sephacryl S-200. The results showed 29% enzyme yield and 29-fold purification in the first step, while in the second step 3.5% enzyme yield and 33-fold purification was obtained. The native molecular mass was estimated by gel-filtration chromatography and resulted in a protein molecular mass of approximately 162 kDa. *Aspergillus* sp. GM4 tannase presented optimum temperature at 40°C and optimum pH at 6.0. The enzyme retained activity in pH 3.0-8.0 and the thermostability assay showed enzyme activity up to 80 °C, but after 30 min it was inactivated. Tannase was stable in the presence of compounds traditionally reported as inhibitors of the enzyme activity, such as β-Mercaptoethanol, EDTA and different metal ions, such as Pb^{2+} and Ba^{2+}.

Keywords: Tannase. Tannase production. Experimental design. Response surface methodology. *Aspergillus* sp.. Purification. Characterization.

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PRIMEIRA PARTE

1 INTRODUÇÃO

As atividades metabólicas realizadas pelos microrganismos produzem diversos produtos com aplicação biotecnológica, dentre os quais se destacam as enzimas. O desenvolvimento de estudos em escala laboratorial para a escolha de microrganismos e enzimas com características desejáveis representa um importante passo no estabelecimento de um processo industrial, por isso, existe a necessidade de prospecção de microrganismos e seleção de enzimas com características mais adequadas à sua aplicação.

Tanase é uma enzima hidrolítica com aplicação nas indústrias de alimentos, bebidas, ração, farmacêutica, química, e com potencial para utilização em processos de biorremediação. A partir da hidrólise de taninos por tanase são produzidos glicose e ácido gálico, um importante precursor na síntese de produtos para a indústria química e farmacêutica. As tanases microbianas são mais estáveis do que as isoladas a partir de plantas ou animais. Além disso, os microrganismos são capazes de produzir enzimas continuamente e os processos de produção podem ser melhorados através de estratégias de otimização.

Apesar do potencial industrial da enzima, atualmente a comercialização de tanase no mundo tem sido realizada por apenas algumas empresas na Índia (Biocon), Alemanha (ASA special enzyme GmbH ; Julich Chiral Solutions GmbH), Japão (Kikkoman; Wako Pure Chemical Industries, Ltd.), Dinamarca (Novo Nordisk) e Estados Unidos (Sigma Aldrich). Isso demonstra a necessidade de buscar microrganismos com alta produção de tanase e enzimas com características e estabilidade apropriadas aos processos. Além disso, é premente o desenvolvimento de estratégias a serem aplicadas em processos

industriais, a fim de que a produção comercial de enzimas seja ampliada e os custos minimizados.

Este trabalho teve como objetivo isolar e caracterizar tanase de *Aspergillus* sp. GM4 e avaliar a influência de alguns fatores visando a otimização da produção desta enzima por fermentação submersa.

2 REFERENCIAL TEÓRICO

2.1 Taninos

Taninos são compostos fenólicos solúveis em água e de alta massa molecular (M_r > 500), contendo hidroxilas fenólicas ou outros grupamentos livres, tais como carboxilas. Podem formar complexos efetivamente fortes com proteínas, carboidratos e minerais, sob determinadas condições ambientais. Juntamente com a lignina, constituem os dois maiores representantes dessa classe de compostos (MACSWEENEY et al., 2001; REED, 1995).

Os taninos podem ocorrer na madeira, casca, folhas, frutos e galhos de plantas e são usualmente subdivididos em dois grupos: taninos hidrolisáveis e taninos condensados. Os taninos hidrolisáveis são divididos em galotaninos e elagitaninos. Os galotaninos são caracterizados pela presença de várias moléculas de ácidos orgânicos, tais como gálico e digálico, esterificados a uma molécula de glicose. Os elagitaninos são compostos por unidades de ácido elágico ligadas a glicosídeos. Estes compostos são facilmente hidrolisados por ácidos ou enzimas em suas unidades monoméricas (AGUILAR et al., 2007; BHAT; SINGH; SHARMA, 1998; MUELLER-HARVEY, 2001; REED, 1995). Por outro lado, os taninos condensados (ou proantocianidinas) compreendem moléculas compostas por unidades flavonóides unidas entre si por ligações carbono-carbono; possuem estruturação complexa e são resistentes à hidrólise, mas podem ser solúveis em solventes orgânicos polares, dependendo de sua estrutura (REED, 1995; SCHOFIELD; MBUGUA; PELL, 2001).

A capacidade de complexar dos taninos confere a eles a possibilidade de serem reativos à parede celular de bactérias e às enzimas extracelulares secretadas pelos microrganismos. Além disso, a interação de taninos com substratos tais como polissacarídeos e proteínas, torna esses complexos

resistentes ao ataque microbiano. Essas interações inibem o transporte de nutrientes para a célula e podem retardar o crescimento dos organismos. Por essa razão, os taninos são, geralmente, reconhecidos como inibidores do crescimento microbiano (LEKHA; LONSANE, 1997; MCSWEENEY et al., 2001).

No entanto, muitos microrganismos evolveram mecanismos e vias de degradação desses compostos em seu habitat natural, tornando-se resistentes ao efeito inibitório dos taninos. A capacidade de metabolizar esses compostos e utilizar a energia para seu próprio crescimento é conferida pela produção de enzimas, tais como galato-descarboxilase e tanino acil hidrolase (ACAMOVIC; BROOKER, 2005; AGUILAR et al., 2007).

2.2 Tanase

2.2.1 Ação e Aplicação de Tanase

Tanase ou tanino acil hidrolase (E. C. 3.1.1.20) é uma enzima que catalisa a hidrólise de ligações éster e depsídicas em taninos hidrolisáveis, tais como ácido tânico, metil-galato, etil-galato, propil-galato e isoamil-galato (AGUILAR et al., 2007; BATTESTIN; MATSUDA; MACEDO, 2004). Tanases geralmente agem sobre os galotaninos. Essas enzimas hidrolisam ácido tânico completamente, liberando glicose e ácido gálico (LEKHA; LONSANE, 1997). Embora a degradação de elagitaninos por tanases já tenha sido demonstrada, os mecanismos de ação das tanases sobre esses compostos não estão bem explicados, principalmente pela complexidade e diversidade dos elagitaninos. Os taninos condensados não são hidrolisados por “tanases clássicas”, sendo os passos iniciais de degradação desses compostos catalisados por mono- ou di-oxigenases (AGUILAR et al., 2007).

A tanase apresenta aplicação na indústria de alimentos, ração, couro, farmacêutica e química. Na produção de bebidas a tanase pode ser utilizada para reduzir a formação de turbidez pelos componentes fenólicos (LEKHA; LONSANE, 1997), com importância no processo de clarificação de cerveja e sucos de frutas, produção de refrigerantes à base de café e na melhoria do *flavour* de vinho de uvas (SETH; CHAND, 2000). No caso dos vinhos, os principais taninos presentes são catequinas e epi-catequinas que podem formar um complexo com galato-catequinas e outros derivados galoil. Parte da coloração do vinho é dada pela presença dos taninos, entretanto, estes compostos são oxidados a quinonas por contato com o ar, podendo promover uma turbidez indesejável, apresentando vários problemas de qualidade. O uso de tanase pode ser uma solução para tais problemas. Na produção de cerveja, a tanase pode ser usada para remoção da turbidez da bebida, causada pela formação de um complexo entre as proteínas da cerveja e os taninos (BELMARES et al., 2004). A descoloração e o desenvolvimento de turbidez na cerveja durante a estocagem pode ser evitada com a hidrólise dos polifenóis do malte com tanase e lacase (LEKHA; LONSANE, 1997).

Outra aplicação da tanase é na produção de ácido gálico, um importante intermediário na síntese da droga antibacteriana, trimetropim, usada na indústria farmacêutica. O ácido gálico também é substrato para a síntese química ou enzimática de propil-galato, um potente antioxidante com importância na indústria alimentícia (AGUILAR et al., 2007; LEKHA; LONSANE, 1994).

Enzimas de fungos envolvidas nas vias degradativas de compostos aromáticos, como os taninos, são consideradas de interesse para a biorremediação e biodegradação de resíduos orgânicos (RAMÍREZ-CORONEL et al., 2003). Os taninos são considerados perigosos poluentes, por essa razão o uso de tanase representa um tratamento natural e de baixo custo para a remoção destes compostos de águas residuais contaminadas por compostos fenólicos,

como os efluentes de curtume (AGUILAR et al., 2001b; BELMARES et al., 2004).

Na indústria de ração, a tanase pode ser usada no pré-tratamento de resíduos de plantas contendo taninos, para remoção de compostos indesejáveis e, assim, aumentar a digestibilidade animal (LEKHA; LONSANE, 1997).

2.2.2 Produção de tanase

Os microrganismos são a fonte mais importante de obtenção da tanase, uma vez que as enzimas produzidas desta forma são mais estáveis do que as proteínas análogas obtidas de fontes animais e vegetais e oferecem uma diversificada produção de enzimas em relação à estrutura química, são de cultivo fácil e rápido e apresentam possibilidade de melhoramento genético. Além disso, microrganismos podem produzir tanase em altas quantidades e de maneira contínua, com conseqüente aumento do rendimento pelo estabelecimento de processos fermentativos otimizados (KAR; BANERJEE, 2000; ROCHA, 2010).

A seleção de linhagens de microrganismos produtores de tanase representa um importante passo no processo de produção industrial da enzima. Apesar de vários microrganismos produzirem tanase, estas não são igualmente ativas com todos os taninos hidrolisáveis. Fungos filamentosos, especialmente espécies do gênero *Aspergillus* e *Penicillium*, têm apresentado importância na produção de tanase, com vantagem na produção de enzimas eficientes na degradação de ácido tânico e outros taninos hidrolisáveis que ocorrem na natureza (BATTESTIN; MATSUDA; MACEDO, 2004; BHAT; SINGH; SHARMA, 1998).

Estudos têm sido conduzidos para avaliar o efeito da concentração de ácido tânico ou outras fontes de carbono nos perfis de produção de tanase (AGUILAR et al., 2001b). Embora tanase possa ser produzida por *Aspergillus*

na ausência de ácido tânico, espécies desse gênero podem tolerar altas concentrações deste substrato sem terem efeito deletério sobre o crescimento e a produção da enzima (RAMÍREZ-CORONEL et al., 2003).

A concentração ótima de galotaninos encontrada para *Aspergillus niger* MTCC282, *Aspergillus fischeri* MTCC150, *Fusarium solani* MTCC350 e *Trichoderma viride* MTCC167 foi de 10, 3, 3 e 2%, respectivamente, em um estudo realizado por Bajpai e Patil (1997). Embora *A. fischeri*, *F. solani* e *T. viride* não tolerem altas concentrações de galotaninos no meio, a produção de tanase foi consideravelmente alta quando comparada à encontrada para *A. niger* que tolera concentrações maiores do que 20%. O potencial de galotaninos, metil-galato, ácido gálico e pirogalol para induzir a atividade de tanase também foi estudado e observou-se que a máxima indução da enzima se deu na presença de ácido gálico para *A. fischerii*, galotanino para *F. solani* e metil galato para *T. viride* (BAJPAI; PATIL, 1997). A indução da produção de tanase por *A. fischerii* na presença de ácido gálico diferencia de outros estudos realizados com isolados do mesmo gênero que demonstraram uma repressão na produção da enzima quando ácido gálico, produto final da hidrólise dos galotaninos, foi introduzido no meio de cultura (AGUILAR et al., 2001a; BRADOO; GUPTA; SAXENA, 1997).

A produção de tanase por *Aspergillus japonicus* foi testada em fermentação submersa na presença de diferentes concentrações de glicose como aditivo no meio contendo ácido tânico como indutor e observou-se que uma concentração de 0,2% de glicose foi favorável tanto para o crescimento do microrganismo como para a produção da enzima. Entretanto, acima de 1% de glicose, a produção de tanase foi drasticamente afetada. A tanase foi produzida em fermentação submersa na presença de diferentes carboidratos, peptona e caseína como substratos, porém o microrganismo duplicou a síntese de enzima na presença de ácido tânico no meio de cultivo. A repressão da produção da

enzima pelo ácido gálico, produto final da hidrólise de galotaninos, foi observada quando a enzima foi produzida com glicose como substrato ou ao ser induzida com ácido tânico como fonte de carbono (BRADOO; GUPTA; SAXENA, 1997).

Os padrões de indução e repressão da síntese de tanase por *Aspergillus niger* Aa-20 foram avaliados em substrato sólido e em cultura submersa. Os resultados de cultura com ácido gálico como fonte de carbono mostrou que este composto reprimiu a atividade de tanase em fermentação submersa, com produção menor do que a atividade basal, sugerindo o mecanismo de repressão pelo produto final. O trabalho mostrou uma forte repressão catabólica com a adição de glicose em concentrações maiores do que 20 $g.L^{-1}$. O ácido tânico apresentou taxas de indução em diferentes concentrações, com a linhagem em estudo tolerando altas concentrações desse composto no meio de cultura (AGUILAR et al., 2001a).

A produção de tanase pode ser via fermentação em superfície líquida, fermentação submersa e fermentação em estado sólido (LEKHA; LONSANE, 1997). As fermentações submersas incluem uma variedade de processos microbianos, nos quais o microrganismo é cultivado em meio de cultura líquido, com o substrato dissolvido ou submerso no líquido. Na fermentação em substrato sólido o substrato é insolúvel em água e não se encontra suspenso no líquido (MURTHY; KARANTH; RAO, 1993). A fermentação em superfície líquida envolve o crescimento de microrganismos na superfície do meio de cultura líquido em profundidade rasa e tem sido uma técnica utilizada em anos recentes para a produção de metabólitos microbianos, mas não tem sido explorada para a produção de tanase (LEKHA; LONSANE, 1994).

Na fermentação em substrato sólido as tanases produzidas são predominantemente extracelulares, podendo ser facilmente extraídas com água ou tampão. A localização da tanase durante a fermentação submersa é

dependente do tempo de cultivo, predominando na forma intracelular no início do cultivo e sendo posteriormente secretada (BELMARES et al., 2004). As tanases extracelulares provavelmente sofrem alterações que envolvem a adição de carboidratos na molécula protéica durante a secreção celular. A glicosilação confere estabilidade à estrutura da proteína, incluindo o sítio ativo da enzima e fornece uma barreira protetora contra a inativação por uma série de agentes desnaturantes (RANA; BHAT, 2005). A tanase ligada ao micélio pode ser extraída após a hidrólise da parede celular com enzimas digestivas, tais como quitinase ou podem ser mecanicamente rompidas para recuperar a tanase que se encontra presa ao micélio (BELMARES et al., 2004). Enzimas extracelulares são preferidas, pois dispensam métodos de ruptura da célula, que são dispendiosos (BATTESTIN; MATSUDA; MACEDO, 2004).

O uso da fermentação submersa é vantajoso devido a facilidade de esterilização do meio e do controle do processo neste sistema (MAHAPATRA et al., 2005), o que o torna ideal para o uso em escala industrial. A produção de tanase em cultura submersa de *Aspergillus* sp. foi melhorada em taxas de aeração elevadas, à temperaturas de 30 a 33 °C nos pHs de 3,5 a 6,5. A atividade enzimática máxima foi alcançada após o tempo de 1 a 3 dias de cultivo (BELMARES et al., 2004).

Para identificar as variáveis que influenciam no crescimento celular, na síntese de enzimas e na acumulação de ácido gálico, o efeito da taxa de agitação, concentração e fontes de carbono e de nitrogênio e pH foram avaliados em *Aspergillus awamorii*. A taxa de agitação, concentração da fonte de carbono e o pH afetaram significativamente a atividade da enzima intracelular, o crescimento celular e o acúmulo de ácido gálico. Além disso, foi observado que o aumento da taxa de aeração levou à oxidação de taninos (SETH; CHAND, 2000). A agitação tem efeitos importantes no cultivo de fungos filamentosos, pois garante o fornecimento de nutrientes, principalmente o oxigênio. Além disso, boa

mistura e transferência de calor, exigem um nível mínimo de agitação. Por outro lado, as taxas de agitação elevadas podem resultar em fragmentação e danos às células e às redes miceliais (KELLY et al., 2004).

2.2.3 Purificação de Tanase

Tanase tem sido purificada de diferentes linhagens de fungos filamentosos, como *Aspergillus awamori* (MAHAPATRA et al., 2005), *Aspergillus japonicus* (GUPTA; BRADOO; SAXENA, 1997), *Aspergillus niger* (RAMIREZ-CORONEL et al., 2003; SHARMA; BHAT; DAWRA, 1999), *Aspergillus oryzae* (PARANTHAMAN; VIDYALAKSHMI; SINGARAVADIVEL, 2009; ZHONG et al., 2004), *Penicillium variable* (SHARMA; AGARWAL; SAXENA, 2008) e *Verticillium* sp. (KASIECZKA-BURNECKA et al., 2007). De forma geral, os passos de purificação envolvem uma etapa inicial com a concentração da enzima e da pré-purificação através da ultrafiltração (SHARMA; AGARWAL; SAXENA, 2008; ZHONG et al., 2004) ou da precipitação com sulfato de amônio (KASIECZKA-BURNECKA et al., 2007) ou acetona (MAHAPATRA et al., 2005). Polietilenoglicol (PEG-6000) também tem sido empregado no processo de concentração da enzima (GUPTA; BRADOO; SAXENA, 1997; SHARMA; BHAT; DAWRA, 1999). Em muitos casos, o passo subsequente de purificação envolve uma etapa de aplicação da amostra enzimática em uma coluna cromatográfica de troca iônica (KASIECZKA-BURNECKA et al., 2007; RAMIREZ-CORONEL et al., 2003; SHARMA; BHAT; DAWRA, 1999; ZHONG et al., 2004) que, geralmente, são de troca aniônica, sendo a tanase conhecida como uma proteína ácida. O passo final empregado nos processos de purificação de tanase geralmente é o emprego de colunas de gel filtração (SHARMA; AGARWAL; SAXENA, 2008).

A tanase de *P. variable* foi purificada por Sharma, Agarwal e Saxena (2008) com dois passos de purificação, utilizando um dispositivo de ultrafiltração, com uma membrana de corte de 100 kDa e, posteriormente, uma coluna de gel filtração Sephadex G-200 e obtendo uma purificação de 135 vezes com 91% de recuperação da atividade da tanase. A partir da utilização das colunas cromatográficas de troca iônica (DEAE-Sephadex A-50) e gel filtração (Sephadex G-150) para purificação de tanase de *A. niger*, Sharma, Bhat e Dawra (1999) obtiveram 2,7% de recuperação da enzima e uma purificação de 29 vezes. As tanases TAH I e TAH II de *Verticillium* sp. foram purificadas por precipitação com sulfato de amônio, seguida por aplicação em coluna cromatográfica DEAE-celulose, obtendo as enzimas purificadas 7,9 e 10,5 vezes, respectivamente (KASIECZKA-BURNECKA et al., 2007).

2.2.4 Caracterização Bioquímica de Tanase

Enzimas com a mesma forma de atuação sob um determinado substrato podem apresentar atividade ótima em pH, temperatura e concentração iônica diferentes (ROCHA, 2010). De forma geral, as tanases microbianas apresentam pH estável na faixa de 3,5 a 8,0; com pH ótimo entre 5,5 a 6,0; temperatura de estabilidade entre 30 °C e 60 °C, ótimo na faixa de 30 a 40 °C, ponto isoelétrico de 4,0 a 4,5 e massa molecular entre 180 kDa e 300 kDa. Estas propriedades dependem das condições de cultivo e também da linhagem utilizada. Além disso, a tanase pode ser inibida por Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} e Mg^{2+} e inativada pela ação de *o*-fenantrolina, EDTA, β-mercaptoetanol, tioglicolato de sódio, sulfatos e cloretos de magnésio e cálcio (BATTESTIN; MATSUDA; MACEDO, 2004).

A diversidade enzimática e os altos custos de produção são fatores limitantes à aplicação industrial da tanase (SETH; CHAND, 2000). Nesse

sentido, estudos tem sido conduzidos objetivando a otimização das condições de cultivo de linhagens produtoras de tanase (BELMARES et al., 2004) e o isolamento e a caracterização de enzimas desejáveis para aplicação industrial (MAHAPATRA et al., 2005).

2.2.4.1 Teor de carboidratos e massa molecular

As tanases fúngicas são caracterizadas como glicoproteínas. O conteúdo de carboidratos pode variar de 25,4 a 66,2% do peso total da enzima (LEKHA; LONSANE, 1997).

Duas tanases extracelulares (TAH I e TAH II) produzidas pelo fungo filamentoso *Verticillium* sp. P9 isolado na Antártica foram purificadas e caracterizadas. As enzimas TAH I e TAH II foram caracterizadas como glicoproteínas multiméricas, contendo 11 e 26% de carboidratos respectivamente, e massa molecular de aproximadamente 155 kDa (KASIECZKA-BURNECKA et al., 2007).

2.2.4.2 Temperatura e pH

As propriedades físico-químicas da tanase intracelular produzida via fermentação submersa (FSbm), fermentação em superfície líquida (FSL) e fermentação em substrato sólido (FSS) por *Aspergillus niger* van Tieghem MTCC 2425 foram estudadas e os resultados mostraram que as tanases produzida pelos três sistemas de fermentação apresentaram propriedades semelhantes, exceto que a produzida durante FSS apresentou uma estabilidade mais ampla em pH de 4,5 a 6,5 e a produzida em FSbm maior termoestabilidade de 20°C a 70°C (RANA; BHAT, 2005).

As tanases extracelulares TAH I e TAH II produzidas por *Verticillium* sp. P9 isolado na Antártida apresentaram uma atividade ótima em pH 5,5 e temperatura de 20 e 25ºC, respectivamente. O uso de enzimas com tais características pode ser interessante no processo produtivo de chás gelados, já que baixas temperaturas podem evitar mudanças negativas nas características sensoriais do produto (KASIECZKA-BURNECKA et al., 2007). Por outro lado, tanases com estabilidade térmica em temperaturas mais altas já foram isoladas, como é o caso da enzima de *Aspergillus niger* van Tieghem. A enzima apresentou temperatura ótima de 60 °C e pH ótimo de 6,0 com um segundo pico em pH 4,5 (SHARMA; BHAT; DAWRA, 1999).

Tanase de *Penicillium variable* apresentou temperatura e pH ótimos de 50 °C e 5,0, respectivamente. Entretanto, a temperatura funcional variou de 25 a 80 °C e o pH funcional de 3,0 a 8,0 (SHARMA; AGARWAL; SAXENA, 2008). A tanase de uma linhagem de *Aspergillus niger*, capaz de tolerar até 20% de ácido tânico no meio de crescimento, mostrou atividade em temperaturas de 30 a 90°C, com maior atividade entre 60 e 70 °C e ativa numa faixa de pH entre 3,5 e 7,0 com um ótimo em pH 6,0 (RAMÍREZ-CORONEL et al., 2003).

2.2.4.3 Efeito de íons metálicos e inibidores

Os íons metálicos Mg^{2+} ou Hg^{+} tiveram um efeito positivo na atividade da tanase de *Rhizopus oryzae*, ativando a enzima, enquanto Ba^{2+}, Ca^{2+}, Zn^{2+}, Hg^{2+}, Ag^{+}, Fe^{3+} e Co^{2+} inibiram a atividade da tanase na mesma concentração (1mM). Os ânions Br^{-} e $S_2O_3^{-2}$, na concentração de 1mM, assim como Tween 40 e Tween 80 aumentaram a atividade da tanase, enquanto Tween 60, Lauril sulfato de sódio e Triton X-100 inibiram a atividade. Uréia estimulou a atividade da tanase em uma concentração de 1,5 M. Entre os quelantes escolhidos para o estudo, 1 mM de EDTA ou *O*-fenantrolina inibiu a atividade de tanase. Dimetil

sulfóxido e β-mercaptoetanol inibiram a atividade, enquanto extrato de soja inibiu a atividade em concentrações variando de 0,05% a 1% (m/v). Entre as fontes de nitrogênio selecionadas, sulfato ferroso amoniacal, sulfato de amônio, nitrato e cloreto de amônio favoreceram a atividade enzimática em concentração de 0,1% (m/v). A presença do íon NH_4^+ mostrou os melhores resultados para atividade de tanase. A adição de 0,1% de NH_4Cl à solução de ensaio aumentou em 401% a atividade enzimática (KAR; BANERJEE; BHATTACHARYYA, 2003).

A atividade de tanase de *Penicillium variable* foi aumentada pela presença dos surfactantes colato de sódio e taurocolato de sódio, enquanto SDS, Tween-60 e Tween-80 inativaram completamente a enzima. Em Triton X-100, ela reteve 100% da atividade residual. No entanto, a atividade da tanase na presença desse surfactante não-iônico diminuiu com o tempo. A inibição pode ser resultado do efeito combinado de fatores, tal como redução nas interações hidrofóbicas, que apresentam um papel crucial na manutenção da estrutura terciária, e a interação direta com a molécula de proteína. A tanase de *P. variable* foi inibida por fluoreto de fenilmetilsulfonil (PMSF) e β-mercaptoetanol, retendo 28% e 39,6% da atividade original, respectivamente, o que sugere que pertença à classe das serinas hidrolases. Além disso, *N*-etilmaleimida mostrou forte inibição com somente 7% de atividade residual, enquanto 1, 10-*O*-fenantrolina foi um inibidor moderado (SHARMA; AGARWAL; SAXENA, 2008).

As tanases TAH I e TAH II do fungo *Verticillium* sp. P9 foram ativadas por íons Mg^{2+} e Br^{-} e por uréia em concentrações variando de 0,5 a 2,0 M. As enzimas foram inibidas pelos íons metálicos Zn^{2+}, Cu^{2+}, K^{+}, Cd^{2+}, Ag^{+}, Fe^{3+}, Mn^{2+}, Co^{2+}, Hg^{2+}, Pb^{2+} e Sn^{2+}, pelo ânion CO_3^{2-} e pelos compostos Tween 20, Tween 60, Tween 80, Triton X-100, dodecil sulfato de sódio (SDS), β-

mercaptoetanol, α-glutationa e 4-cloromercuriobenzoato (KASIECZKA-BURNECKA et al., 2007).

Em razão do potencial para aplicação industrial de tanases e as características físicos químicas destas enzimas, este trabalho teve como objetivo produzir, isolar e caracterizar a enzima de *Aspergillus* sp. GM4. O maior conhecimento sobre a diversidade de tanase pode contribuir na busca de enzimas com maior eficiência e aplicação mais direcionada a determinados processos, o que pode levar a uma redução de custos comercial para a aplicação industrial da enzima.

REFERÊNCIAS

ACAMOVIC, T.; BROOKER, J. D. Biochemistry of plant secondary metabolites and their effects in animals. **Proceedings of the Nutrition Society**, Cambridge, v. 64, n. 3, p. 403-412, Apr. 2005.

AGUILAR, C. N. et al. Induction and repression patterns of fungal tannase in solid-state and submerged cultures. **Process Biochemistry**, London, v. 36, n. 6, p. 565-570, Jan. 2001a.

______. Microbial tannases: advances and perspectives. **Applied Microbiology and Biotechnology**, Berlin, v. 76, n. 1, p. 47-59, Jan. 2007.

______. Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid-state fermentation: influence of glucose and tannic acid. **Journal of Industrial Microbiology and Biotechnology**, Hampshire, v. 26, n. 5, p. 296-302, May 2001b.

BAJPAI, B.; PATIL, S. Induction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of fungi imperfecti. **Enzyme and Microbial Technology**, London, v. 20, n. 8, p. 612-614, June 1997.

BATTESTIN, V.; MATSUDA, L. K.; MACEDO, G. A. Source and application of tannin and tannase in foods. **Brazilian Journal of Food and Nutrition**, Araraquara, v. 15, n. 1, p. 63-72, 2004.

BELMARES, R. et al. Microbial production of tannase: an enzyme with potential use in food industry. **Lebensmittel-Wissenschaft und-Technologie**, London, v. 37, n. 8, p. 857-864, Dec. 2004.

BHAT, T. K.; SINGH, B.; SHARMA, O. P. Microbial degradation of tannins: a current perspective. **Biodegradation**, Dordrecht, v. 9, n. 5, p. 343-357, Oct. 1998.

BRADOO, S.; GUPTA, R.; SAXENA, R. K. Parametric optimization and biochemical regulation of extracellular tannase from *Aspergillus japonicus*. **Process Biochemistry**, London, v. 32, n. 1, p. 135-139, Mar. 1997.

GUPTA, R.; BRADOO, S.; SAXENA, R. K. Rapid purification of extracellular tannase using polyethylene glycol-tannic acid complex. **Letters in Applied Microbiology**, Oxford, v. 24, n. 4, p. 253-255, Apr. 1997.

KAR, B.; BANERJEE, R. Biosynthesis of tannin acyl hidrolase from tannin-rich forest residue under different fermentation conditions. **Journal of Industrial Microbiology and Biotechnology**, Hampshire, v. 25, n. 1, p. 29-38, Jan. 2000.

KAR, B.; BANERJEE, R.; BHATTACHARYYA, B. C. Effect of additives on the behavioral properties of tannin acyl hydrolase. **Process Biochemistry**, London, v. 38, n. 9, p. 1395-1401, Sept. 2003.

KASIECZKA-BURNECKA, M. et al. Purification and characterization of two cold-adapted extracellular tannin acyl hydrolases from an Antarctic strain *Verticillium* sp. P9. **Applied Microbiology and Biotechnology**, Berlin, v. 77, n. 1, p. 77-89, Jan. 2007.

KELLY, S. et al. Agitation effects on submerged growth and product formation of *Aspergillus niger*. **Bioprocess and Biosystem Engineering**, New York, v. 26, n. 5, p. 315-323, Oct. 2004.

LEKHA, P. K.; LONSANE, B. K. Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus* niger PKL 104 in solid-state, liquid surface and submerged fermentations. **Process Biochemistry**, London, v. 29, n. 6, p. 497-503, Aug. 1994.

______. Production and application of tannin acyl hydrolase: state of the art. In: NEIDLEMAN, S.; LASKIN, A. (Ed.). **Advances in applied microbiology**. San Diego: Academic, 1997. p. 215-260.

MAHAPATRA, K. et al. Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori nakazawa*. **Process Biochemistry**, London, v. 40, n. 10, p. 3251-3254, Oct. 2005.

MCSWEENEY, C. S. et al. Microbial interactions with tannins: nutritional consequences for ruminants. **Animal Feed Science and Technology**, Amsterdam, v. 91, n. 1, p. 83-93, Feb. 2001.

MUELLER-HARVEY, I. Analysis of hydrolysable tannins. **Animal Feed Science and Technology**, Amsterdam, v. 91, n. 1, p. 3-20, Feb. 2001.

MURTHY, R. M. V.; KARANTH, N. G.; RAO, R. K. S. Biochemical engineering aspects of solid state fermentation. **Advances in Applied Microbiology**, San Diego, v. 38, n. 1, p. 99-149, 1993.

PARANTHAMAN, R.; VIDYALAKSHMI, R.; SINGARAVADIVEL, K. Comparative study on the suitability of different substrates for Tannin Acyl Hydrolase production using *Aspergillus oryzae*. **Journal of Pharmaceutical Sciences and Research**, Chandigarh, v. 4, n. 1, p. 36-42, Apr. 2009.

RAMÍREZ-CORONEL, M. A. et al. A novel tannase from *Aspergillus niger* with β-glucosidase activity. **Microbiology**, New York, v. 149, n. 10, p. 2941-2946, Oct. 2003.

RANA, N. K.; BHAT, T. K. Effect of fermentation system on the production and properties of tannase of *Aspergillus niger* van Tieghem MTCC 2425. **Journal of General and Applied Microbiology**, Tokyo, v. 51, n. 4, p. 203-212, 2005.

REED, J. D. Nutritional toxicology of tannins and related polyphenols in forage legumes. **Journal of Animal Science**, Champaign, v. 73, n. 5, p. 1516-1528, May 1995.

ROCHA, C. P. **Otimização da produção de enzimas por *Aspergillus* niger em fermentação em estado sólido**. 2010. Dissertação (Mestrado em Engenharia Química) - Universidade Federal de Uberlândia, Uberlândia, 2010.

SCHOFIELD, P.; MBUGUA, D. M.; PELL, A. N. Analyses of condensed tannins: a review. **Animal Feed Science and Technology**, Amsterdam, v. 91, n. 1, p. 21-40, Jan. 2001.

SETH, M.; CHAND, S. Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori*: optimisation of process parameters. **Process Biochemistry**, London, v. 36, n. 1, p. 39-44, Feb. 2000.

SHARMA, S.; BHAT, T. K.; DAWRA, R. K. Isolation, purification and properties of tanase from *Aspergillus niger* van Tieghem. **World Journal of Microbiology and Biotechnology**, Oxford, v. 15, n. 6, p. 673-677, Dec. 1999.

SHARMA, S.; AGARWAL, L.; SAXENA, R. K. Purification, immobilization and characterization of tannase from *Penicillium variable*. **Bioresource Technology**, Essex, v. 99, n. 7, p. 2544-2551, May 2008.

ZHONG, X. et al. Secretion, purification and characterization of a recombinant *Aspergillus oryzae* tannase in *Pichia pastoris*. **Protein Expression and Purification**, New York, v. 36, n. 2, p. 165-169, Aug. 2004.

SEGUNDA PARTE – ARTIGOS

ARTIGO 1 PRODUCTION OF TANNASE BY *Aspergillus* sp. GM4 IN SUBMERGED FERMENTATION

Artigo redigido conforme as normas da revista

PRODUCTION OF TANNASE BY *Aspergillus* sp. GM4 IN SUBMERGED FERMENTATION

Abstract

Tannase is a hydrolytic enzyme with production induced in tannic acid presence. It presents biotechnology application in the pharmaceutical, chemical, food and beverage industries. Tannase catalyses the hydrolysis reaction of ester and depside bonds present in hydrolysable tannins releasing gallic acid and glucose. Microorganisms, especially filamentous fungi, present an important role in tannase production. The aims of this work were to find a potential tannase producer and increase the cultivation conditions. In this work, three *Aspergillus* species; *A. japonicus* 246A, *A. tamarii* 3 and *Aspergillus* sp. GM4, had their ability to produce tannase tested in Adams, Czapeck, Khanna, M5 and Vogel culture medium. *Aspergillus japonicus* and *Aspergillus* sp. GM4 were the best producers. *Aspergillus* sp. GM4 and Adams medium were selected. In addition, tannase production was tested in the presence of 1% and 2% tannic acid; 1% green tea; 1% methyl gallate; 1% gallic acid as inducers. The highest induction ratio was in the presence of 2% tannic acid. Gallic acid as sole carbon source also induced the enzyme production, which may be interesting because tannase production by *Aspergillus* sp. GM4 can continue even with the product increase in the culture medium. The Plackett-Burman screening design was performed with six variables. Thus, $MgSO_4$ and agitation rate were selected for the complete factorial. The Central Composite Rotatable Design allowed an increase of 2.66-fold in the enzyme production with small modifications in the medium composition and the selection of a low-cost medium.

Keywords: Tannase, *Aspergillus* sp., tannic acid, inducers, submerged fermentation.

Introduction

Tannase (EC. 3.1.1.20) is a hydrolytic enzyme involved in biodegradation of tannins. It catalyzes the hydrolysis of ester and depside bonds present in hydrolysable tannins releasing gallic acid and glucose (Lekha and Lonsane, 1997). Industrial bioconversion of tannic acid is generally accomplished by the tannase enzyme for the production of gallic acid, which is mostly used in pharmaceutical industry for production of the anti-bacterial drug Trimethoprim (Bajpai and Patil, 1997). It is also used as an important substrate for synthesis of propyl gallate in the food industry as an antioxidant (Lekha and Lonsane, 1997). Tannase has wide applications in the food, beverage, pharmaceuticals and chemical industries, and even in bioremediation (Chávez-González et al., 2012; Sharma et al., 2008). These enzymes are gaining more attention because of their hydrolytic as well as synthetic capability in suitable solvent systems and the complex catalytic property of the tannases has enhanced their commercial importance (Chávez-González et al., 2012). Microorganisms can produce tannase in large quantities and continuously, resulting in increased yield with the establishment of optimized fermentation methods. Tannase is produced in the presence of tannic acid by several filamentous fungi, mainly *Aspergillus* and *Penicillium* species (Bajpai and Patil, 1997). Biodegradation by certain microorganism and enzymes is one of the most efficient ways to degrade large tannin molecules into small molecules with high value bio-activity. Depending on the strain and fermentation conditions, tannase can be produced either constitutively or by substrate induction. Therefore, there are some controversies about the tannase regulation mechanism and the specific role of some compounds in the induction and repression of the tannase expression (Chávez-González et al., 2012). Industrial production of microbial tannase is obtained by submerged culture because it makes the sterilization and process

control easier (Kar and Banerjee, 2000). Studies aiming to understand the cultivation of tannase-producing strains represent an important step to choose strains and enzymes with desirable characteristics for industrial application. Thus, in the present study, an *Aspergillus* specie tannase producer and a suitable medium were selected. In addition, the effects of different inducers were verified and the improved tannase production by *Aspergillus* sp. GM4 in submerged fermentation was investigated using response surface methodology.

Materials and methods

Selection of microorganism and culture medium

Three *Aspergillus* species; *A. japonicus* 246A, *A. tamarii* 3 and *Aspergillus* sp. GM4; belonging to the culture collection of the Laboratory of Prospection and Genetics of Fungi (Department of Biology, Federal University of Lavras), were previously selected due to their ability to produce tannase in tannic acid agar. Fermented media used for tannase production were Khanna (Khanna *et al.*, 1995), Czapeck (Wisemam, 1975), M5 (Peralta *et al.*, 1990), Adams (Adams, 1990) and Vogel (Vogel, 1964), each one containing 2% tannic acid as the main carbon source. Erlenmeyer flasks containing 25 mL of culture medium were inoculated with a spore suspension at 1 x 10^5 spores per milliliter of medium and incubated at 30°C for 72 h on a rotary shaker (100 rpm). Biomass was separated by filtration through Whatman N° 1 paper filter by vacuum and a cell-free culture broth was used to measure extracellular tannase activity.

Effect of incubation time in the tannase production

A spore suspension of *Aspergillus* sp. GM4 (1 x 10^5 conidia per milliliter) was inoculated in modified Adams medium (pH 6.0) with the

composition (gL^{-1}): yeast extract, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; tannic acid, 20.0. Erlenmeyer flasks were incubated at 30°C, at 100 rpm for 24, 48, 72, 96 and 120 hours. After each incubation period, the culture filtrate was analyzed for tannase activity.

Effect of inducers on the tannase activity

The fungus was grown initially for 48 h in the Adams medium containing 2% glucose. Subsequently, the mycelia mass was washed with sterilized distilled water and transferred in aseptic conditions to Adams medium with appropriate inducers (1% and 2% tannic acid; 1% green tea; 1% methyl gallate; 1% gallic acid) and incubated for 48 h more. The mycelial mass was transferred to flasks containing 1% glucose and to a control flask without carbon source. After incubation time, tannase activity was measured and the induction ratio calculated dividing the induced tannase activity by the basal tannase activity from the medium containing glucose as sole carbon source (Aguilar et al., 2001).

Enzyme assay

The tannase activity was estimated by the procedure of Deschamps et al. (1983). To 0.5 mL of the reaction mixture, 0.25 mL of 1% tannic acid (in 0.1 M sodium acetate buffer, pH 5.0) and 0.25 mL of crude filtrate were added. This mixture was incubated at 40 °C for 30 min in water bath and the reaction was stopped by adding 1 mL of bovine serum albumin (BSA) (1 $mg.mL^{-1}$) solution (prepared in 0.17 M NaCl in 0.1 M sodium acetate buffer, pH 5.0). For the control, BSA was added in the reaction mixture before the incubation. The tubes were kept for 20 min at room temperature to precipitate the residual tannins and then centrifuged at 3000*g* for 20 min. The supernatants were diluted in distilled water between 40 and 100-fold, and the absorbance was read at a wavelength

that corresponds to the optimal absorption of gallic acid, 260 nm. The amount of gallic acid produced in the reaction mixture was estimated from the standard curve of gallic acid prepared in the range of 10-100 μg mL^{-1}. One unit of tannase activity was defined as the amount of enzyme required to release one μmol of gallic acid per minute under the standard assay conditions.

Protein estimation

The protein content in the culture filtrate was estimated according to Bradford (1976), with BSA as standard.

Plackett-Burman experimental design

To determine which variables significantly influenced in the tannase production by *Aspergillus* sp. GM4 under submerged fermentation a Plackett-Burman screening design was used with six selected independent variables ($MgSO_4.7H_2O$, KH_2PO_4, yeast extract, tannic acid, agitation rate and salts solution) listed in Table 3. The initial concentration of the salts was ($g.L^{-1}$) $ZnSO_4.7H_20$, 0.0035; $CuSO_4.5H_20$, 0.0031; $MnSO_4.H_20$, 0.0069; $FeSO_4$, 0.0033 and KCl, 0.049. A 1000-fold concentrate solution was prepared and the appropriate dilution was performed to test the original concentration and the double of the concentration. Two levels, the minimum and maximum for each variable and three more repetitions at the central point were studied. Production was valued by inoculating the media with a spore suspension at 1×10^5 spores per milliliter of medium; the medium conditions as suggested by the matrix (Table 3) and incubated for specified agitation rate (80, 100 and 120 rpm), at 30ºC for 72 hours. For each assay, the tannase production was measured and calculated in terms of U/mL. The software STATISTICA 10.0 (Stat Soft. Inc.®, Tulsa – Ok, U.S.A) was used to analyze the experimental data. The variables with significant effect on the tannase production were those with significance

level less than 10% (p-value < 0.1). These variables were selected to conduct a Central Composite Rotatable Design.

Central composite rotatable design

The experiments were based on a Central Composite Rotatable Design (CCRD) with the two variables selected in the PB design. A CCRD with a total of 11 experiments was adopted and included four factorial points and three central points to check the curvature of the experiment region and to provide additional degrees of freedom for error, which are important to test the significance of effects. The independent variables, their levels and real values are presented in Table 4. In addition, to fit a second order model, four axial points (at a distance ±1.41 from the central point) were added at the matrix for this design. The tannase activity was taken as dependent variable (Y). The tannase production data were subjected to analysis of variance (ANOVA) to check the adjustment of the model and to calculate the deviation between predicted and observed values. The proportion of variance explained by the model is given by the coefficient of multiple determination (R^2). The mathematical relationship between the independent and dependent variables was calculated by the second order polynomial equation:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ij} x_i x_j + \Sigma\beta_{ii} x_i^2$$

where Y is predicted value, represented by the enzymatic activity; β_0 is the middle term; β_i is the linear effect; β_{ij} is the interaction effect; β_{ii} is the quadratic effect; X_i and X_j are levels of the independent variables. The quadratic equation is used to form the response surfaces for the variables studied. After this step, the response surface and contour curve were generated to define the optimal operating ranges of each variable involved in the process.

Results and discussion

In the screening, three species of *Aspergillus* genera were cultured in Adams, Czapeck, Khanna, M5 and Vogel culture media. After the incubation period, *Aspergillus japonicus* 246A was the strain with maximum production of tannase in Adams (19.24 $U.mg^{-1}$) and *Aspergillus* sp. GM4 in Adams (12.17 $U.mg^{-1}$ (Table 1). According to assay results, Adams medium was the more favorable for enzyme production for *Aspergillus japonicus* 246A and *Aspergillus* sp. GM4, followed by Khanna and Czapeck. M5 medium was not favorable for the tannase production, which could have been due to composing of the medium with yeast extract and peptone as additional carbon sources. *A. tamarii* 3 presented the lower tannase activity compared to others and maximum production of 3.09 $U.mg^{-1}$ in Czapeck medium. Protein content and tannase production by *Aspergillus japonicus* and *Aspergillus* sp. GM4 were very similar. In addition, many studies have been conducted with *A. japonicus* for tannase production. Therefore, we selected *Aspergillus* sp. GM4 for our next steps.

Table 1 Production of tannase by *Aspergillus* sp. GM4, *A. japonicus* 246A and *A. tamarii* 3 in five different culture medium.

		Units	Adams	Czapeck	Khanna	M5	Vogel
Aspergillus sp. GM4	Tannase activity	U.mL^{-1}	0.202	0.162	0.149	0.051	0.060
	Proteins	mg.mL^{-1}	0.017	0.015	0.015	0.083	0.017
	Specific activity	U.mg^{-1}	12.17	10.64	9.90	0.61	3.44
A. japonicus 246A	Tannase activity	U.mL^{-1}	0.178	0.102	0.146	0.091	0.077
	Proteins	mg.mL^{-1}	0.009	0.012	0.010	0.026	0.009
	Specific activity	U.mg^{-1}	19.24	8.79	14.78	3.52	8.28
A. tamarii 3	Tannase activity	U.mL^{-1}	0.124	0.085	0.177	0.071	0.023
	Proteins	mg.mL^{-1}	0.069	0.028	0.061	0.040	0.014
	Specific activity	U.mg^{-1}	1.79	3.09	2.89	1.80	1.69

The potential of some substrates to induce tannase activity was valued (Table 2). The fungus was initially cultured in Adams medium containing 2% glucose and it presented basal tannase activity (1.29 U.mg^{-1}). The mycelial mass was transferred to Adams medium containing gallic acid, green tea, methyl gallate, tannic acid and glucose to check induction or repression of these carbon sources. Tannase activity increased 3.25-fold when *Aspergillus* sp. GM4 was induced with 2% tannic acid compared to 1%. Methyl gallate presented a good induction ratio, only lower than 2% tannic acid. In the presence of green tea, *Aspergillus* sp. GM4 presented a low tannase activity. After 48h, in the medium containing glucose, no activity was detected suggesting that tannase production by *Aspergillus* sp. GM4 is substrate induced and the production is repressed in the glucose presence. Tannase can be produced either constitutively or by substrate induction, depending of the strain and fermentation conditions. *A. japonicus* produced tannase constitutively in culture medium containing simple or complex sugars, but the production increased with tannic acid as sole carbon

source (Bradoo et al., 1997). In addition, some studies have shown that up to 0.2% glucose concentration may favor the production of tannase, but above 2%, this carbon source has a strong catabolic repression in the enzyme synthesis (Aguilar et al., 2001 b).

Table 2 Specific enzyme activity and induction ratio in the presence of different inducers

Inducer	*Aspergillus* sp. GM4	
	Specific Enzyme Activity $U.mg^{-1}$	Induction ratio
Basal activity	1.29	1.0
1% Gallic acid	5.27	4.1
1% Green tea	1.17	0.9
1% Methyl gallate	8.79	6.8
1% Tannic acid	2.87	2.2
2% Tannic acid	9.32	7.2

Aspergillus sp. GM4 produced tannase in the presence of gallic acid as sole carbon source in the medium. This result was interesting because it suggests that the tannase production by *Aspergillus* sp. GM4 can continue even with the increase of product content in the culture medium, differently from some fungal strains that have presented the tannase production repressed in the acid gallic presence suggesting that the enzyme production in these strains has been regulated by the end-product (Aguilar et al., 2001 b; Bradoo et al., 1997). There are some controversies about the tannase regulation mechanism and the specific role of some compounds in the induction and repression of the tannase expression. The tannic acid molecule is very large and reactive to penetrate the cell membrane of microorganisms. In addition, the gallic acid has been used as inducer for the production of tannase but has also been linked to its regulation. Therefore, it has been generally accepted that tannic acid cannot act directly as

inducer and that the tannase production is induced by intermediary compounds produced during the hydrolysis of tannins (Chávez-González et al., 2012).

Adams medium, containing 2% tannic acid was used to cultivate *Aspergillus* sp. GM4 at 24, 48, 72, 96 and 120 hours. The samples filtrated were measured for tannase activity (Figure 1). The extracellular tannase activity was higher at 72 hours of incubation with decreasing in activity in the following hours. This decrease may be due to the change in pH of the medium and secretion of proteases that can act on secreted tannase. Therefore, 72 hours was fixed as the best incubation time for tannase production from *Aspergillus* sp. GM4.

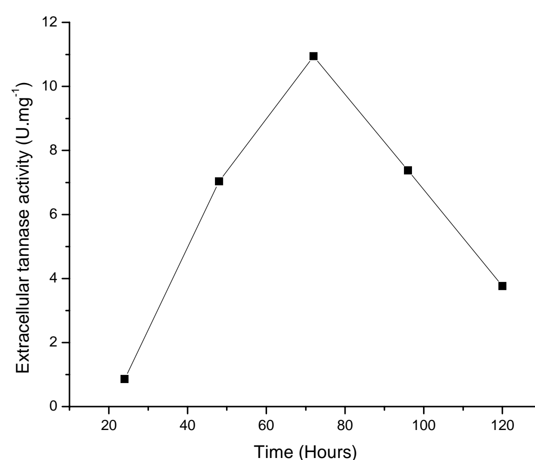


Figure 1 Effect of incubation time in the tannase production

The positive or negative effects of independent variables $MgSO_4.7H_2O$, potassium phosphate monobasic (KH_2PO_4), yeast extract, tannic acid, agitation rate and salt solution were valued on tannase production by Plackett- Burman screening design. Twelve assays in the lower and upper level and three assays in

the central points were performed and tannase activity was the response variable. Table 3 shows the results of the PB design.

Table 3 Plackett-Burman design matrix with six independent variables containing real and coded values and tannase activity as response

Run	$MgSO_4.7H_2O$	KH_2PO_4	Yeast Extract	Tannic acid	Agitation rate	Salt solution	Activity
	g L^{-1}	g L^{-1}	g L^{-1}	g L^{-1}	rpm	mL.L^{-1}	(U.mL^{-1})
1	1 (0.5)	-1 (0)	1 (2.0)	-1 (20)	-1 (80)	-1 (0)	0.439
2	1 (0.5)	1 (1.0)	-1 (0.2)	1 (40)	-1 (80)	-1 (0)	0.268
3	-1 (0)	1 (1.0)	1 (2.0)	-1 (20)	1 (120)	-1 (0)	0.234
4	1 (0.5)	-1 (0)	1 (2.0)	1 (40)	-1 (80)	1 (2.0)	0.434
5	1 (0.5)	1 (1.0)	-1 (0.2)	1 (40)	1 (120)	-1 (0)	0.358
6	1 (0.5)	1 (1.0)	1 (2.0)	-1 (20)	1 (120)	1 (2.0)	0.200
7	-1 (0)	1 (1.0)	1 (2.0)	1 (40)	-1 (80)	1 (2.0)	0.273
8	-1 (0)	-1 (0)	1 (2.0)	1 (40)	1 (120)	-1 (0)	0.146
9	-1 (0)	-1 (0)	-1 (0.2)	1 (40)	1 (120)	1 (2.0)	0.067
10	1 (0.5)	-1 (0)	-1 (0.2)	-1 (20)	1 (120)	1 (2.0)	0.379
11	-1 (0)	1 (1.0)	-1 (0.2)	-1 (20)	-1 (80)	1 (2.0)	0.280
12	-1 (0)	-1 (0)	-1 (0.2)	-1 (20)	-1 (80)	-1 (0)	0.340
13	0 (0.25)	0 (0.5)	0 (1.1)	0 (30)	0 (100)	0 (1.0)	0.194
14	0 (0.25)	0 (0.5)	0 (1.1)	0 (30)	0 (100)	0 (1.0)	0.202
15	0 (0.25)	0 (0.5)	0 (1.1)	0 (30)	0 (100)	0 (1.0)	0.210

Yeast extract, salt solution, KH_2PO_4 and tannic acid showed statistically non significant effect on tannase production. From the data displayed in Figure 2, it was found that the presence of low concentrations of KH_2PO_4, tannic acid and salt solution increased the tannase production but the effect was not significant. Furthermore, the experimental design employed indicated that the enzyme production by *Aspergillus* sp. GM4 is not much affected by yeast extract concentration in the used range (0.2 to 2.0 g.L^{-1}).

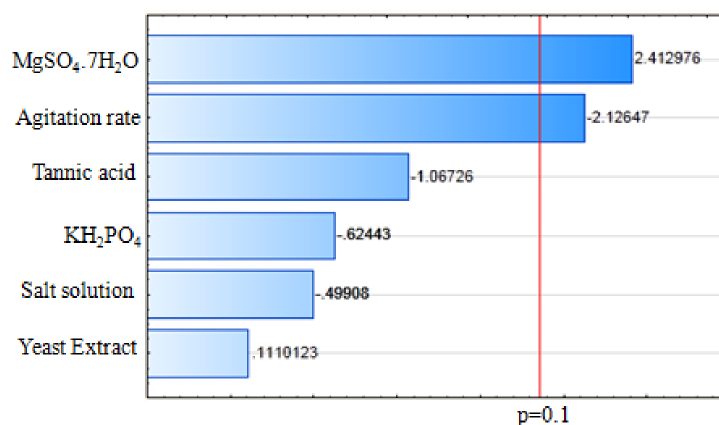


Figure 2 Pareto chart of the effect of variables about tannase production

Only the variables $MgSO_4$ and agitation rate presented significant effect on tannase production and these variables were selected for the CCRD. $MgSO_4$ has shown a positive effect on enzyme production, indicating that the increase of this compound in the culture medium can favor the enzyme production. Mg^{2+} is a well known enzyme cofactor. The supplementation of the culture medium with salts containing magnesium is important for adequate action of magnesium-dependent enzymes which are present in the general metabolic pathways and in the nucleic acid biochemistry. Furthermore, some studies have demonstrated that tannase can have improved activity by the presence of this divalent cation in the reaction medium (Chávez-González et al., 2012).

The agitation rate also has shown significant effect, but negative, which has indicated that a greater production of tannase occurs at lower agitation levels. Agitation has been an important effect in the supply of nutrients, especially oxygen, in filamentous fungi cultivation. Furthermore, good mixing, mass and heat transfer require a threshold level of agitation. On the other hand, high agitation rates lead to high energy dissipation rates connected with high

shear stress, which may result in fragmentation and cell and mycelial network damage (Kelly et al., 2004).

As the salt solution was not part of the initial composition of the culture medium, it was removed from the medium for the next step to minimize the enzyme production costs. From the tannase characterization of *Aspergillus* sp. GM4 (data not shown), it was observed that this tannase activity has been slightly inhibited in the presence of some metal ions (1mM) as K^{+}, Zn^{2+}, Mn^{2+}, and Fe^{3+}, with greater effect by Mn^{2+}. Although the medium supplementation with trace elements can be important for fungal growth, the presence of these metal ions may present negative effect on the secreted enzyme activity. Yeast extract, KH_2PO_4 and tannic acid were fixed. From this point, the medium was modified to the following composition (g.L^{-1}): tannic acid, 20.0; KH_2PO_4, 0.5 and yeast extract, 1.0. The pH was adjusted to 6.0 and the medium was incubated at 30 °C.

$MgSO_4$ and agitation rate were studied. The matrix containing the assays and the predicted and observed for each assay of the CCRD is presented in Table 4. The estimated effect for each variable, as well as the interactions between them, were determined and reported in Table 5. It can be seen that both variables were relevant concerning the tannase production at a 95% confidence level. The correlation analysis has shown that the $MgSO_4$ interaction versus agitation rate and the $MgSO_4$ quadratic term were not statistically significant. The agitation rate quadratic term and both linear terms were shown to be significant, which affected the curvature in the response surface plot. The analyses of the results show that the deviations were low in the desirable range where the activity value presented high. Therefore, in Assay 2 and 6 the deviations were higher, which suggest that the model does not adjust for a low response value.

Table 4 Levels of the two independent variables and Central Composite Rotatable Design of the variables with tannase activity as response

Run	$MgSO_4.7H_2O$	Agitation rate	Activity ($U.mL^{-1}$)		Deviation	Relative
	gL^{-1}	rpm	Experimental	Predicted		Deviation (%)
1	-1 (0.4)	-1 (52)	0.219	0.198	0.021	9.510
2	1 (1.1)	-1 (52)	0.136	0.102	0.034	25.080
3	-1 (0.4)	1 (108)	0.431	0.423	0.008	1.929
4	1 (1.1)	1 (108)	0.394	0.375	0.020	4.983
5	-1.41 (0.25)	0 (80)	0.211	0.223	-0.012	-5.686
6	1.41 (1.25)	0 (80)	0.092	0.122	-0.030	-32.368
7	0 (0.75)	-1.41 (40)	0.169	0.199	-0.030	-17.949
8	0 (0.75)	1.41 (120)	0.539	0.550	-0.011	-2.104
9	0 (0.75)	0 (80)	0.161	0.168	-0.007	-4.306
10	0 (0.75)	0 (80)	0.170	0.168	0.003	1.535
11	0 (0.75)	0 (80)	0.173	0.168	0.005	2.831

Table 5 Effect estimates for tannase activity

Factor	Effect	Std.Err.	t(5)	p	-95% Cnf.Limt	95% Cnf.Limt
Mean	0.168	0.017	10.023	0.0002	0.125	0.211
1 $MgSO_4.7H_2O$ (L)*	-0.072	0.021	-3.511	0.0171	-0.125	-0.019
$MgSO_4.7H_2O$ (Q)	0.005	0.025	0.193	0.8549	-0.058	0.068
2 Agitation rate (L)*	0.249	0.021	12.122	0.0001	0.196	0.302
Agitation rate (Q)*	0.208	0.025	8.495	0.0004	0.145	0.271
1L by 2L	0.023	0.029	0.794	0.4633	-0.052	0.098

* Significant factors (p>0.05)

The Table 6 shows the analysis of variance (ANOVA) for tannase production. The results of ANOVA show good reproducibility of the data observed by very low value of the pure error. The determination coefficient (R^2) explaining 97.9% of the variability of the data and the *F*-test results

demonstrated that the model was adequate to represent the relationship between the response and the variables involved in the tannase production process.

Table 6 Analysis of variance for tannase production as response

Source of variation	Sum of squares	Degrees of Freedom	Mean squares	F test
Regression	48.55045	5	9.7100903	11543.6
Error	0.004206	5	0.0008412	
Total	0.204195	10		

$R^2 = 97.94\%$; $F_{5,5;\,0.05} = 5.05$

The regression analysis of the experimental data obtained after of the ANOVA resulted in the second order polynomial equation below:

Tannase activity (Y) = $+ 0.168 - 0.036*A + 0.002*A^2 +$
$0.124*B + 0.104*B^2 + 0.012*A*B$

where Y is the tannase produced as a function of the coded levels of $MgSO_4.7H_2O$ concentration (A) and agitation rate (B). The equation allows to calculate the predicted values for each assay and the relative deviation between the real and the predicted response. Furthermore, the model generated allows fitting the response surface to find the best range for tannase production. Figure 3 presents the response surface and contour curve with the influence of the variables $MgSO_4$ and agitation rate on enzyme activity.

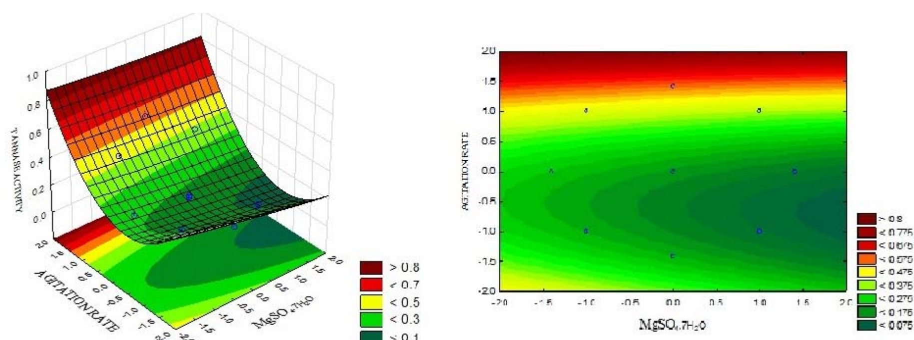


Figure 3 Response surface (a) and contour curve (b) for tannase activity

According to the range of the variables studied, the results have indicated that the optimum conditions for tannase production are at the highest levels of agitation and the variations in the $MgSO_4$ levels have not shown greater influence on the tannase activity. This result was different from that presented in the Plackett-Burman and may be due to less range used in the screening design that could have affected the effects of the variables.

Aspergillus sp. GM4 tannase produced in this simplified low cost medium possessed activity (0.539 $U.mL^{-1}$) representing a 2.66-fold increase compared to that obtained previously, before applying the experimental design. The experimental design allowed to reduce some medium components and a slight increase in the tannase production. Other experiments should be conducted in order to find the greater optimal region for *Aspergillus* sp. GM4 tannase production in submerged fermentation.

Conclusion

The Adams medium favored the production of tannase by *Aspergillus* sp. GM4 and it is a low cost medium when compared with the other four culture media tested. *Aspergillus* sp. GM4 produced tannase in the presence of tannic

acid and methyl gallate inducers. Gallic acid as sole carbon source in the medium also induced the enzyme production, which may be interesting because tannase production by *Aspergillus* sp. GM4 can continue even with the product increase in the culture medium and it was not regulated by the end-product. In addition, the experimental design allowed reducing some nutrients used in the medium composition, and increased 2.66-fold tannase production.

Acknowledgements

The authors sincerely thank the "Fundação de Amparo à Pesquisa do Estado de Minas Gerais" (FAPEMIG, Brazil), CAPES and CNPq for the financial aid that made the development of the present work possible. We also would like to thank Professor Luis Henrique Souza Guimarães from the University of São Paulo (USP, Brazil) for the cooperation and support given during the experiments performed in his laboratory.

References

ADAMS, P. R. Mycelial amylase activities of thermophilic species of Rhizomucor, Humicola and Papulaspora. Mycopathologia, 112: 35-37, 1990.

AGUILAR, C. N.; AUGUR, C.; FAVELA-TORRES, E.; VINIEGRA-GONZÁLEZ, G. Induction and repression patterns of fungal tannase in solid-state and submerged cultures. Process Biochemistry 36: 565–570, 2001.

BAJPAI, B.; PATIL, S. Induction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of fungi imperfecti. Enzyme and Microbial Technology, 20: 612–614, 1997.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254, 1976.

CHÁVEZ-GONZÁLEZ, M.; RODRÍGUEZ-DURÁN, L.V.; BALAGURUSAMY, N.; PRADO-BARRAGÁN, A.; RODRÍGUEZ, R.; CONTRERAS, J. C.; AGUILAR, C. N. Biotechnological Advances and Challenges of Tannase: An Overview. Food and Bioprocess Technology, 5 (2): 445-459, 2012.

DESCHAMPS, A. M., OTUK, G., LEBEAULT, J. M. Productions of tannase and degradation of Chesnutt tannis by bacteria. Journal fermentation technology, 61: 55-59, 1983.

KAR, B.; BANERJEE, R. Biosynthesis of tannin acyl hydrolase from tannin-rich forest residue under different fermentation conditions. Journal of Industrial Microbiology and Biotechnology, 25: 29-38, 2000.

KELLY, S.; GRIMM, L. H.; HENGSTLER, J.; SCHULTHEIS E.; KRULL, R.; HEMPEL, D. C. Agitation effects on submerged growth and product formation of *Aspergillus niger*. Bioprocess and Biosystem Engineering, 26: 315–323, 2004.

KHANNA, P.; SUNDARIL, S. S.; KUMAR, N. J. Production, isolation and partial purification of xylanases from *Aspergillus* sp. World Journal of Microbiology and Biotechnology, 11: 242-243, 1995.

LEKHA P. K., LONSANE B. K. Production and application of tannin acyl hydrolase; state of the art. Advances in Applied Microbiology; 44:215–60, 1997.

PERALTA, R. M.; TERENZI, H. F.; JORGE, J. A. β-D-glycosidase activities of *Humicola grisea*: biochemical and kinetic characterization of a multifunctional enzyme. Biochimica et Biophysica Acta, 1033: 243-249, 1990.

RAMÍREZ-CORONEL, M. A.; VINIEGRA-GONZÁLEZ, G.; DARVILL, A.; AUGUR, C. A novel tannase from *Aspergillus niger* with β-glucosidase activity. Microbiology, 149: 2941–2946, 2003.

SHARMA, S.; AGARWAL, L., SAXENA, R. K. Purification, immobilization and characterization of tannase from *Penicillium variable*. Bioresource Technology, 99: 2544–2551, 2008.

VOGEL, H. F. Distribution of lysine pathways among fungi: evolutionary implications. American Naturalist, 98: 435-446, 1964.

WISEMAN, A. Handbook of enzyme biotechnology. New York: John Wiley & Sons, p. 148, 1975.

ARTIGO 2

PURIFICATION AND CHARACTERIZATION OF TANNASE FROM *Aspergillus* sp. GM4

Artigo redigido conforme as normas da revista

PURIFICATION AND CHARACTERIZATION OF TANNASE FROM *Aspergillus* sp. GM4

Abstract

Tannase catalyzes the hydrolysis reaction of tannic acid, producing gallic acid and glucose. This enzyme has presented importance in the food, beverage, feed, chemical and pharmaceutical industries. The industrial application of tannase depends of the adequate enzyme choice for the process. To know the characteristics of the enzymes it is necessary isolate and characterize them. The objective of this work was to purify tannase from *Aspergillus* sp. GM4 and to characterize the enzyme. The purification of tannase from *Aspergillus* sp. GM4 was performed by filtration using 100 kDa molecular mass cut-off membrane and gel-filtration chromatography using Sephacryl S-200. The enzyme was purified 29-fold with yield of 29% in filtration and 3.5% enzyme yield and 33-fold purification by gel-filtration chromatography. The native molecular mass estimated by gel-filtration chromatography was 162 kDa. *Aspergillus* sp. GM4 tannase presented optimum temperature of activity at 40°C and optimum pH 6.0. The enzyme retained its activity in pH 3.0-8.0 and the thermostability assay showed enzyme activity up to 80 °C, but after 30 min it was inactivated. Tannase was stable in the presence of compounds traditionally reported as inhibitors of the enzyme activity, such as β-Mercaptoethanol, EDTA and different metal ions, such as Pb^{2+} and Ba^{2+}. This tannase has shown different characteristics which may be interesting for industrial application of this enzyme.

Keywords: *Aspergillus.* Tannase. Purification enzyme. Biochemistry characterization.

Introduction

Tannase (E.C.3.1.1.20) catalyses the hydrolysis reaction of ester and depside bonds present in hydrolysable tannins releasing gallic acid and glucose. Tannase is used in the food, feed, beverage, brewing, pharmaceutical and chemical industries (Sharma et al., 2008). This enzyme has potential application in the clarification of beer and fruit juices, manufacture of coffee flavored soft drinks, and flavor improvement of grape wine and as an analytical probe for determining the structure of naturally occurring gallic acid esters (Seth and Chand, 2000). *Aspergillus* species are one of the most important sources of tannase for industrial application, but a large number of other microbes, such as bacteria, yeast and other filamentous fungi, are also exploited for tannase production (Beena, 2010). Tannases reported in the literature vary greatly in molecular mass, ranging from 50 kDa to 300 kDa (Ramirez-Coronel et al., 2003; Kasieczka-Burnecka et al., 2007; Chávez-González et al., 2012). In general, tannases produced by submerged fermentation (SmF) have an isoelectric point around 4.0; an optimum temperature between 30 and 40°C and their optimum pH is between 5.0 and 7.0 (Ramirez-Coronel et al., 2003). The high production cost is a limiting factor in the industrial application of tannase (Seth and Chand, 2000). Thus, the realization of studies aimed at optimization of culture conditions for tannase-producing strains and the isolation and characterization of enzymes desirable for industrial application is important. For tannase purification, common protocols of two or three steps include fractional precipitation (with salts, organic solvents, tannic acid, or pH), ion exchange or gel permeation chromatography. The enzyme purification is performed to increase its catalytic power, improve stability or prevent unwanted reactions (Chávez-González et al., 2012). In the present study, tannase from *Aspergillus* sp. GM4 was purified and characterized. The pre-purified preparation was

characterized biochemically, having its molecular mass, optimum temperature and pH determined. In addition, assays were performed to evaluate the effects of some metal ions, chelator and inhibitor in tannase activity and we have found an enzyme containing interesting characteristics for industrial application.

Materials and methods

Growth and maintenance of Aspergillus sp. *GM4*

Aspergillus sp. GM4 belonging to the culture collection of the Laboratory of Prospection and Genetics of Fungi (Department of Biology, Federal University of Lavras), was grown on potato dextrose agar (PDA) slanted at 30 ºC for seven days, the cultures maintained at 4 ºC.

Enzyme production

The spore suspension was obtained from a 7-day-old culture. Approximately, 1 x 10^5 spores per milliliter of medium were inoculated in 1000 mL of sterilized Adams medium (Adams, 1990) (pH 6.0) with the composition (gL^{-1}): yeast extract, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; tannic acid, 20.0. Erlenmeyer flasks were incubated at 30°C, at 100 rpm for 72 hours. After the incubation period, the biomass was separated by filtration and the filtrate was assayed for tannase activity.

Enzyme assay

The tannase activity was estimated by procedure of Deschamps et al. (1983). To 0.5 mL of the reaction mixture, 0.25 mL of 1% tannic acid (in 0.1 M sodium acetate buffer, pH 5.0) and 0.25 mL of culture filtrate were added. This mixture was incubated at 40 °C for 30 min in water bath and the reaction was terminated by adding 1 mL of bovine serum albumin (BSA) solution (1 $mg.mL^{-1}$). For the control, BSA was added in the reaction mixture prior to incubation.

The tubes were left for 20 min at room temperature to precipitate the residual tannins and then centrifuged at 3000 *g* for 20 min. The supernatant was suitably diluted in distilled water between 50 and 100-fold, and the absorbance was read at wavelength of 260 nm that corresponds to the optimal absorption of gallic acid. The amount of gallic acid produced in the reaction mixture was estimated from the standard curve of gallic acid prepared in the range of 2-20 and 10-100 $\mu g\ mL^{-1}$. One unit of tannase is defined as the amount of enzyme required to release one µmol of gallic acid per minute under the standard assay conditions.

Protein contents

The protein content in the culture filtrate was estimated according to Bradford (1976), using BSA as standard.

Enzyme purification procedure

Filtration

The cell-free culture broth (1000 mL) containing tannase was filtered using an Amicon RA 2000 apparatus with a 100 kDa molecular mass cut-off membrane. The samples retained and the filtrate collected after this step were analyzed for tannase activity and total protein contents.

Gel-filtration chromatography

The volume retained after filtration (50 mL) was concentrated by lyophilization and resuspended to a final volume of 2 mL. The sample was then loaded in the Sephacryl S-200 column with a bed size of 1.0 x 80.0 cm. The column was equilibrated with 0.05 M sodium acetate buffer, pH 5.0 added with 0.05 M NaCl and was eluted with the same buffer with 0.05 M NaCl at the flow rate the 1.4 $mL\ min^{-1}$. Three hundred fractions of 1.4 milliliters each were collected in a fraction collector (Pharmacia LKB – FRAC 100, USA). The

protein content in the fractions collected was estimated at 280 nm using a spectrophotometer (Shimadzu UV mini – 1240, Tokyo, Japan). The tannase activity was estimated using the Deschamps et al. (1983) procedure. The fractions corresponding to higher tannase activity were pooled, dialyzed, concentrated by lyophilization and then evaluated for purity in gel.

Electrophoretic procedure

The purification process was monitored by electrophoresis in polyacrylamide gel in denaturing conditions (10% SDS-PAGE) (Laemmli, 1970), using a power source set to 120V and 40 mA. After the electrophoretic running, the gel was stained with Coomassie Brilliant Blue – R250. The molecular mass markers used were αZ-macroglobulin (168 kDa), β-galactosidase (112 kDa), lactoferrin (91 kDa), pyruvate kinase (67 kDa), lactic dehydrigenase (36 kDa) and triosephosphate isomerase (31 kDa).

Tannase biochemical characterization

To determine the stability and optimum pH and temperature; and the effect of metal ions, β-mercaptoethanol and EDTA, the pre-purified enzyme was used.

Molecular mass of native tannase

The molecular mass of native tannase was determined by gel-filtration chromatography using Sephacryl S-200 with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) as molecular mass markers. The mixture of high non-denaturing molecular mass markers was loaded on Sephacryl S-200 and eluted with 0.05 M Tris-HCl buffer (pH 7.5) at a flow rate of 1.4 mL min^{-1}. The void volume (V_o) of 103.74 mL was determined using Blue Dextran 2000. The

sample containing proteins was collected at their elution volume (V_e) and the graph of $V_e V_o^{-1}$ against log of molecular mass (M_w) was plotted for the estimation of native molecular mass of tannase.

Optimum temperature and pH for tannase activity

To determine the optimum temperature for tannase activity, the reaction mixture was incubated at different temperatures ranging from 30°C to 80°C. The optimum pH was determined by diluting the substrate of the enzymatic reaction in 100 mM citrate-phosphate buffer, pH 3.0 to 8.0. The enzymatic activity was measured.

Temperature stability

The thermostability was determined by incubating aliquots of tannase at temperatures of 40 °C to 80 °C at intervals from 0 to 60 minutes. After each interval, the enzyme aliquots were removed and kept in an ice bath for the determination of tannase activity.

pH stability

The pH stability of tannase was observed by incubating the enzyme in an ice bath, in 100 mM citrate-phosphate buffer with a pH from 3.0 to 8.0. The enzyme and buffer were added with 1:1 (v/v) and after incubation, aliquots were removed at different time intervals and the relative enzyme activity was determined.

Effect of metal ions, chelator and inhibitor on tannase activity

The effect of different salts (Table 2), β-mercaptoethanol and EDTA on the tannase activity was investigated. The compounds were used at final

concentrations of 1mM in the reaction medium for tannase activity determination.

Results and discussion

The purification of tannase from *Aspergillus* sp. GM4 was carried out in two-steps. In the first, the cell-free culture broth containing the enzyme was concentrated by filtration using a 100 kDa molecular mass cut-off membrane, resulting in 29% enzyme yield and 29-fold purification. After this, the concentrated enzyme was purified by gel-filtration chromatography using Sephacryl S-200, which resulted in 3.5% enzyme yield and 33-fold purification (Table 1). The similar results found in purification terms in the first and second step, added to loss in yield suggest that the utilization of pre-purified enzyme may be more interesting in terms of yield, without major losses in purification. Some studies have found, in the second purification step, values less than or equal to those found in our first step (Sharma et al., 1999; Kasieczka-Burnecka et al., 2007). The elution profile of tannase in Sephacryl S-200 is represented in Figure 1. The *Aspergillus* sp. GM4 tannase on SDS-PAGE showed a singular band in the gel (Figure 2) and the native molecular mass of this enzyme estimated by gel-filtration chromatography was 162 kDa. Fungal tannases are generally glycoprotein and the carbohydrate content is relatively high and contributes to its high molecular mass. It is believed that the carbohydrate content of tannase may protect the core-protein from the denaturing action of hydrolysable tannins and also to direct the substrate to the active-site region, where the cleavage of tannin molecules occurs into their respective components, gallic acid and glucose (Lekha and Lonsane, 1997).

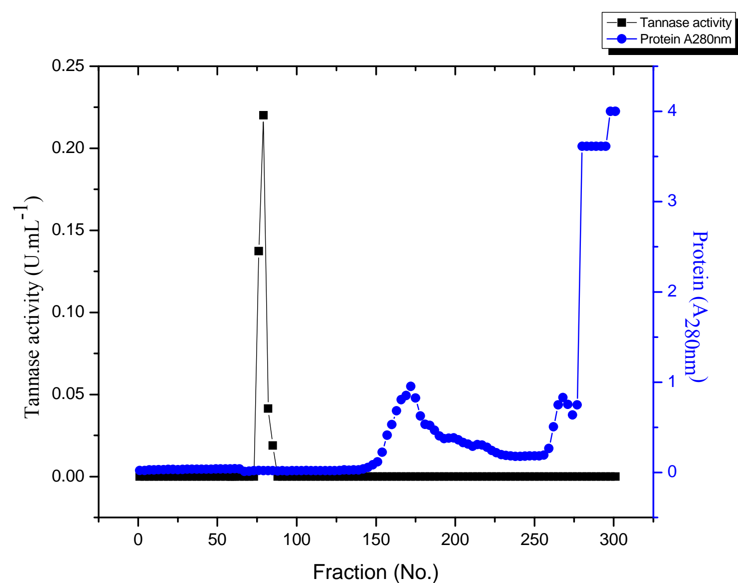


Figure 1 Elution profile of protein and activity of tannase from Sephacryl S-200 column

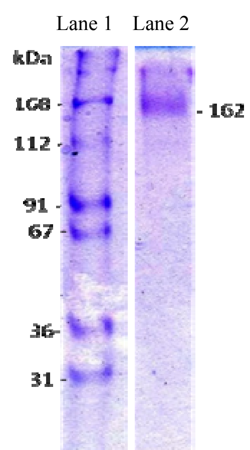
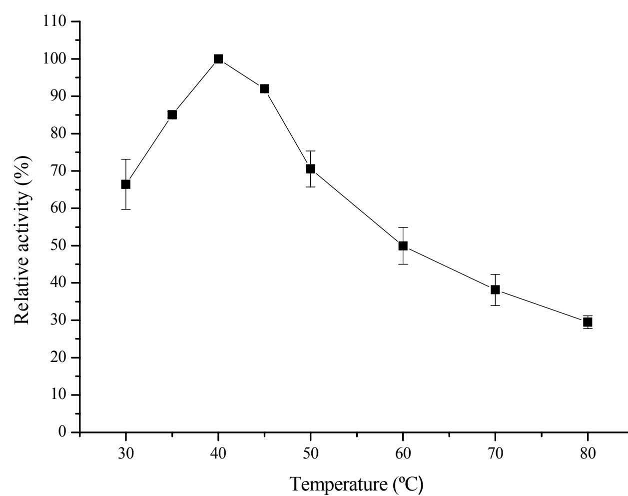


Figure 2 Analysis of Sephacryl S-200-purified tannase by SDS-PAGE (lane 2) and molecular markers (lane 1), Coomassie Brilliant Blue – R250 stained

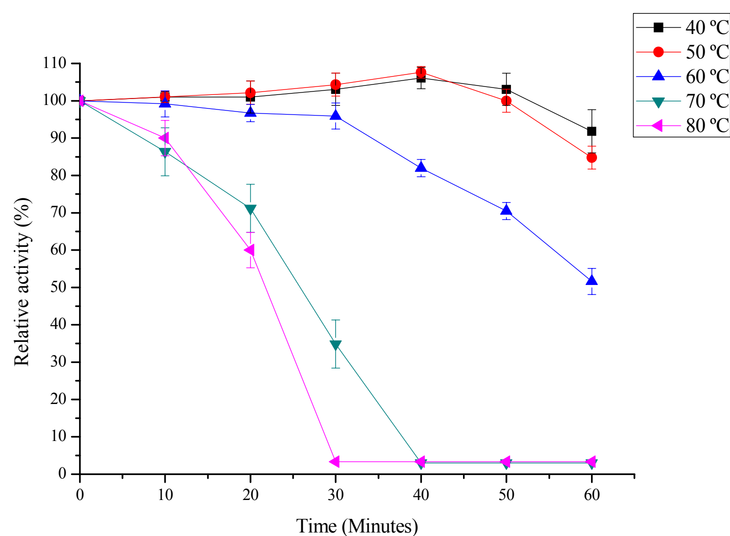
Table 1 Purification of tannase produced by *Aspergillus* sp. GM4

Steps	Volume (mL)	Total tannase (U)	Total protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold purification
Culture Filtrate	1000	112.9	56.52	2.00	100	1
Filtrate 100 kDa	50	32.6	0.57	57.2	29	29
Gel-filtration chromatography	13	3.9	0.06	65	3.5	33

The optimum temperature for tannase activity of *Aspergillus* sp. GM4 was 40 °C (Figure 3a). This enzyme showed activity up to 80 °C, but the thermostability assays showed that after 40 and 30 min incubated in the temperatures of 70 and 80°C, respectively, the relative activity declined drastically and the enzyme lost its activity (Figure 3b). After 60 min of incubation, tannase retained 50% of its activity at 60 °C and it was stable at 40 and 50 °C, losing only 10% of its activity when incubated at these temperatures. The thermostability may be an interesting characteristic in the application of tannase in heat-treated processes and products.



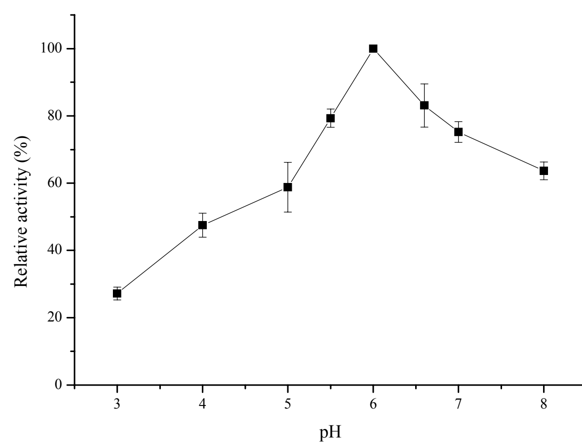
(a)



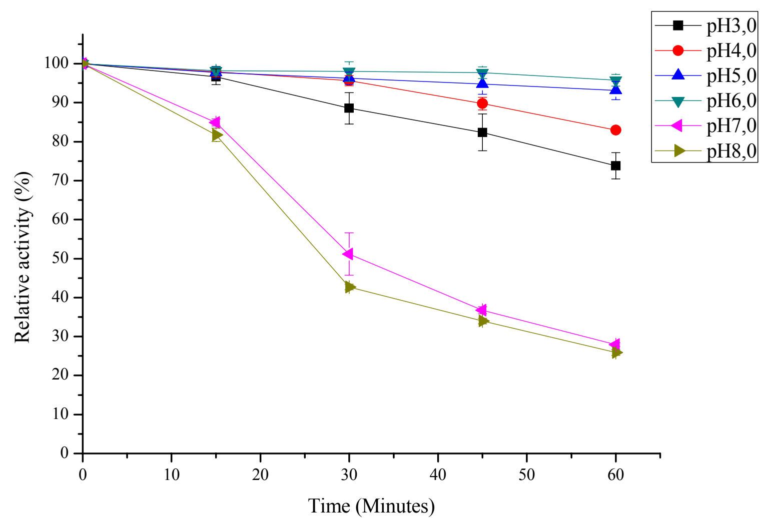
(b)

Figure 3 Optimum temperature for tannase activity (a) and temperature stability of *Aspergillus* sp. GM4 tannase (b)

The tannase was optimally active at pH 6.0 (Figure 4a) and the range with higher activity was between 5.5 and 6.5. The pH stability was tested in the pH range of 3.0-8.0. Although tannase has showed considerable relative activity in pH 7.0 and 8.0, it retained only 30% of the relative activity with 60 min incubation. However in acid pH this enzyme showed stability, losing only 25 and 15% of the relative activity after 60 min incubation in pH 3.0 and 4.0, respectively (Figure 4b). In pH 5.0 and 6.0 the tannase retained approximately 100% activity, presenting high stability in this range. Tannase has been reported to be an acidic protein with an optimum pH around 5.5 (Lekha and Lonsane, 1997). The presence of tannins in the medium causes the pH decreases; therefore the stability of tannase at low pH is a desirable characteristic for enzyme application. Furthermore, the enzyme activity at acidic pH has potential for various industrial applications such as tea cream solubilisation (Beena, 2010).



(a)



(b)

Figure 4 Optimum pH for tannase activity (a) and pH stability of tannase from *Aspergillus* sp. GM4 (b)

Table 2 Effect of diverse compounds on *Aspergillus* sp. GM4 tannase activity.

Addition (1mM)	Relative activity (%)	Addition (1mM)	Relative activity (%)
Control *(no addition)*	100 ± 0.0	*Metal ions (continued)*	
Metal ions		$ZnCl_2$	76.8 ± 5.77
NaCl	77.9 ± 0.99	CoCl	96.2 ± 7.47
$AgNO_3$	94.1 ± 4.10	$Pb(C_2H_3O_2)_2 . 3H_2O$	204.8 ± 0.43
$CaCl_2$	81.3 ± 5.67	$FeCl_3$	88.4 ± 0.28
$MgCl_2$	128.8 ± 6.41	$HgCl_2$	84.8 ± 6.54
$MnCl_2$	52.5 ± 7.86	$BaCl_2$	119.8 ± 0.95
KCl	98.5 ± 3.14	*Chelator*	
NH_4Cl	95.5 ± 0.96	EDTA	104.4 ± 4.13
$AlCl_3.6H_20$	105.7 ± 6.98	*Inhibitor*	
CuCl	120.5 ± 5.37	β-Mercaptoethanol	119.4 ± 3.58

The presence of metal ions has been required by many enzymes to express and increase their catalytic activity. Tannase activity from *Aspergillus* sp. GM4 was stimulated in the presence of the ions Mg^{2+}, Cu^{+}, Ba^{2+} and Pb^{2+} (Table 2). Different from another study in literature in which tannase activity of *Verticillium* sp. P9 was inhibited by Pb^{2+} (Kasieczka-Burnecka et al., 2007), tannase of *Aspergillus* sp. GM4 had activity stimulated in its presence. This ion presented greater induction effect on tannase activity compared with the others. The action of Pb^{2+}, enhancing tannase activity, has not been reported. Generally divalent cations, that enhance the enzyme activity, may act interacting with the enzyme catalytic site and alter its structure or by interaction with the substrate. The manner that Pb^{2+} acts on tannase activity is not known, but tannase from *Aspergillus* sp. GM4 has demonstrated this particularity in relation to other enzymes. From the metal ions studied, Na^{+}, K^{+}, Zn^{2+}, Ca^{2+}, Mn^{2+} and Hg^{2+} inhibited the tannase activity, with greater effect by Mn^{2+}. A study about the effect of metal ions on tannase activity suggested that Ag^{+}, Zn^{2+} and Hg^{2+}

present a type of competitive inhibition upon the enzyme (Kar et al., 2003). Ag^{+}, K^{+}, NH_4^{+}, Al^{3+}, Co^{+} and Fe^{3+} unchanged the tannase activity.

β-Mercaptoethanol has been reported as a tannase inhibitor (Kasieczka-Burnecka et al., 2007; Sharma et al., 2008), but it did not presented inhibitory effect on the tannase activity of *Aspergillus* sp. GM4. The inhibitory effect of β-Mercaptoethanol indicates the presence of a serine residue in the catalytic site, suggesting that tannase is a serine hydrolase which needs this amino acid residue for its catalytic function (Beena, 2010). Differently, the results of this study have showed that this compound did not inhibit the tannase activity and its presence has slightly stimulated the enzyme activity, indicating that the tannase of *Aspergillus* sp. GM4 presents different characteristics and stability if compared to others reported in the literature. Similarly, in the presence of the EDTA chelator, enzymatic activity was not inhibited. EDTA is a potent inhibitor of metal-dependent enzymes, which is generally used commercially as a protease inhibitor, where it acts as chelator of metals like lead and zinc. An enzyme which is not affected by action of this compound may be interesting for industrial application.

Conclusion

Tannase produced by *Aspergillus* sp. GM4 was purified in two purification steps using filtration and a gel filtration column. The first step of purification provided good results in terms of purification and yield. It presented molecular mass of 162 kDa. *Aspergillus* sp. GM4 tannase retained activity in pH 3.0-8.0, but its optimum pH was 6.0. The optimum temperature for tannase activity was 40°C. The enzyme presented activity up to 80 °C, but after 30 min it was inactivated. *Aspergillus* sp. GM4 showed stability in the presence of different compounds traditionally reported as inhibitors of the enzyme activity at

the concentration tested, such as β-Mercaptoethanol and EDTA and different metal ions. The different characteristics of tannase of *Aspergillus* sp. GM4 in terms of pH, temperature and additive stability, may be interesting and a potential for industrial application of the enzyme.

Acknowledgements

The authors sincerely thank the “Fundação de Amparo à Pesquisa do Estado de Minas Gerais” (FAPEMIG, Brazil), CAPES and CNPq for the financial aid that made the development of the present work possible. We also would like to thank Professor Luis Henrique Souza Guimarães from the University of São Paulo (USP, Brazil) for the cooperation and support given during the experiments performed in his laboratory.

References

ADAMS, P. R. Mycelial amylase activities of thermophilic species of *Rhizomucor*, *Humicola* and *Papulaspora*. Mycopatology, 112: 35-37, 1990.

BEENA, P.S. Production, purification, genetic characterization and application studies of tannase enzyme from marine fungus *Aspergillus awamori*. Thesis - Cochin University of Science and Technology. Doctor of Philosophy and Biotechnology, 2010.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochemistry, 72:248-254, 1976.

CHÁVEZ-GONZÁLEZ, M.; RODRÍGUEZ-DURÁN, L.V.; BALAGURUSAMY, N.; PRADO-BARRAGÁN, A.; RODRÍGUEZ, R.; CONTRERAS, J. C.; AGUILAR, C. N. Biotechnological Advances and Challenges of Tannase: An Overview. Food and Bioprocess Technology, 5 (2): 445-459, 2012.

DESCHAMPS, A. M., OTUK, G., LEBEAULT, J. M. Productions of tannase and degradation of Chesnutt tannins by bacteria. Journal fermentation technology, 61: 55-59. 1983.

DUBOIS, M.; GILLES, K. A.; HAMILTON, J. K.; REBERS, P. A.; SMITH, F. Colorimetric method for determination of sugars and related substances. Analytic Chemistry, 28: 350-356, 1956.

KAR, B.; BANERJEE, R. Biosynthesis of tannin acyl hydrolase from tannin-rich forest residue under different fermentation conditions. Journal of Industrial and Biotechnology, 25: 29-38, 2000.

LAEMMLI, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685, 1970.

MAHAPATRA K.; NANDA R. K.; BAG S. S.; BANERJEE R.; PANDEY A.; SZAKACS G. Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori nakazawa*. Process Biochemistry 40: 3251–3254, 2005.

RAMÍREZ-CORONEL, M. A.; VINIEGRA-GONZÁLEZ, G.; DARVILL, A.; AUGUR, C. A novel tannase from *Aspergillus niger* with β-glucosidase activity. Microbiology, 149: 2941–2946, 2003.

SETH M.; CHAND, S. Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori* — optimization of process parameters. Process Biochemistry, 36: 39–44, 2000.

SHARMA, S.; AGARWAL, L., SAXENA, R. K. Purification, immobilization and characterization of tannase from *Penicillium variable*. Bioresource Technology, 99: 2544–2551, 2008.

SHARMA, S.; BHAT, T. K.; DAWRA, R. K. Isolation, purification and properties of tannase from *Aspergillus niger* van Tieghem. World Journal of Microbiology and Biotechnology, 15: 673-677, 1999.