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ANDERSON JOSÉ PAULO

PRODUÇÃO, CARACTERIZAÇÃO E PURIFICAÇÃO PARCIAL DE UMA NOVA ß-GALACTOSIDASE PRODUZIDA POR *Cladosporium tenuissimum* URM 7803

Recife

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas do Centro de Biociências da Universidade Federal de Pernambuco como parte dos requisitos parciais para obtenção do título de doutor em Ciências Biológicas.

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Orientadora: Profª Drª Ana Lúcia Figueiredo Porto

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Aprovada em: 28/02/2018

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Ao Universo e aos meus Pais, com Amor,

Dedico

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RESUMO

A β-galactosidase (EC3.2.1.23) é classificada como uma hidrolase, e realiza reações de catalisação a partir do resíduo terminal β-galactopiranosil da lactose (Galβ1-4Glc) para formar glicose e galactose. Esta enzima se destaca na indústria de alimentos e farmacêutica, sendo utilizada para hidrólise da lactose e produção de produtos destinados a intolerantes à lactose, além de ser usada na síntese de galacto-oligossacarídeos (GOS). Neste sentido, este trabalho objetiva a produção, caracterização e purificação parcial de uma nova β-galactosidase produzida por um isolado da espécie *Cladosporium tenuissimum* URM 7803. O sequenciamento genético do fator de alongamento e tradução (TEF-1) apresentou 88% de suporte para a espécie *C. tenuissimum* URM 7803 dentro do complexo de espécies *C. cladosporioides*. Após confirmação da espécie, a produção de β-galactosidase por *C. tenuissimum* URM 7803 foi realizada utilizando fermentação líquida conduzida a 28 °C, pH 6.5 e 180 rpm por 312h. Um sistema de duas fases aquosas composto por polietilenoglicol-citrato (SDFA–PEG-Citrato) foi feito por planejamento fatorial $2^{4,}$ utilizando como variáveis a massa molar do PEG (400, 3350 e 8000); concentração do PEG (g/L) (20, 22 e 24); concentração de citrato (g/L) (15, 17,5 e 20) e pH (6, 7 e 8). Os resultados mostraram que a maior produção volumétrica de β-galactosidase (462,13 U/mL) ocorreu em 264h. No SDFA-PEG/Citrato o ensaio 2 (MM-PEG 8000, %PEG 20, %citrato 15 e pH 6,0) apresentou um valor significativo de fator de purificação (*FP*) de 12,82, e a produção da enzima nessas condições foi selecionada para a etapa de caracterização enzimática. A caracterização do extrato bruto e do extrato parcialmente purificado por SDFA, demonstrou respectivamente temperatura e pH ótimos de 35-60º C e 3,0-4,5 para o extrato bruto e, 40-60º C e 3,0-4,5 para o extrato parcialmente purificado. O íon Fe^{2+} aumentou em 38% a atividade enzimática do extrato parcialmente purificado, e os íons Zn^{2+}, Cu^{2+} e Co^{2+} reduziram em média, 25% da atividade. Em relação ao efeito dos inibidores e detergentes a atividade enzimática foi aumentada ou mantida, exceto para o Fenilmetilsulfonilflúor (PMSF) que reduziu em 20 e 12% a atividade no extrato bruto e parcialmente purificado, respectivamente. Pode-se concluir que a nova β-galactosidase produzida por C. *tenuissimum* URM 7803 foi parcialmente purificada pelo SDFA PEG-Citrato, sendo o primeiro estudo utilizando este tipo de sistema para purificação de β-galactosidase, além de ter apresentado boa atividade enzimática durante sua caracterização físico-química, sendo necessário novos estudos para aumentar o grau de pureza.

Palavras-chave: β-galactosidase. *Cladosporium tenuissimum*. SDFA. purificação. caracterização.

ABSTRACT

β-galactosidase (EC3.2.1.23), is classified as a hydrolase, and performs catalytic reactions from the β-galactopyranosyl terminal residue of lactose (Galβ1-4Glc) to form glucose and galactose. This enzyme excels in the food and pharmaceutical industry, being used for hydrolysis of lactose and production of products destined to lactose intolerant, besides being used in the synthesis of galacto-oligosaccharides (GOS). In this sense, this work aims at the production, characterization and partial purification of a new β-galactosidase produced by an isolate of the species *Cladosporium tenuissimum* URM 7803. The genetic sequencing of the elongation and translation factor (TEF-1) presented 88% support for the species *C. tenuissimum* URM 7803 within the *C. cladosporioides* species complex. After confirmation of the species, β-galactosidase production by *C. tenuissimum* URM 7803 was performed using liquid fermentation conducted at 28º C, pH 6.5 and 180 rpm for 312 h. A two-phase aqueous system composed of polyethylene glycol-citrate (SDFA-PEG-Citrate) was done by factorial design 24, using as variables the PEG molar mass (400, 3350 and 8000); concentration of PEG (g / L) (20, 22 and 24); concentration of citrate (g / L) (15, 17,5 and 20) and pH (6, 7 and 8). The results showed that the highest volumetric production of β-galactosidase (462.13 U / mL) occurred in 264h. In SDFA-PEG/Citrate assay 2 (MM-PEG 8000, %PEG 20, %citrato 15 e pH 6,0) presented a purification factor (PF) of 12.82, and the production of the enzyme under these conditions was selected for the enzymatic characterization step. The characterization of the crude extract extract and partially purified by SDFA showed respectively optimum temperature and pH of 35-60 ° C and 3,0-4,5 for crude extract and 40-60 ° C and 3,0-4,5 for the partially purified extract. The Fe2 + ion increased the enzymatic activity of the partially purified extract by 38%, and the Zn2 +, Cu2 + and Co2 + ions reduced, on average, 25% of the activity. Regarding the effect of the inhibitors and detergents the enzymatic activity was increased or maintained, except for Phenylmethylsulfonylfluoride (PMSF), which reduced the activity in the crude and partially purified extract by 20 and 12%, respectively. It can be concluded that the new β-galactosidase produced by *C. tenuissimum* URM 7803 was partially purified by SDFA PEG-Citrate, being the first study using this type of system for purification of β-galactosidase, besides showing good enzymatic activity during its physico-chemical characterization, being necessary new studies to increase the degree of purity.

Keywords: β-galactosidase. *Cladosporium tenuissimum.* ATPS. purification. characterization.

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1 INTRODUÇÃO

As enzimas microbianas têm elevada importância no cenário biotecnológico, sendo as fontes mais empregadas para obtenção de uma série de produtos destinados a diversos setores industriais e comerciais. Um exemplo importante é a β-galactosidase ou lactase (EC 3.2.1.23; β-D-galactosil galactohidrolase) uma enzima responsável pela reação de catalisação na hidrólise das ligações β-1,4-D-galactosídicas da lactose, um carboidrato dissacarídeo encontrado no leite. O emprego inicial desta enzima teve destaque na área clínica, visto que é a enzima utilizada para hidrolisar a lactose, o que beneficia o grupo populacional que sofre de intolerância a este carboidrato. Entretanto, outras aplicações importantes surgiram para β-galactosidase, sendo uma dessas a ligada a reação alternativa de transgalactosilação, o que permite a produção galacto-oligossacarídeos (GOS), importantes prebióticos amplamente utilizados na indústria de alimentos funcionais e que desempenham efeitos pleiotrópicos no organismo (ASHOK, et al., 2016; LATORRE et al., 2014; HUSAIN, 2010).

Boa parte das enzimas comercializadas, dentre elas a β-galactosidase, são oriundas de fontes microbianas, destacando-se entre elas os fungos filamentosos. O gênero *Cladosporium* spp. se destaca entre os vários táxons, sendo considerado um dos fungos mais cosmopolitas e de maior concentração na atmosfera, em especial nas regiões temperadas (ZOPPAS et al., 2011). O gênero *Cladosporium* spp. foi criado por Link, em 1816, sendo um dos maiores e mais heterogêneos gêneros de Hyphomycetes (DUGAN et al., 2004). Este gênero compreende mais de 189 espécies, estando entre os fungos que são mais frequentemente encontrados em ambientes externos e internos, matéria orgânica estragada e, são considerados contaminantes importantes para os alimentos (BENSCH et al., 2012). Algumas espécies são conhecidas por serem patógenos de plantas, causando manchas foliares e outras lesões, ou parasitando outros fungos. As espécies de *Cladosporium* spp. são também conhecidos por serem endófitos comuns (REVANKAR e SUTTON, 2010). As espécies mais isoladas são *C. sphaerospermum*, *C. cladosporioides*, *C. herbarum* e *C. elatum.*

Os fungos deste gênero ainda são pouco explorados biotecnlogicamente, entretanto muitas espécies de *Cladosporium* também são capazes de produzir alguns metabólitos secundários, como antibióticos inibidores de *Bacillus subtilis*, *Escherichia coli* e *Candida albicans* (GALLO et al., 2004). Além disso, algumas espécies de *Cladosporium* são inseticidas biológicos eficientes, particularmente contra insetos que desenvolveram resistência a inseticidas químicos (ABDELBAKY e ABDEL-SALAM, 2003).

Uma das etapas mais importantes no processo de produção de enzimas, seja em escala laboratorial ou industrial, é voltada aos processos de purificação e recuperação da biomolécula. Alternativas eficientes e econômicas vêm sendo estudadas nos últimos anos, e dentre estas se destaca o sistema de duas fases aquosas (SDFA). Estes sistemas se mostram como uma tecnologia atraente para purificação de biomoléculas por oferecer vantagens como: simples e rápida separação, clarificação do extrato, baixa desnaturação das enzimas devido ao alto teor de água em ambas as fases, rápida transferência de massa, partição seletiva e custo baixo. Desta forma, os SDFAs tem sido utilizados em vários campos da biotecnologia para separar as moléculas de interesse das moléculas contaminantes (YUZUGULLU; DUMAN, 2015).

Deste modo, este trabalho se propõe a produzir, caracterizar e purificar parcialmente uma nova β-galactosidase a partir do fungo *Cladosporium tenuissimum* URM 7803, utilizando um sistema de duas fases aquosas PEG-citrato para o processo de purificação parcial da enzima.

1.1 OBJETIVOS

1.1.1 Geral

- Produzir, caracterizar e purificar parcialmente uma nova ß-galactosidase produzida por *Cladosporium Tenuissimum* URM 7803.

1.1.2 Específicos

- Realizar identificação molecular do fungo para definir sua espécie.
- Produzir ß-galactosidase por *Cladosporium Tenuissimum* URM 7803.
- Avaliar os parâmetros de produção (tempo de cultivo, consumo do substrato, temperatura e curva de pH) de ß-galactosidase por *C. Tenuissimum* URM 7803.
- Utilizar um sistema de duas fases aquosas PEG/ctrato para realizar purificação parcial da enzima.
- Caracterizar a enzima em relação ao efeito do pH, da temperatura, da presença de íons, detergentes e inibidores utilizando o extrato bruto e o extrato parcialmente purificado por sistema de duas fases aquosas.

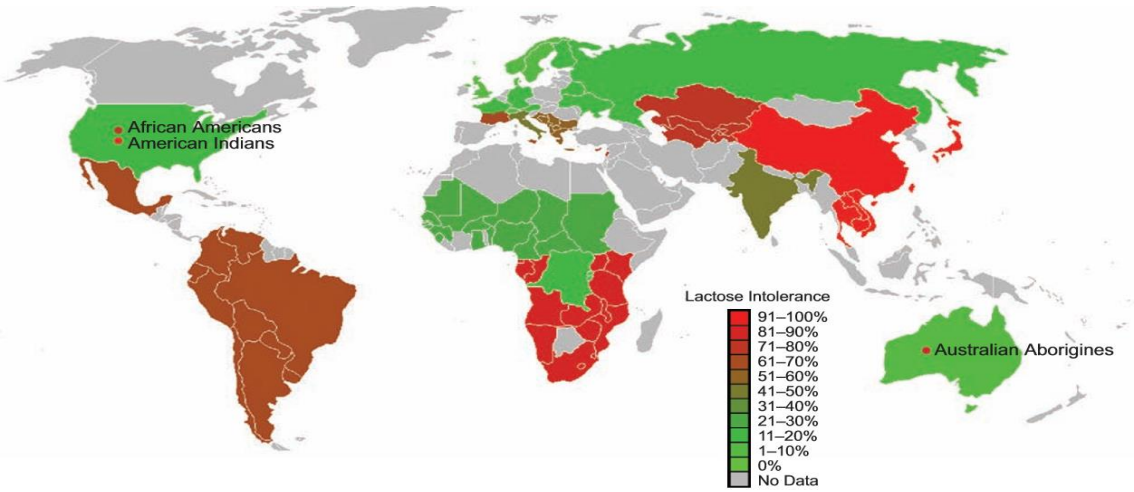
2 REVISÃO DE LITERATURA

2.1 LACTOSE

A lactose (O-β-d-galactopiranosil-(1-4)-β-d-glucopiranose) é um dissacarídeo natural e abundante que só é obtido do leite de mamíferos (composto por cerca de 3 a 8% (p/v) de lactose e 70 a 80% dos componentes sólidos no soro de queijo são formados por lactose), sendo o principal carboidrato e fonte de energia desta matriz. A síntese da lactose ocorre nas células epiteliais mamárias a partir da glicose e da galactose absorvidas da circulação sanguínea. Esta síntese é catalisada por um complexo de proteínas denominado lactase-sintase, no qual o componente catalítico, UDP-galactosiltransferase, irá catalisar a transferência da porção galactose da UDP-galactose para glicose e o componente não catalítico α-lactalbumina, atuará reduzindo a constante de Michaelis da transferase em várias vezes (VOET, 2006).

A lactose apresenta um baixo grau de doçura (possui cerca de 15% de sacarose), tem menor solubilidade em relação aos seus componentes monossacarídicos, e de forma geral é menos solúvel do que a maioria dos açúcares. Um outro fator muito importante ligado à lactose são os eventos de intolerância a este carboidrato, que afeta uma parcela significativa da população mundial. A estimativa é de que cerca de dois terços da população sofram de má digestão e intolerância à lactose, sendo verificado que existe uma forte condição étnica e dependente da idade (SCHAAFSMA, 2008), de modo que há uma distribuição geográfica acentuada, como visto na Fig.1 (HEYMAN, 2006; LATORRE et al., 2014).

Figura 1 Percentual de intolerância à lactose em diferentes populações espalhadas pelo mundo



Fonte: HUSAIN (2010).

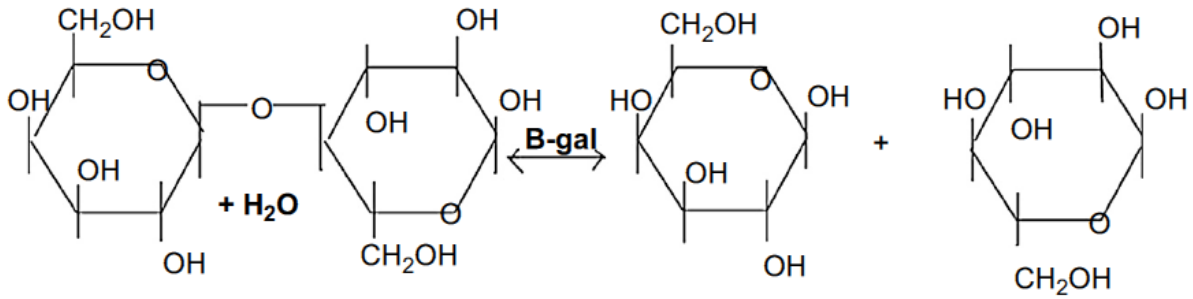
As enzimas vêm sendo usadas como catalisadores industriais por mais de um século. Como forma de diminuir os inconvenientes de problemas como, por exemplo, a intolerância a lactose, o importante emprego das β-galactosidases, enzimas responsáveis, dentre outras funções, pela hidrolise da lactose em glicose e galactose, tem ganhado relevância na produção de leites lactose *free* e outros produtos derivados lácteos (PANESAR et al., 2010). A hidrólise da lactose nos seus componentes monossacarídicos provoca vários efeitos que foram bastante apreciados pelos produtores de alimentos, como o aumento da solubilidade, aumento da doçura dentre outras características organolépticas, além do principal fator que é a ausência de intolerância (REHMAN, 2009).

A redução do teor de lactose no leite e no soro de leite pode ser conseguida por fracionamento de membrana, cromatografia ou métodos enzimáticos. A hidrólise de lactose por meio da β-galactosidase, é o método mais frequentemente utilizado, e com a redução nos preços de comercialização das enzimas, este método pode continuar sendo o preferível por um período maior de tempo (PANESAR et al., 2010).

2.2 β-GALACTOSIDASE

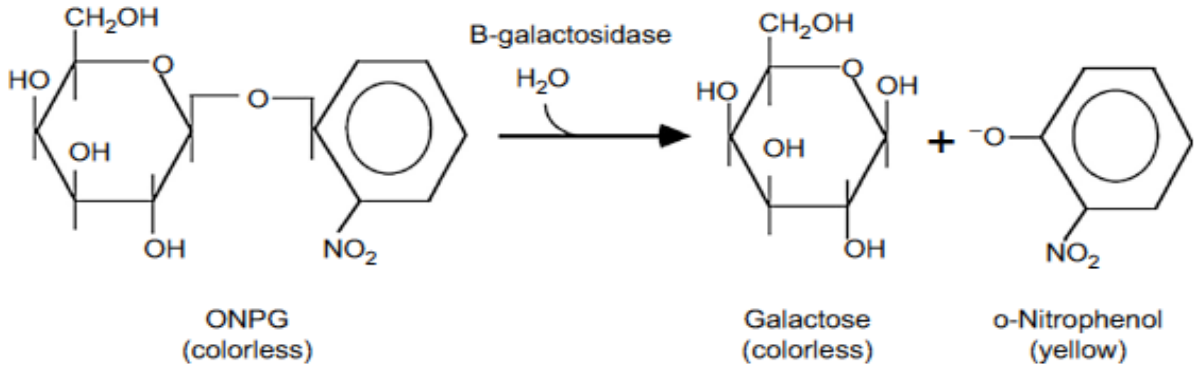
A β-galactosidase ou lactase (EC 3.2.1.23; β-D-galactosil galactohidrolase) é a enzima responsável pela reação de catalisação na hidrólise das ligações β-1,4-D-galactosídicas da lactose, um carboidrato dissacarídeo encontrado no leite contendo glicose e galactose ligadas através de uma ligação β-1,4 glicosídicas (Fig. 2), além de atuar na hidrólise de cromógenos como o-nitrofenil-β-D-galactopiranosídeo (ONPG) (Fig.3), o p-nitrofenil-β-D-galactopiranosídeo (PNPG), 6-bromo-2-naftil galactopiranosídeo (BNG) (GUL-GUVEN et al., 2007) e 4-metilumbeliferil-β-D galactopiranosídeo (LU et al., 2007). Além destas características, esta enzima também realiza a síntese de certos oligossacarídeos pela reação de transferência galactosil. Esta transferência de galactose da lactose para um composto aceitador é chamada de transgalactosilação ou transglicosilação e os seus produtos reacionais são designados por galacto-oligossacáridos (GOSs), que são úteis na saúde humana como constituintes alimentares prébióticos (RICHMOND et al., 1981; ASHOK, et al., 2016). Estes prebióticos não são digeríveis, mas podem modificar a microbiota intestinal em favor de bactérias promotoras da saúde, tais como *Bifidobacterium* sp. e *Lactobacillus* sp.

Figura 2 Ação da enzima β-galactosidase sobre a lactose e formação dos monossacarídeos galactose e glicose



Fonte: Precepta (2016).

Figura 3 Mecanismo de ação da enzima β-galactosidase sobre o o-nitrofinil β-D-galactosidase (ONPG)



.Fonte: ASHOK et al., (2016).

2.3 FONTES PRODUTORAS DE β-GALACTOSIDASE

A enzima β-galactosidase pode ser encontrada em uma grande variedade de organismos, destacando-se os micro-organismos, plantas e tecidos animais. Quando são comparadas às fontes de origem animal e vegetal, é bastante notável que os micro-organismos produzem a β-galactosidase em quantidade superior, justificando desta forma, o forte uso das fontes microbianas na indústria biotecnológica (Tab 1). A β-galactosidase ocorre em uma variedade de micro-organismos, destre estes destacam-se as fungos filamentosos e leveduras, bactérias e actinobactérias (MILICHOVA E ROSENBERG, 2006; HUSAIN, 2010).

Tabela 1 Alguns exemplos de β-galactosidases comerciais

Fonte da enzima	Nome comercial	Fornecedor
Bactérias		
Bacillus sp.	Novozym 231	Novozymes A/S, Dinamarca
Escherichia coli	β-Galactosidase	Sigma-Aldrich, Reino Unido
Leveduras		
Candida pseudotropicalis	Neutral lactase	Pfizer, EUA
Kluyveromyces lactis	Maxilact	DSM Food Specialties, Holanda
	Lactase	SNAM Progetti, Itália
	β-Galactosidase	Sigma-Aldrich, Reino Unido
Kluyveromyces marxianus	Lactozyme	Novozymes A/S, Dinamarca
Kluyveromyces sp.	Lactase NL	Enzyme Development Corporation, EUA
Saccharomyces fragilis	β-Galactosidase	Sigma-Aldrich, Reino Unido
Fungos		
Aspergillus oryzae	Fungal lactase	Enzyme Development Corporation, EUA
	Biolactase	Biocon Inc., EUA
	Lactase 2214C	Rohm, Darmstadt, Alemanha
	β-Galactosidase	Sigma-Aldrich, Reino Unido
Aspergillus niger	Sumylact	Sumitomo Chemical, Japão
	Lactase	Valio Laboratory, Finlândia
Não informada		
	Lactaid	McNeil Nutritional, Canadá
	Dairycare	Plainview LLC, EUA
	Lacteeze	Gelda Scientific, ON, Canadá
	Lifeplan	The Natural Medicine Co., Irlanda

Fonte: O'CONNELL & WALSH (2006); PANSEAR et al., (2006).

2.3.1 Fungos Filamentosos

As β-galactosidases fúngicas são normalmente do tipo extracelular e termoestáveis, entretanto são susceptíveis à inibição do produto final, que ocorre principalmente por galactose. Em estudo apontado po Husain (2010), existem duas estratégias básicas utilizadas pelos fungos para realizar o catabolismo da lactose: (1) hidrólise extracelular seguida da captação de monômeros resultantes e (2) captação de dissacarídeos. Na busca por novas β-galactosidases de interesse biotecnológico, Chen e colaboradores (2008) relataram a produção de uma β-galactosidase termoestável proveniente de *Aspergillus niger*. Ja nos anos 2013 e 2015, respectivamente, foi descrita uma nova β-galactosidase intracelular acidófila de *Teratosphaeria acidotherma* por Isobe et al., (2013) e Chiba et al., (2015). De acordo com a literatura, um numero reduzido de fungos, tais como *Aspergillus oryzae*, *Aspergillus*

aculeatus, Penicillium expansum, Penicillium chrysogenum e *Paecilomyces aerugineus* são os principais produtores de β-galactosidase. Neste sentido, faz-se útil a realização de novos estudos com vistas à descoberta de novas fontes fúngicas produtoras de β-galactosidase com novas características de interesse à industria biotecnológica (BAILEY & LINKO, 1990; NAGY et al., 2001; KATROLIA et al., 2011 e FRENZEL et al., 2015).

2.3.2 Leveduras

As β-galactosidases de leveduras na maioria das vezes são enzimas intracelulares. Dentre este grupo de micro-organismos, uma fonte com destaque na indústria biotecnológica é a β-galactosidase da levedura *Kluveromyces lactis*, que tem como habitat natural o ambiente lácteo (TELLO-SOLI et al., 2005 e HUSAIN, 2010). Esta β-galactosidase possui uma potente capacidade na hidrólise de lactose, sendo portanto utilizada comercialmente para a produção de leite reduzido de lactose consumido por indivíduos que sofrem com intolerância a lactose (RECH e YAUB, 2007). Outra levedura do mesmo gênero, a *K. marxianus*, por exemplo, tem a capacidade de crescer em vários substratos incluindo a lactose como a única fonte de carbono e energia, o que pode facilitar seu cultivo e custos operacionais na indústria (RIBEIRO et al., 2007).

2.3.3 Bactérias

β-galactosidases produzidas por *Escherichia coli* são uma das enzimas mais estudadas, uma vez que o operon Lac (em especial o gene Lac Z, responsável por codificar a síntese de β-galactosidase) tem desempenhado um papel central na regulação e expressão bacteriana. No entanto, os diversos estudos realizados em torno dos seus possíveis efeitos tóxicos associados aos coliformes mostram que é imprópria para utilização nos processos alimentares (MILICHOVA E ROSENBERG, 2006).

Dentre as fontes bacterianas, as bactérias do tipo ácido-láctico (BAL), que compreendem um diverso grupo dos gêneros, como os lactococos, estreptococos e lactobacilos, têm recebido uma atenção especial em várias pesquisas científicas relacionadas à produção de β-galactosidase, principalmente devido a estes micro-organismos possuírem status GRAS (geralmente reconhecido como seguro), que permite a possibilidade de usar as enzimas sem passar por processos de purificação extensiva. Também deve ser considerado,

que as pessoas intolerantes à lactose podem consumir produtos lácteos fermentados por estes micro-organismos, com poucos ou nenhum efeitos adversos, visto que a atividade probiótica dos BAL confere uma melhor digestão da lactose (HUSAIN, 2010; VINDEROLA & REINHEIMER, 2003).

Neste contexto, *Bifidobacterium* sp. e *Lactobacillus* sp. são as bactérias que têm sua utilização mais difundidas como probióticos em alimentos e sistemas alimentares por conta dos seus promissores potenciais benéficos para a saúde do hospedeiro. As *Bifidobacterium* foram selecionadas como modelo bacteriano para estudar a fermentação da lactose por colônias da microbiota intestinal (ARUNACHALAM, 2004; HUSAIN, 2010).

2.4 APLICAÇÕES BIOTECNOLÓGICAS DA β-GALACTOSIDASE

Esta enzima possui ampla e interessantes aplicações nas indústrias de alimentos e fermentação, médica, analítica (formulação de biossensores para determinação específica de lactose no diagnóstico da intolerância à lactose) e de biogás (MILICHOVA E ROSENBERG, 2006; PANESAR *et al*. 2006). Do ponto de vista industrial, a termoestabilidade da β-galactosidase é uma propriedade muito importante, sendo bastante considerada em suas aplicações. As β-galactosidases que possuem atividade em baixas temperaturas possuem um elevado interesse biotecnológico como enzimas da indústria alimentar, visto que tem como destino a retirada da lactose de leite e outros produtos lácteos em baixas temperaturas, como também a biorremediação do soro de queijo e produção de bioetanol. Estes tipos de β-galactosidases são extraídas principalmente, de leveduras psicrófilas, e que quando aplicadas em processos industriais, facilitam o tratamento do leite e demais produtos lácteos, preservando as propriedades organolépticas e nutricionais destes alimentos (FERNANDES et al., 2002; MLICHOVA & ROSENBERG, 2006; ASRAF & GUNASEKARAN, 2010).

De acordo com Husain (2010), o desenvolvimento de métodos analíticos, como a formulação de biossensores com a β-galactosidase, tem grande importância, visto que o controle da qualidade do leite e dos seus derivados é um campo muito exigente e a necessidade de métodos analíticos sensíveis, que economizam tempo e são precisos para a hidrólise da lactose presente no leite e soro de leite é crescente.

2.5 SISTEMA DE DUAS FASES AQUOSAS (SDFA)

O sistema de duas fases aquosas (SDFA), é caracterizado por um método de fracionamento envolvendo uma composição líquido-líquido e, que foi reconhecido como um método muito simples e de fácil execução, considerado como uma abordagem eficiente para purificação e recuperação de ampla gama de biomoléculas, em especial as enzimas (ALBERTSSON, 1986; MOHAMMADI e OMIDINIA, 2013). O SDFA tem a vantagem de ser um método alternativo (econômico) e bastante promissor, já que se pode estudar em uma única etapa, características como a concentração e purificação parcial da molécula em questão. Geralmente, os SDFA são compostos de solução aquosa de dois polímero incompatíveis e polímero/sais. Quando a concentração destes componentes aumenta acima de um determinado valor crítico, ocorre a formação de duas fases.

A extração de enzimas com SDFA é uma alternativa interessante aos métodos de separação tradicional principalmente, no que diz respeito ao baixo custo, retenção da atividade catalítica, manutenção da estabilidade enzimática devido a redução das condições desnaturantes e alta biocompatibilidade, menores danos ao meio ambiente em relação ao seu descarte (o que pode ser observado com o sistema Polietileno glicol-Citrato), baixo consumo de energia e, relativa confiabilidade no processamento em larga escala (NEELWARNE, 2012). Além disso, esses processos de separação em um único passo, permite a purificação simultânea de moléculas alvo e remoção de contaminantes, proporcionando deste modo um tempo de processamento mais curto.

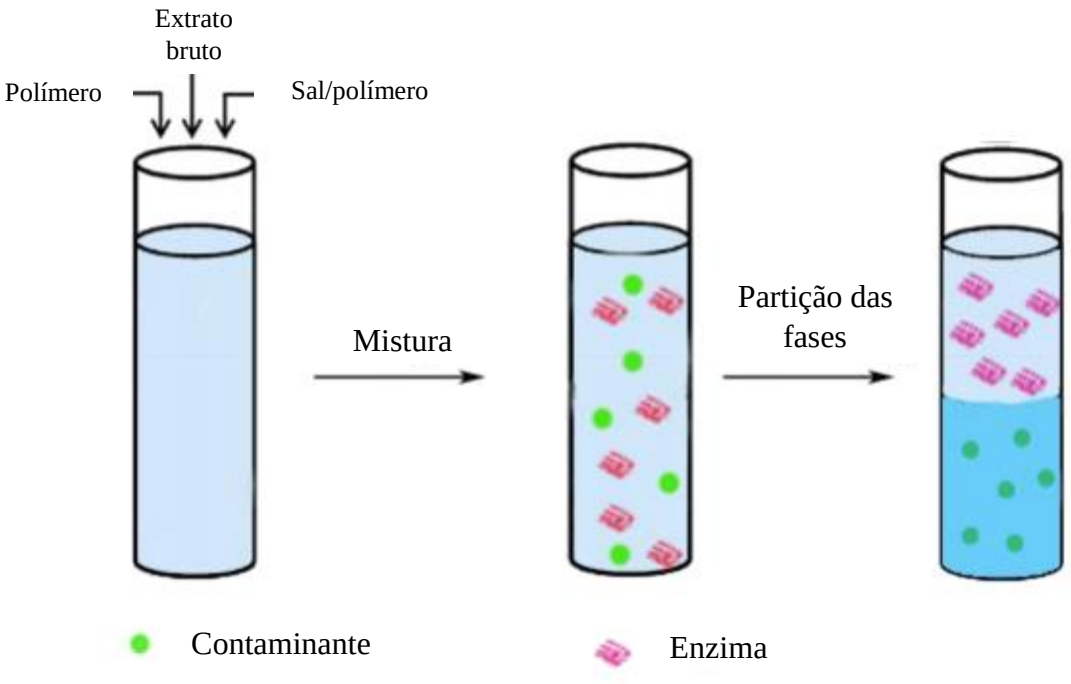
O sucesso dos sistemas de duas fases aquosas depende da capacidade de manipular os parâmetros do sistema (composição de fases) de modo a obter coeficientes de partição (*K*) apropriados (que é a proporção da concentração de biomoléculas na fase superior e na fase inferior) e seletividade para a adequada separação da proteína alvo. No entanto, os mecanismos de partição ainda não estão completamente elucidados (BENAVIDES e RITO-PALOMARES, 2008).

As diretrizes para um maior particionamento da molécula em estudo dependem não apenas das propriedades das biomoléculas, mas também das propriedades do sistema. No que diz respeito às propriedades das biomoléculas, elas incluem muitas variáveis, tais como: hidrofobicidade, conformação molecular, massa molecular, concentração da proteína, carga elétrica da proteína e ponto isoelétrico (pi) (SALGADO et al., 2008). Além disso, as diferentes interações químicas e físicas, como a de Van der Waals e interações iônicas das biomoléculas com a fase circundante, atrelado à ligações de hidrogênio e interações

hidrofóbicas, também desempenha um papel importante na partição das moléculas. Já as propriedades do sistema dependem do massa molar e da concentração de polímero formador de fase, tipo de sal formando a fase, concentração de sal e relação do volume das fases (GLYK et al., 2015; SARAVANAN, et al., 2008).

Geralmente, o desenvolvimento de estratégias úteis para a purificação de enzimas pelo SDFA é dividido em quatro etapas principais (Figura 4). O primeiro passo é a caracterização físico-química da matéria-prima (propriedades da enzima e dos contaminantes), seguido da segunda e terceira etapas em que o tipo de SDFA é definido (polímero/sal ou polímero/polímero) e os parâmetros do sistema (massa molar e concentração, curva binodal, relação de volume e pH). O passo final é avaliar a influência dos parâmetros do processo (concentração da amostra, adição dos componentes no sistema, reciclagem de produtos químicos) no alvo da recuperação e pureza do produto (BENAVIDES e RITO-PALOMARES, 2008, ROSA et al., 2010).

Figura 4 Estratégia prática para o desenvolvimento de um SDFA para a separação e purificação de enzimas



Fonte: o autor (adaptado)

Diferentes combinações de componentes formadores de fase são aplicados para partição e recuperação de várias enzimas. Uma vez que o produto de interesse é caracterizado (verificado para qual fase se particionou), uma das fases que forma o sistema deve ser selecionada e, executadas as etapas posteriores para análise da enzima estudada, de acordo com a especificidade de cada biomolécula.

2.6 O GÊNERO *Cladosporium*

Os fungos pertencentes ao gênero *Cladosporium*, criado por Link em 1815, são considerados um dos maiores e mais diversificados gêneros de Hyphomycetes. Abrangendo várias espécies, boa parte dos fungos são contaminantes e oportunistas, dematiáceos e são bastante cosmopolitas, atuando como saprófitos no solo e em material em decomposição. Muitas espécies são conhecidas por serem patógenos de plantas, já outras são frequentemente relacionados como contaminantes e agentes de deterioração em alimentos ou produtos industriais, além de provocarem quadros asmáticos pela inalação de seus esporos aéreos (EL-MORSY, 2000; SCHUBERT, 2005).

O gênero *Cladosporium* é destacado entre os fungos mais comumente isolados do ambiente em quase todas as partes do globo terrestre. Dentre as espécies mais isoladas se destacam: *C. elatum*, *C. herbarum*, *C. spherospermum*, *C. cladosporioides*, *C. carrionii*, *C. oxysporum* (TASIC, TASIC, 2007). Durante sua fase saprófita, estes fungos formam hifas septadas com tonalidade escura, com conidióforos nas laterais e nos pontos terminais de tamanhos variados. A conidiação é do tipo *Cladosporium*, isto é, em geral, os conidióforos produzem cadeias longas e ramificadas de conídios de coloração castanha, com forma ovalada e, de paredes lisas e delgadas. Normalmente aparecem nos tecidos como células grande e redondas, septadas e escuras, com 5-12 µm de diâmetro (BENSCH et al., 2012).

Os conídios podem ser produzidos em cadeias, sendo catenulados, mas também podem ser, muitas vezes, solitários em algumas espécies onde os conídios são mais largos e algumas vezes são ramificados, simples, cilíndricos, ovóides, doliformes, fusiformes, elipsóides, esféricos ou sub-esféricos. Possuem coloração marrom olivácea escuro ou marrom, a superfície do conídio pode ser lisa, verrugosa ou equinulada com 0–3 septos que podem surgir ocasionalmente (ELLIS, 1971).

Os conídios de *Cladosporium* apresentam boa adaptação para espalhar-se com facilidade e de forma numerosa, por longas distâncias. São importantes agentes alérgenos, e quando inalados em grandes quantidades, podem afetar gravemente as pessoas asmáticas e com doenças respiratórias. Além disso, exposições constantes e prolongadas podem enfraquecer o

sistema imunológico (MULLINS, 2001; FLANNIGAN, 2001). Estes fungos também apresentam crescimento lento, atingindo a maturidade em um período médio de 21 dias. São caracterizados pela produção de colônias efusas ou ocasionalmente puntiformes, com superfícies planas, aveludadas, circulares, de crescimeto lento e enrrugado, que variam do verde oliva ao marrom escuro e reverso preto (Figura 5) (TAMSIKAR, NAIDU, SINGH, 2006).

Figura 5 – Macromorfologia da colônia de C. tenuissimum URM 7803 apresentando superfície aveludada, circular e de tonalidade cinza chumbo, cultivado no meio de cultura Agar-Sabouraud com 14 dias de crescimento.

Fonte: o autor.

Do ponto de vista biotecnológico os fungos deste gênero ainda são pouco explorados, principalmente no que diz respeito à produção de enzimas. Dentre as espécies deste gênero, a que apresenta maiores aplicações biotecnológicas é a espécie *Cladosporium cladosporioides*, sendo relatada na literatura como produtora de algumas enzimas, como a pectina metil esterase (PME) e a poligalacturonase (ALMATAR e MAKKY, 2016).

3 RESULTADOS

3.1 *Cladosporium tenuissimum* URM 7803: a promising new β-galactosidase producer

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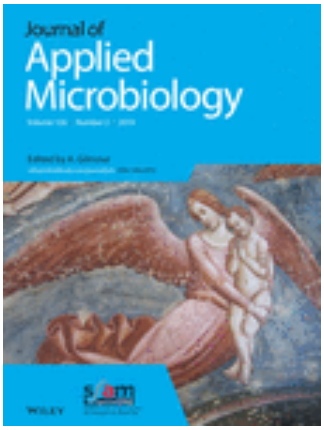

Cladosporium tenuissimum **URM 7803: a promising new β-galactosidase producer**

Anderson José Paulo

Bioscience Center, Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Av. Prof. Moraes Lins do Rego, s/n, 50670-901, Recife, PE, Brazil

Maria Carolina de Albuquerque Wanderley

Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Av. Prof. Moraes Lins do Rego, s/n, 50670-901, Recife, PE, Brazil

Rafael José Vilela de Oliveira

Departament of Micology Prof. Chaves Batista, Federal University of Pernambuco, Av. Prof. Moraes Rego, s/n, Bioscience Center, 50670-901, Recife, PE, Brazil,

Willie Anderson dos Santos Vieira

Department of Agranomy, Federal Rural University of Pernambuco, Av. Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil

Luiz Carlos Alves

Institute Aggeu Magalhães-IAM/FIOCRUZ, Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Av. Prof. Moraes Lins do Rego, s/n, 50670-901, Recife, PE, Brazil

Ana Lúcia Figueiredo Porto*

Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, Av. Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil

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(*)Corresponding author:

Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, Av. Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil

analuporto@yahoo.com.br

Telephone number: +55 81 33206391

Fax number: +51 81 21268485

Cladosporium tenuissimum URM 7803: a promising new producer β-galactosidase

Abstract

β-Galactosidase (β-d-galactohydrolases, EC 3.2.1.23) is an abundant glycosyl hydrolase enzyme that catalyzes the hydrolysis of terminal β-d-galactosyl units to monosaccharides by breaking the glycosidic bond of substrates such as lactose. The objective of present study is to evaluate the β-galactosidase production by a new isolate of *Cladosporium tenuissimum* URM 7803 identified by rDNA sequencing. The maximum production of β-galactosidase by *C. tenuissimum* URM 7803 was displayed at 264 h, with enzymatic activity of 462,13 U/mL, at 28 °C and pH 6,5. The phylogenetic analysis demonstrated the isolated strain placed together with other *C. tenuissimum* isolates showing very strong support of 88%, belonging to the *C. cladosporioides* complex species. According to results *C. tenuissimum* URM 7803 had its species confirmed through sequencing of elongation factor 1-α, confirming that it is a new β-galactosidase producer isolate with potential application in biotechnology industry. Further studies should be performed to characterize the enzyme, purify it and apply it to prebiotics production, such as galactooligosaccharides (GOS).

Keywords: β-Galactosidase, production, phylogenetic, *Cladosporium tenuissimum.*

1. Introduction

β-Galactosidase (β-d-galactohydrolases, EC 3.2.1.23) is an abundant glycoside hydrolase enzyme that catalyzes the hydrolysis of terminal β-d-galactosyl moieties to monosaccharides by breaking the glycosidic bond of substrates such as disaccharides, diverse glycoconjugates, and polysaccharides (Husain, 2010). β-galactosidase not only hydrolyzes lactose but also carries out a transgalactosylation reaction to produce galacto-oligosaccharides (Gosling et al., 2010; Mussatto and Mancilha, 2007). Galactooligosaccharides (GOS) are non-digestible oligosaccharides that are consumed by humans as a food component, classified as prebiotic, providing increased growth of some bacteria from intestinal microbiota, such as *Bifidobacterium sp*. and *Lactobacillus sp.* These microorganisms provide positive effects on the host health, reducing cholesterol levels and stimulating the production of different vitamins in addition to possess anticancer properties (Grosová et al., 2008).

In pharmaceutical and dairy industries, β-galactosidase has significant applications to reduce crystallization effect of lactose in condensed milk and frozen dairy products. This enzyme can also be applied to reduce water pollution caused by whey from cheese processing industry and its derivatives, as well as its use in milk digestibility improvement for people suffering from lactose intolerance or allergy (Elnashar and Yassin, 2009; Haider and Husain, 2008; Ansari and Husain, 2011).

β-galactosidases can be derived from a number of different sources; however, enzymes of microbial origin, including bacteria, yeasts and filamentous fungi, are the most studied and used for various purposes in biotechnology industry, especially in the food and pharmaceutical industries (Hsu, et al., 2005, Park and Oh, 2010, Oliveira, et al., 2011). Several filamentous fungi as *Teratosphaeria acidotherma* (Isobe et al., 2013), *Aspergilus tubengensis* (Raol et al., 2014), *Aspergillus nidulans* (Kamran, et al., 2016), *Aspergillus lacticoffeatus* (Cardoso, et al., 2017) and *Papiliotrema terrestris* (Ke, et al., 2018) were reported as producers of β-galactosidase, but new studies on the discovery of new microbial sources produced of β-galactosidase with biotechnologically interesting characteristics have been encouraged in recent years.

Candidate as a new β-galactosidase producer, fungi of the genus *Cladosporium* are still little explored biotechnologically. The genus *Cladosporium* spp, divided by Link in 1816, is one of the largest and most heterogeneous genera of Hyphomycetes (Dugan et al., 2004). This genus comprises more than 189 species, being among the most commonly isolated fungi in the environment and almost everywhere. *Cladosporium* spp. are called black yeast, because

they are naturally brownish in color due to the presence of melanic pigment (dihydroxynaphthalenomelanin) in their cell wall. Among the species of this genus that have largely biotechnologically applied, *Cladosporium cladosporioides* is reported as a producer of some enzymes such as Pectin methylesterase and polygalacturonase (Almatar and Makky, 2016).

The present study evaluated the production of β-galactosidase by a new isolate of *Cladosporium tenuissimum* URM 7803 identified by rDNA sequencing, for possible application for lactose hydrolysis and prebiotics production, such as galactooligosaccharides (GOS).

2. Materials and Methods

2.1 Microorganism

The *Cladosporium tenuissimum* strain (URM 7803) was isolated from culture medium saturated with lactose and deposited at "Micoteca - URM" of Mycology Department of the Federal University of Pernambuco (UFPE), Recife-PE, Brazil, preserved in mineral oil and maintained at 28ºC in Czapek Dox Agar.

2.2 DNA extraction, PCR amplification and DNA sequencing

Extraction of rDNA - Cultures grown in test tubes containing malt extract were incubated at 28°C for six days to obtain the fungal biomass. The material was transferred to 2mL microtubes with screw caps. To each tube, 0.5 g of acid-washed glass beads, with two different diameters (150–212 µm and 425–600 µm, 1:1; Sigma, USA), were added. The material was crushed by stirring at high speed in a FastPrep homogenizer. The genomic DNA extraction procedure was conducted as described by Góes-Neto et al. (2005). The mycelium was washed with chloroform: isoamyl alcohol (24:1) and then homogenized in 2% cetyltrimethylammonium bromide buffer. The DNA was precipitated in isopropanol, washed with 70% ethanol, and resuspended in 50 µL ultrapure water.

For amplification of the translation elongation factor 1-a (TEF1) the primer pairs EF1-728F/EF1-986R (Carbone and Kohn 1999) were used. PCR reactions were carried out in a volume of 50 µl containing 75 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 200 µM each dNTPs, 1 µM each primer, and 2 units Taq DNA polymerase (Fermentas, MD, USA). Thermal cycling parameters were set as follows: an initial denaturation temperature of 94°C for 5 min, followed by 40 cycles of denaturation

temperature of 94°C for 45 s, primer annealing at 52 °C for 30 s, primer extension at 72°C for 90 s and a final extension step at 72°C for 6 min.

The final amplicons were purified with the kit Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). Sequencing was performed by the Laboratory of Molecular Biology and Evolutionary Biology of UFPE. Sequence assembly and editing were performed using the tools called Pregap4 and Gap4, which belong to the Staden package (Staden et al., 1998).

2.3 Phylogenetic analysis

Forward and reverse sequences were assembled using the Staden package. The sequence generated in the current study was deposited in GenBank (Table 1). The sequence was compared to GenBank sequences using BLAST to query the NCBI sequence database for approximate species identification.

Sequences of type and representative strains of *C. cladosporioides* species complex of elongation factor 1-α were downloaded from GenBank and combined with the newly generated sequence. Multiple sequence alignment was performed in MEGA 7.0.14 (Kumar et al. 2016).

The phylogeny was inferred under the maximum likelihood (ML) criterion. The ML analyses were done in PhyMl 3,1 aLRT (Guindon & Gascuel 2003) implemented on Phylogeny.fr web server (Dereeper et al. 2008, available in http://www.phylogeny.fr/). ML tree searchs were performed under the HKY85 substitution model with 1000 pseudoreplicates.

Tabela 1 GenBank and culture collection accession numbers of Cladosporium species employed in phylogeny.

Species (1)	**Culture accession numbers (2)**	**ef1-α GenBank accession number**
*Cladosporium angustisporum**	CBS 125983; CPC 12437	HM148236
*C. australiense**	CBS 125984; CPC 13226	HM148240
*C. cladosporioides**	CBS 112388	HM148244
*C. colocasiae**	CBS 386.64; ATCC 200944; MUCL 10084	HM148310
*C. cucumerinum**	CBS 171.52; MUCL 10092	HM148316
*C. feniculosum**	CBS 122129; ATCC 38010; IFO 6537; JCM10683	HM148338
*C. gamsianum**	CBS 125989; CPC 11807	HM148339
*C. grevilleae**	CBS 114271; CPC 2913	JF770472
*C. phaeocomae**	CBS 128769; CPC 18223	JF499875
*C. pseudocladosporioides**	CBS 125993; CPC 14189	HM148402
*C. subuliforme**	CBS 126500; CPC 13735	HM148441
C. tenuissimum	SRRC1616	KT950252
C. tenuissimum	SRRC1634	KT950253
*C. tenuissimum**	CBS 125995; CPC 14253	HM148442
C. tenuissimum	URM 7803	
*C. verrucocladosporioides**	CBS 126363; CPC 12300	HM148472

1 : "*" represents holotype isolates. 2: ATCC- American Type Culture Collection, Virginia, U.S.A.; CBS - CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC - Culture collection of Pedro Crous, housed at CBS; IFO - Institute for Fermentation, Osaka, Japan; MUCL - Mycotheque de l'Universite catholique de Louvain, Laboratoire de Mycologie Systematique et Appliquee, Universite catholique de Louvain, Louvain-la-Neuve, Belgium; SRRC - Southern Regional Research Center, New Orleans, Louisiana, USA.

2.4 Scanning electron microscopy

The *Cladosporium tenuissimum* URM 7803 were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer 0.1 M, pH 6.8 (Sigma-Aldrich, São Paulo, Brazil). Then the samples were post-fixed in 1% osmium tetroxide (OsO4) (Sigma-Aldrich, São Paulo, Brazil) in cacodylate buffer (Sigma-Aldrich, São Paulo, Brazil) for 1 h in the absence of light at 25ºC. After this step, washing and dehydration were performed in a series of increasing ethanol for 15 min each (30%, 50%, 70%, 90% and 100%). The samples were subjected to critical point

using liquid CO2, coated with colloidal gold and examined under a microscope JEOL-5600LV (Jeol, Tokyo, Japan).

2.5 Inoculum preparation and β-galactosidase production

Spore suspensions for inoculum were prepared in sterile saline solution 0.85% (w/v) NaCl containing 0.01% (w/v) Tween 80, and conidia density was adjusted to 10^7 conidia/mL. The fermentation medium is composed of (% w/v): lactose (2), peptone (0.4), yeast extract (0.4) and salts (KH_2PO_4 (0.2), $Na_2HPO_4.12H_2O$ (0.8) and $MgSO_4.7H_2O$ (0.025)) conducted at 28 °C, pH 6.5 and 180 xg for 13 days. An Erlenmeyer (50 mL) was collected every 24 hours and β-galactosidase production was evaluated.

2.6 β-galactosidase activity

Extracellular β-galactosidase activity was determined by incubating samples (50 µL), at 37 °C for 30 minutes, with 50 µL of ONPG solution (3 mM) prepared in sodium-citrate buffer (50 mM pH 4.5). The reaction was stopped by the addition of 200 µL of sodium carbonate (0.1 M) (Nagy et al., 2001). The released *O*-nitrophenol was determined spectrophotometrically at 420 nm. One unit (U) of enzyme was defined as the amount of enzyme that liberates 1 µmol of *O*-nitrophenol from ONPG per minute under the assay conditions.

3. Results and Discussion

3.1 β-galactosidase production

The production of β-galactosidase by *C. tenuissimum* URM 7803 was evaluated in this study. The fermentation was carried out for a period of 312 h, samples were taken at each 24 h interval to determine the best time of enzyme production. It was verified that the maximum production of β-galactosidase by *C. tenuissimum* URM 7803 was 462.13 U/mL in the time of 264 h, and was maintained stable in periods of 288 h and 312 h (459.91 and 451.69 U/mL) respectively (Fig. 1). Fungi of the genus Cladosporium spp. have a slow growth, reaching maturity between 14 and 21 days, as verified by Tamsikar et al. (2006). The maximum concentrations of β-galactosidase were obtained in this period of time, and it was also verified that the fungus entered the cell death period after 212 h growth, at which time β-galactosidase concentrations increased, suggesting that the enzyme has a greater production in the intracellular level, a characteristic that has also been verified in studies of Isobe et al., (2013)

e Nath et al., (2014) with *Teratosphaeria acidotherma* and *Bacillus safensis* respectively, the former having evaluated the multiple forms and activity profile of β-galactosidases produced intracellularly and extracellularly at different pH ranges.

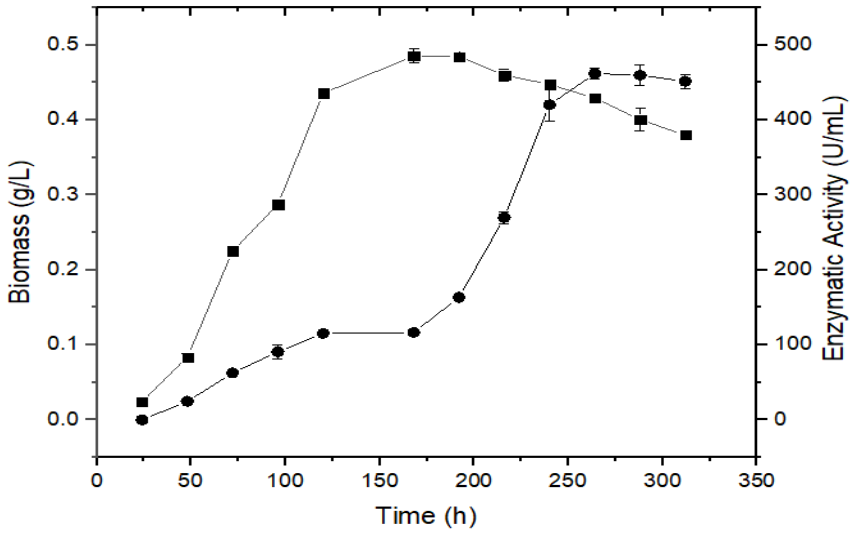

Figura 1 Influence of time on the production of β-galactosidase (●) and biomass (■). The highest volumetric activity (462.13 U/mL) was verified with 264 h of fermentation and cellular concentration of 0.42 g/L.

The β-galactosidase enzyme is produced by several microorganisms, however, the β-galactosidase produced by *Cladosporium tenuissimum* URM 7803 presented higher volumetric enzymatic activity when compared to other studies with filamentous fungi (Table 2), proving to be a new enzyme with potential for future biotechnological applications.

Table 2 Microorganisms producing β-galactosidase in submerged fermentation

Microorganism	**Enzymatic Activity**	**Reference**
Aspergillus tubengensis GR-1	352 U/mL	Raol et al., (2014)
Aspergillus terreus	250 U/mL	Vidya et al., (2014)
Aspergillus uvarum	13,77 U/mL	Silverio et al., (2017)
Aspergillus nidulans	60 U/mL	Kamran et al., (2016)

3.2 – Morphological and phylogenetic analyzes

According to Menezes et al., (2017) *Cladosporium* spp. forms septate and dark hyphae with lateral and terminal conidiophores of varying sizes. Their conidia can be produced in

chains and often solitary in some species, being able to assume cylindrical forms, eggshaped, fusiforms, ellipsoids or spherical. They present a dark (Fig. 2) or brown olive color, and the surface of the conidium can be smooth or verrucous (Fig. 3) macro and micromorphological characteristics observed in *C. tenuissimum* URM 7803.

Figure 2 Macromorphology of C. tenuissimum URM 7803 colony in Agar-Sabouraud culture medium with 14 days of growth

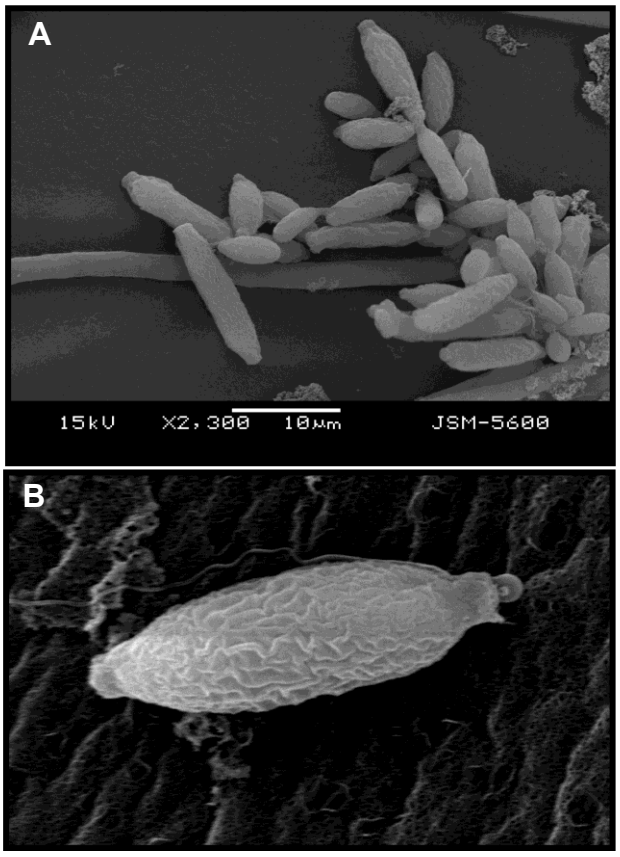

Figure 3 Scanning electron micrographs from Cladosporium tenuissimum URM 7803. (A) ramoconidia and conidia; (B) detail of conidia. Scale bars = 10µm (A), 1µm (B).

Phylogenetic analyzes showed that ef1-α partial sequence of *Cladosporium tenuissimum* URM 7803 β-galactosidase producer was similar to sequences of the *C. cladosporioides* species complex according to BLAST search. The isolate was placed together

with *C. tenuissimum* isolates with very strong support (88%) in ML tree (Fig. 4) and was confidently assigned to this species.

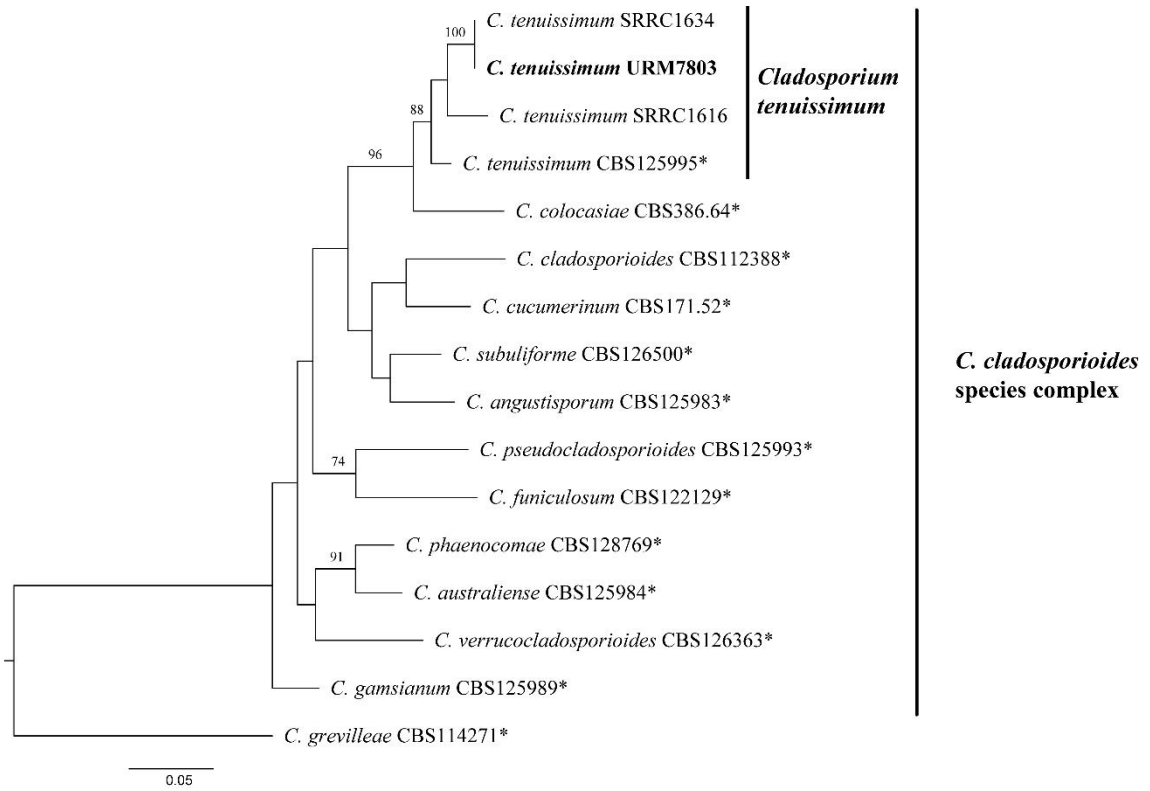

Figure 4 - Maximum likelihood tree of Cladosporium cladosporioides species inferred from an alignment of ef1-α. Supports ≥ 70% are shown above the nodes. Ex-types are marked with "*". Isolate from present study is emphasized in bold. Cladospsorium grevilleae was used as outgroup taxa. The scale bar indicates the average number of substitutions per site.

The genus *Cladosporium* spp. presents species with very significant morphological and genetic similarities, being the sequencing of elongation factor 1-α an important mechanism to distinguish species (Bensch et al., 2012), and thus confirms the species *C. tenuissimum* URM 7803 as a new fungal isolate producing β-galactosidase not reported in the literature.

4. Conclusion

The *C. tenuissimum* URM 7803 had its species confirmed through the elongation factor 1-α sequencing, confirming that it is a new β-galactosidase producer isolate with potential application on biotechnology industry. Further studies should be performed to characterize the enzyme, purify it and apply it for prebiotics production, such as galactooligosaccharides (GOS).

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Ethical Statement/Conflict of Interest

The authors are aware of the ethical responsibilities and the manuscript has no conflict of interest.

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3.2 Partial purification of a new β-galactosidase from *Cladosporium tenuissimum* URM 7803 using poly(ethylene glycol)-citrate aqueous two-phase system

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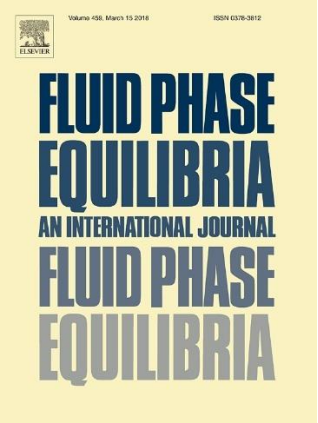

Partial purification of a new β-galactosidase from *Cladosporium tenuissimum* **URM 7803 using poly(ethylene glycol)-citrate aqueous two-phase system**

Anderson José Paulo

Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Av. Prof. Moraes Lins do Rego, s/n, 50670-901, Recife, PE, Brazil

Maria Carolina de Albuquerque Wanderley

Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Av. Prof. Moraes Lins do Rego, s/n, 50670-901, Recife, PE, Brazil

Daniela Araújo Viana Marques

Serra Talhada Campus, University of Pernambuco (UPE), Av. Afonso Magalhães, s/n, Serra Talhada, PE, Brazil

Ana Lúcia Figueiredo Porto*

Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, Av. Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil

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(*)Corresponding author:

Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, Av. Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil

analuporto@yahoo.com.br

Telephone number: +55 81 33206391

Fax number: +51 81 21268485

Partial purification of a new β-galactosidase from *Cladosporium tenuissimum* URM 7803 using poly(ethylene glycol)-citrate aqueous two-phase system

Abstract

β-galactosidases (EC 3.2.1.23) are enzymes with the ability to hydrolyze milk sugar, lactose, into its components d-glucose and d-galactose, being an are one of the main enzymes of industrial interest, and can be applied in health and food technology sectors. Purification and recovery of biomolecules is part of the critical phase of the production process, in this context, Aqueous Two Phase System (ATPS) methodology has emerged as an alternative technique for extracting and purifying enzymes. In this work, experiments were performed according to a 2^4 -full factorial design using PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), citrate concentration (C_{CIT}) and pH as independent variables; and purification factor (PF), partition coefficient (K) and activity yield (Y) as responses. β-galactosidases was partitioned into salt-rich phase. The highest yield (56.61) and purification factor (12.84) were obtained in run 2 using MPEG = 8000 g/mol, 20% (w/w) CPEG, 15% (w/w) CCIT at pH 6.0. Consequently, the PEG-Citrate ATPS has not yet been reported in the literature for β-galactosidase purification having been shown in this study to be efficient and an alternative to be used as a first step for β-galactosidase pre-purification from in-state fermentation liquid of *C. tenuissimum* URM 7803.

Keywords: β-galactosidases, Aqueous two-phase systems, *C. tenuissimum*, purification, yield.

1 Introduction

β-galactosidases (EC 3.2.1.23) are enzymes with the ability to hydrolyze the milk sugar, lactose, into its components d-glucose and d-galactose (HEMAVATHI; RAGHAVARAO, 2011; SILVERIO et al., 2018). β-galactosidases are one of the main enzymes of industrial interest, and can be applied in health and food technology sectors. Patients who are lactose intolerant can take artificial beta-galactosidase supplementation in order to consume lactose daily without presenting intestinal disorder (KHAYATI; ANVARI; SHAHIDI, 2015). The food industry can work with these enzymes to hydrolyze and reduce the lactose content of foods, increasing sweetness, flavor and solubility (SILVERIO et al., 2018). Besides these applications, β-galactosidases can be used to lactose-based prebiotics synthesis, such as galacto-oligosacharides (GOS) (CARDOSO et al., 2017).

Rcovery of biomolecules is part of the critical phase of the production process. The purification of a product aimed at the industrial market must contain some characteristics such as: robustness, reliability, ease of process scheduling and removal of impurities, process speed, high recovery yield and low cost (ROSA et al., 2011). For the manufacture of a biomolecule, the product recovery rate and its purification, where the process challenge is currently dominated by several chromatographic steps, must be taken into account (PRZYBYCIEN; PUJAR; STEELE, 2004).

Literature reports several techniques for enzymes separation and purification. Ammonium sulphate precipitation, ultrafiltration, successive chromatography steps, dialysis are common methods (LI; YOURAVONG; H-KITTIKUN, 2006; WU et al., 2010). However, for industrial scale, these procedures are considered in infeasible due high cost, time consuming and difficult to scale up (DA SILVA et al., 2017; MCMASTER, 2007; PORTO et al., 2008).

It is necessary to develop alternative methods, low cost and high yield, replacing those already mentioned. In this context, Aqueous Two Phase System (ATPS) methodology has emerged as an alternative technique for extracting and purifying enzymes (DUARTE et al., 2015; KHAYATI; ANVARI; SHAHIDI, 2015; MALHOTRA; JESHREENA; CHAPADGAONKAR, 2016). The liquid-liquid extraction using ATPS is one of the most promising processes bioseparation (ROSSO et al., 2012), due the use of simple and economic components. It is composed of a mixture of two polymers or a polymer and a salt, which is separated into two phases in an aqueous medium. The advantages of these systems include the

processing time, low material cost, low toxicity of phase-forming chemicals, biocompatibility and low power consumption (LEMES et al., 2014; YAVARI et al., 2013).

The literature has already reported the use of ATPS for the separation of several biomolecules as lipases (PIMENTEL et al., 2013; RAMAKRISHNAN et al., 2016), collagenases (LIMA et al., 2013; ROSSO et al., 2012; WANDERLEY et al., 2017) and others. The use of ATPS for β-galactosidase purification was reported by some authors, all using a two-phase system consisting of polyethylene glycol (PEG) and potassium phosphate (HEMAVATHI; RAGHAVARAO, 2011; KHAYATI; ANVARI; SHAHIDI, 2015; LEMES et al., 2014; SILVA; FRANCO, 1999).

The aim of this work was to determine the best conditions for partial purification of β-galactosidase produced by *Cladosporium tenuissimum* URM 7803 using ATPS formed by PEG and sodium citrate. For this purpose, a 2^4 full factorial design was applied to the process to characterize the optimal levels of PEG molar mass, pH, sodium citrate and PEG concentrations at β-galactosidase extraction parameters.

2 Material and methods

2.1 Microorganism

Cladosporium tenuissimum strain URM 7803 was obtained at "Micoteca - URM" of Mycology Department of the Federal University of Pernambuco (UFPE), Recife-PE, Brazil, preserved in mineral oil and maintained at 28ºC in potato dextrose agar.

2.2 Inoculum preparation and β-galactosidase production

Spore suspensions for inoculum were prepared in sterile saline solution 0.85% (w/v) NaCl containing 0.01% (w/v) Tween 80, and conidia density was adjusted to 10^7 spore/mL. Fermentation medium is composed of (% w/v): lactose (2), peptone (0.4), yeast extract (0.4) and salts (KH_2PO_4 (0.2), $Na_2HPO_4.12H_2O$ (0.8) and $MgSO_4.7H_2O$ (0.025). Cultures were incubated at 28 °C, pH 6.5, 180 xg for 9 days.

2.3 Enzymatic activity determination

β-Galactosidase activity was determined by incubating samples (50 µL), at 37 °C for 30 minutes, with 50 µL of o-nitro-phenyl-,B-D-galactopyranoside (ONPG) solution (3 mM) prepared in sodium-citrate buffer (50 mM pH 4.5). The reaction was stopped by the addition

of 200 µL of sodium carbonate (0.1 M). The released *O*-nitrophenol (ONP) was determined spectrophotometrically at 420 nm. One unit of D-galactosidase activity was defined as the amount of the enzyme required to liberate 1 µmol of ONP per minute under assay conditions. The experiments were done in triplicate (Nagy et al., 2001).

2.4 Protein determination

Total protein concentration was determined by the bicin-choninic acid (BCA) method using the Thermo Scientific Pierce BCA Protein Kit (Life Technologies, Carlsbad, CA, EUA) and bovine serum albumin (BSA) as the standard. The experiments were done in triplicate (SMITH et al., 1985).

2.5 Aqueous two-phase systems

A concentrated solution of sodium citrate (30% w/w) was prepared by mixing appropriate quantities of equimolar solutions of dihydrate tri-sodium citrate and monohydrate citric acid so as to give different pH values (6.0, 7.0 or 8.0) at 25 ± 1°C. The required amounts of the above solutions were mixed in 15 mL-graduated tubes provided with conical tips and containing 50% (w/w) polyethylene glycol (PEG) solutions with different molar masses (400, 3350 or 8000 g/mol) and the crude extract accounting for 20% (w/w) of total mass. After water addition up to a final mass of 10 g, the suspensions were then homogenized in a vortex for 1 min, and the two phases were separated by settling after 1 h. The volume of each phase was then measured, and protein concentration and enzymatic activity were determined. To avoid interference of PEG and citrate salt, all the samples were compared with protein-free standard solutions, with the same phase composition.

2.5 Experimental design

A 2^4 full factorial design was utilized to evaluate the influence of four independent variables, namely PEG molar mass (x_1), PEG concentration (x_2), citrate concentration (x_3), and pH (x_4) on parameters of partition coefficient, activity yield and purification factor. Experimental design included 16 runs and 4 repetitions at the central point, which were necessary to calculate the pure error (Table 1).

Table 1 Factor levels of the 24-full factorial design used to investigate □-galactosidase partition and purification by ATPS

Factor	Level		
	Low (-1)	Center (0)	High (+1)
PEG molar mass (M_{PEG})	400	3350	8000
PEG concentration (C_{PEG})	20	22	24
Citrate concentration (C_{CIT})	15	17.5	20
pH	6.0	7.0	8.0

The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA); the first-order model equation was determined by Fischer's test. The experimental and predicted values were compared in order to determine the validity of the developed model by the Statistica 8.0, statistical program package (Statsoft Inc, 2015).

2.6 Determinations of the partition coefficient, activity yield and purification factor

The β-galactosidase partition coefficient was determined as the ratio of the β-galactosidase activity in the top phase (A_T) to that in the bottom phase (A_B):

$$K = \frac{A_T}{A_B}$$

The activity yield was defined as the ratio of A_B to the initial activity in the fermentation broth (A_F) and expressed as a percentage:

$$Y = \left(\frac{A_B}{A_F}\right) x\ 100$$

The purification factor was calculated as the ratio of the specific activity in the selected phase (A_B/C_B) to the initial specific activity in the fermentation broth before partition (A_F/C_F):

$$PF = \frac{A_B/C_B}{A_F/C_F}$$

where C_B and C_F are the protein concentrations, expressed in mg/mL, in the bottom phase and the fermentation broth, respectively.

3 Results and Discussion

Several factors can interfere on biomolecules partitioning between two phases. It can be modified by changing systems conditions, as the type, concentration and molar mass of the polymer or salt used, as well as co-solute salts, pH and temperature, biomolecules characteristics and surface properties are important influencing factors on separation (LIMA, 2013).

The influence of independent variables, M_{PEG}, C_{PEG}, C_{CIT}, and pH on parameters of partition coefficient, activity yield and purification factor for β-galactosidase extraction used on ATPS is described on Table 2. It should be noticed that the runs 1, 3, 9 and 11 did not form any biphasic system; probably because of components concentrations were below or very near the critical point of the binodal curve (PORTO et al., 2008). It seems that using lower molecular mass PEG, along with a difficulty on forming biphasic systems, the enzyme had a tendency to partition to the bottom phase (K between 0.12 and 0.18) when compared to runs using high molecular mass PEG (K between 0.13 and 3.32). This β-galactosidase behavior was the opposite of *Aspergillus tamarii* protease behavior on a similar system (SILVA, O. et al. 2017). Although large sized polymers can create a volume repulsive effect, along with higher hydrophobicity, leading to lower K values (Wanderley, M. et al. 2017), the synergistic effect of high mass PEG with the other factors in runs 2 and 4 lowered the K values and achieved higher purification factors (PF). Which seems that, in these conditions, different components of the extract partition to the top phase while the β-galactosidase behaves the opposite way.

Table 2 Factor level combinations and results of the 24 factorial design employed to investigate the extraction of □-galactosidase from Cladosporium tenuissimum URM 7803 by PEG/citrate ATPS. No biphasic system was observed in the runs 1, 3, 9, and 11

Run	M_{PEG}[a] (g/mol)	C_{PEG}[b] (% w/w)	C_{CIT}[c] (%. w/w)	pH	A_e[d] (U/mg)	A_T[e] (U/ml)	K[f]	Y[g] (%)	PF[h]
1	400	20	15	6	-	-	-	-	-
2	**8000**	**20**	**15**	**6**	**261.52**	**33.69**	**0.13**	**56.61**	**12.82**
3	400	24	15	6	-	-	-	-	-
4	**8000**	**24**	**15**	**6**	**238.91**	**65.65**	**0.27**	**51.71**	**12.94**
5	400	20	20	6	368.47	66.95	0.18	79.76	2.57
6	8000	20	20	6	97.17	138.26	1.42	21.03	14.86
7	400	24	20	6	327.6	40.86	0.12	70.91	1.91
8	8000	24	20	6	101.52	112.82	1.11	21.97	11.98
9	400	20	15	8	-	-	-	-	-
10	8000	20	15	8	198.26	38.91	0.20	42.91	9.35
11	400	24	15	8	-	-	-	-	-
12	8000	24	15	8	34.34	84.78	2.47	7.43	1.57
13	400	20	20	8	276.08	38.47	0.14	59.76	1.28
14	8000	20	20	8	79.13	130	1.64	17.13	5.95
15	400	24	20	8	274.34	43.04	0.16	59.38	1.35
16	8000	24	20	8	43.47	144.34	3.32	9.41	3.48
17(C)	**3350**	**22**	**17.5**	**7**	**198.69**	**56.95**	**0.29**	**43.01**	**14.24**
18(C)	**3350**	**22**	**17.5**	**7**	**191.95**	**64.78**	**0.34**	**41.55**	**12.60**
19(C)	**3350**	**22**	**17.5**	**7**	**194.78**	**71.52**	**0.37**	**42.16**	**13.80**
20(C)	**3350**	**22**	**17.5**	**7**	**182.17**	**71.52**	**0.39**	**39.43**	**10.71**

[a] PEG molar mass. [b] PEG concentration. [c] Citrate concentration. [d] β-galactosidase activity of bottom phase. [e] β-galactosidase activity of top phase. [f] Partition coefficient. [g] Activity yield of bottom phase. [h] Purification factor of bottom phase. - , no biphasic system. The best results are shown in boldface.

3.1. Partition coefficient and activity yield responses on the β-galactosidase extraction using ATPS

According to observed in Table 2, the majority of runs presented *K* values under 1.0 Although, Lemes (2014) and Silva and Franco (1999) also obtained the partition of respective β-galactosidase for bottom phase, it is not an unusual behavior for β-galactosidase partitioning. Several studies report *K* values over 1.0, partitioning selectively this enzyme for top phase (KOHLER, K., VEIDE, A., ENFORS, S. 1991; HEMAVATHI, A. B. & RAGHAVARAO, K. S. M. S. 2011; KHAYATI, M. et al. 2015), however, none of them used citrate as phase-forming salt. The selection of most appropriate phase-forming salt is one of

the key-ponts of ATPS technique and directly affects the extraction process by influencing on systems environment (KHAYATI, M. et al. 2015). Concerting PEG-Salt ATPS, based on cation and anion effects on relative hydrophobicity of the phases, studies on dinitrophenyl-amino acids partitioning, indicate the advantage of citrate salts over tartarate salts as well as sodium-based salts over potassium-based salts (CHOONIA, H. & LELE, S. 2013).

Statistical analysis of these results revealed that most of the independent variables effects, namely PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}) citrate concentration (C_{CIT}) and pH, and its interactions were statistically significant at a 95% confidence level (Table 3).

For *K* response, all variables are positive effect; meantime M_{PEG} was significantly higher and interaction of C_{PEG} and C_{cit} (x_2x_3) was negative, it means that at high C_{PEG} values and at low C_{cit}, increase the enzyme extraction to bottom phase (Table 2). Although several factors affects the efficiency of partitioning such as hydrophobic properties, addition of salts, electrical potential between the phases, molecular size and molecular conformation. (HERCULANO, et al. 2012).

The use of affinity ligands in ATPS can result in high recovery yields and high purification factors of biological products as it is a primary stage recovery technique. Thus, the overall yield is an important feature of a purification process. The effects of independent variables on activity yield response had similar results of K response (Table 3); however, with highlight for PEG molar mass, citrate concentration and pH. C_{CIT} and pH interaction and M_{PEG} displayed negative and positive effects, respectively, indicating that the decrease on C_{CIT} and pH value and an increase on M_{PEG} causes an improvement on β-galactosidase partition to bottom salt rich phase. It implies that at high M_{PEG} values, the physical space is not enough for the enzyme in the top phase, resulting in the exclusion volume theory for bottom phase; and still, in combination with low C_{CIT} and pH values, occurred *salting on* phenomenon, i.e., an increase in the protein - protein interactions, favoring protein solubility in aqueous medium (Wanderley, M. et al. 2017).

Table 3 Statistical effects calculated for the responses of □-galactosidase extraction from Cladosporium tenuissimum URM 7803 by PEG/citrate ATPS performed according to the 24-experimental design of Table 2.

Variable or Interaction	**A_E[a] (U/mg)**	**K[b]**	**Y[c] (%)**	**PF[d]**
(1) M_{PEG}[e]	2.121*	1.245*	-5.199*	8.230*
(2) C_{PEG}[f]	-0.438	0.468*	-7.047*	-1.700
(3) C_{CIT}[g]	0.215	0.629*	22.585*	0.836
(4) pH	-1.098*	0.585*	**-13.246***	-4.262*

x_1x_2	-0.400	0.478*	-4.741*	-1.552
x_1x_3	-0.242	0.478*	**-44.866***	-0.942
x_1x_4	-0.980*	0.588*	-5.364*	-3.802*
x_2x_3	0.055	**-0.136***	3.047*	0.216
x_2x_4	-0.218	0.524*	-3.847*	-0.845
x_3x_4	-0.142	0.020	1.247	-0.552

[a] Specific activity, [b] Partition coefficient. [c] Activity yield. [d] Purification factor. [e] PEG molar mass. [f] PEG concentration. [g]Citrate concentration. * Statistically significant values ($p < 0.05$)

3.2 Purification factor and β-galactosidase specific activity responses on the β-galactosidase extraction using ATPS

PEG molar mass (x_1) and pH (x_4) main effects of independents variables and the interaction between this two effects were statistically significant at 95% confidence level, which means that. In conformity with the results, pH value decrease and at high M_{PEG} has a positive effect in enzyme extraction, i.e. these both significant interactions showed negative effects, its means that when pH value (x_4) is low and M_{PEG} (x_1) is higher, the enzyme extraction was increased (Figure 1).

An increase in PEG molar mass seems to reduced the space available for β-galactosidase in the top phase. Several authors, in studies using PEG for partitioning different proteins, found relations between *K* and PEG molar mass and that higher molar masses could increase the exclusion effect and then decrease *K* (LIMA, et al. 2013).

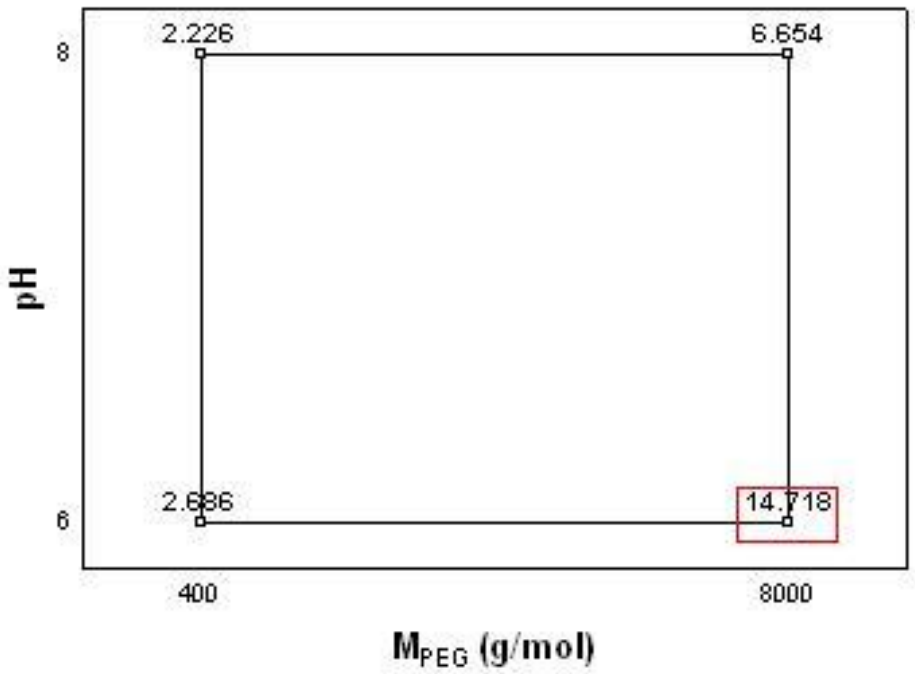

Figure 1 Square plot of the means of purification factor response at different levels of pH and PEG molar mass (MPEG) values according to the fractional factorial design of Table 2. Pure error= 0.002

The effect of different combinations of M_{PEG}, C_{cit} at different pH values on β-galactosidase specific activity response are summarized in Fig. 2. It is noteworthy that the two highest values of A_e (3.851 and 3.736 U/mg) are located on the bottom face of the diagram, which corresponds to pH 6.0, to opposite level combinations (−1 and +1) for C_{cit} (20 and 15%, respectively), and to 8000 g/mol of M_{PEG}. The specific activity values demonstrated that the M_{PEG} and pH had a significant antagonist effect on partitioning. This parameter increased with lower values of pH and at high M_{PEG}. C_{cit} was not statistically significant at 95% confidence level, close to observed for PF response, due to this, using both the lower and the highest concentrations did not interfere on enzyme specific activity.

The pH of the system influences ionizable groups of a protein and alters the protein surface charges. At high pH values, the protein is more negatively charged than at low pH, and therefore, the partition coefficient of the protein increases with increasing the pH [29], which may be due to the electrostatic interactions between the protein and the PEG units [30].

Some studies indicate that negatively charged substances preferentially partition to the top and negatively charged substances to the bottom phases, also that a higher pH can influence the partition coefficient and yield when separating biomolecules in ATPS (HERCULANO, P. et al. 2012). The decrease in the partition coefficient of β-galactosidase

may be associated with the increase of the hydrophobicity of the top phase coupled with the volume exclusion effect resulting from the use of higher PEG molar mass [34]. Babu et al., indicate that there is a relation between the MPEG and partition coeffcicient (*K*) as the increase on molar mass reduces the free space (excluded volume) available for biomolecules in the top phase leading to partition to the bottom phase.

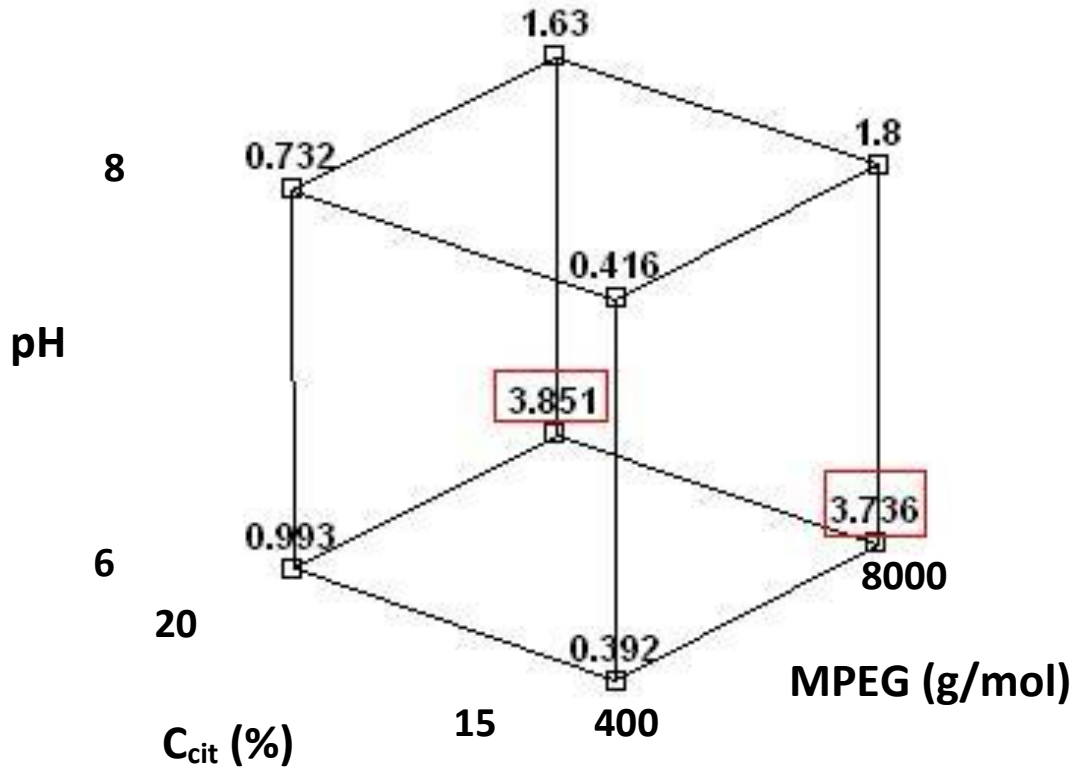

Figure 2 Cubic plot of the means of β-galactosidase specific activity response at different levels of pH, PEG molar mass (MPEG) values and Citrate concentration (Ccit) according to the fractional factorial design of Table 2. Pure error=0.002

Based on the purification factor and β-galactosidase specific activity responses, the best conditions were obtained when the PEG/citrate system was composed of C_{PEG} 20% (w/w), M_{PEG} 8000 (g/mol) and 15% (w/w) sodium citrate at pH 6.0.

Taking this into account, runs 2, 4 along with the center points (17, 18, 19 and 20) highlighted in Table 2 were selected as the best enzyme partition in ATPS, mainly for the results combinations of yield, purification factor and β-galactosidase specific activity (A_e) in bottom phase.

According to previous studies, Silva and Franco (1999) obtained a purification factor equivalent to 2.8, and Hemavathi (2011), only 1.8. In the best condition obtained through experimental design, as can be seen in Figure 1, it is considered that the runs where the mass

molar of PEG were the largest (8000) and pH were the lowest (6), ie, runs 2 and 4, were the best results with respect to purification factor (12.82 and 12.94, respectively).

Silva and Franco (1999) obtained 39% yield using PEG 4000 (6% w/w) and 8% (w/w) dextran. Hemavathi (2011) reported 50% yield with PEG 1500 (14% w/w) and 13% (w/w) ammonium sulfate. Lemes (2014) obtained yield of 101.7% using PEG 4000 (14% w/w) and potassium phosphate pH 7 (15% w/w), however, the purification factor was only 3.8. In addition, Khayati (2015) obtained 85% yield and a purification factor of 38.17 using PEG 2000 (15.24% w/w) and $(NH_4)_2HPO_4$) pH 7 (17.14% w/w). Despite the good results, as mentioned previously, the partition of the enzyme from Khayati (2015) went to the PEG-rich phase, which hinders more its subsequent recovery.

The present work obtained as a purification factor of run 2 (M_{PEG} 8000, 20% w/w, C_{CIT} 15% w/w, pH 6) equal to 12.82, and run 4 (M_{PEG} 8000, 24% w/w C_{CIT} 15% w/w, pH 6) equal to 12.94. Indicating that the concentrations of PEG and citrate were not relevant in obtaining the values found. Also the purification factors obtained were higher than other β-galactosidase ATPS purification methods, and the enzyme partition to the salt phase, which facilitates its recovery.

The validity of the model was verified by analysis of variance presented in Table 4. For *K*, PF and A_e responses, *F* test was significant for all independent variables and the model is adequate to describe the results, i.e., *F* calculated (6.4 for *K*, 4.6 for PF and for A_e 4.6) was higher than *F*-tabulated value (3.06). In relation to Y, F test was not significant at 95% of confidence level, due the lack of fit. For determination coefficient-R^2 obtained by ANOVA analyses for all independent variables effects in responses were between 0.75 to 0.91, a value close to 1 indicated agreement between the experimental and model predicted. The estimated effects and the corresponding *p*-values indicate that independent variables have a significant effect on the response studied ($p<0.05$).

Table 4 Analysis of multiple regression described for the response, K, PF and Ae over the independent variables according to 24 full statistical design.

Response	Df	SS model	MS model	*F-calc*	*F-tab*	R^2	$R^2_{ajusted}$	*p* value
K								
Regression	4	10.032	2.508	6.402	3.061	0.794	0.630	0.003
Resid	15	5.876	0.391					
Total	19	15.908	-					
PF								
Regression	4	357.939	89.484	4.587	3.061	0.550	0.430	0.013
Resid	15	292.604	19.507					
Total	19	650.543	-					
$\boldsymbol{A_e}$								
Regression	4	23.777	5.944	4.587	3.06	0.742	0.550	0.013
Resid	15	19.437	1.296					
Total	19	43.214	-					

All values are statistically significant at 95% confidence level. df= degree of freedom SS model= model quadratic sum MS model= model quadratic mean.

4 Conclusions

The purification technique via aqueous two-phase system (ATPS) was effective for the partition and extraction of β-galactosidase produced by *Cladosporium tenuissimum* URM 7803. The purification factor, yield values and partition coefficient were satisfactory compared to those in the literature relating ATPS to purify β-galactosidase. This is the first record of PEG/Citrate ATPS applied for β-galactosidase extraction. The runs with best values of purification factor, yield and partition coefficient were runs 2 and 4, with higher PEG molar mass (8000) and lower citrate pH (6.0). The β-galactosidase where purified close to 13 folds and partition to the salt fase, facilitating its posterior recovery. The results indicate that ATPS was a fast, efficient, feasible methodology with very interesting results regarding the activities of β-galactosidases desired by the industrial enzymatic market.

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Ethical Statement/Conflict of Interest

The authors are aware of the ethical responsibilities and the manuscript has no conflict of interest.

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3.3 Partial Characterization of a new β-galactosidase Produced by *Cladosporium tenuissimum* URM 7803

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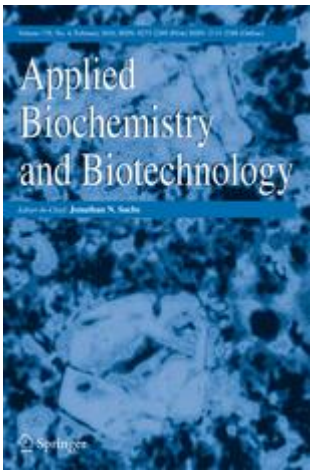

Partial Characterization of a new β-galactosidase Produced by *Cladosporium tenuissimum* URM 7803

Anderson José Paulo

Bioscience Center, Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Av. Prof. Moraes Lins do Rego, s/n, 50670-901, Recife, PE, Brazil

Maria Carolina de Albuquerque Wanderley

Laboratory of Immunopathology Keizo Asami (LIKA), Federal University of Pernambuco, Av. Prof. Moraes Lins do Rego, s/n, 50670-901, Recife, PE, Brazil

Ana Lúcia Figueiredo Porto*

Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, Av. Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil

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(*)Corresponding author:

Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, Av. Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil

analuporto@yahoo.com.br

Telephone number: +55 81 33206391

Fax number: +51 81 21268485

Partial Characterization of a new β-galactosidase Produced by *Cladosporium tenuissimum* **URM 7803**

Abstract

Enzymes have played an important role in many aspects of life since the dawn of time. The β-galactosidase enzyme (EC3.2.1.23) is classified as a hydrolase, capable of transferase to galactosyl groups, catalyzing the β-galactopyranosyl terminal residue of lactose (Galβ1-4Glc) to form glucose and galactose. The objective of this work was characterize partially the crude extract and partially purified extract by PEG-Citrate aqueous two-phase system (ATPS) of a new β-galactosidase produced by *C. tenuissimum* URM 7803. The results showed that the enzyme presented optimum temperature in the range of 35 to 60°C (crude extract) and 40 to 60°C in ATPS extract. The optimal pH for this β-galactosidase was found in the ranging from 3.0 to 4.5, and the enzymatic activity decreased significantly at higher pH values. In relation to the effects of ions in the ATPS extract the Fe^{2+} ion promoted a 38% increase in the hydrolytic activity, while the Zn^{2+}, Cu^{2+} and Co^{2+} ions negatively affected, reducing approximately 25% in its activity. The enzymatic activity was improved or maintained by all the additives and detergents used, except phenylmethylsulfonyl fluoride (PMSF), having reduced by 20 and 12% the activities in the crude extract and ATPS extract, respectively. Therefore, β-galactosidase of *C. tenuissimum* URM 7803 presents good profile for application in the production of prebiotics such as, galactooligosaccharides (GOS), for their behavior in acidic bands of pH and higher temperatures.

Keywords: β-galactosidase, characterization, crude extract, partial purification

1. Introduction

Enzymes are organic biocatalysts, which govern, initiate and control biological reactions important for life processes. The β-galactosidase enzyme (EC3.2.1.23) is classified as a hydrolase, capable of transferase to galactosyl groups, catalyzing the β-galactopyranosyl terminal residue of lactose (Galβ1-4Glc) to form glucose and galactose, and can also enrich the product hydrolyzed with galactooligosaccharides (GOS). They act as a biological reaction catalyst that hydrolyzes β-galactosyl linkages in glycoproteins, polysaccharides, disaccharides, and compounds such as ortho and para-nitrophenyl-β-D-galactosides, the latter two being used to determine β-galactosidase activity. Also known as lactase, it is one of the enzymes most studied and reported in the literature (Santiago, 2002; Awan, 2010).

These enzymes can be produced by large number of microorganisms, such as filamentous fungi, bacteria and yeasts, but are also found among vegetables, particularly almonds, peaches, apricots, apples; in organs of animals such as intestine, brain and placenta (Gekas, López-Leiva, 1985). This enzyme is industrially important because it can be used to avoid lactose crystallization in sweetened, condensed and frozen dairy products such as ice cream and condensed milk and solve problems associated with whey utilization and disposal. In addition, it is used to avoid lactose intolerance complications in individuals who are lactase deficient. Transgalactosylation capable β-galactosidases are used in medical applications such as treatment of disorders and development of digestive supplements. It also has lots of potential applications in food processing, bioremediation, biosensors, diagnosis and treatment of disorders (Asraf, 2010; Pansear et al., 2010).

Different β-galactosidases properties depend on their origin. In general, filamentous fungus lactases have an optimal pH in an acidic range (2.5-4.5) while the optimal pH of lactases from yeasts and bacteria is in a more neutral region (6.0-7.0 and 6.5-7.5, respectively). These different optimum pH conditions allow the selection of the most appropriate lactase for a specific application. In this aspect, filamentous fungus lactases are more suitable for hydrolysis of acid whey, for example (Gekas; López-Leiva, 1985).

Regarding temperature, they are reported as optimal in the range of 35-40° C for bacteria and yeast enzymes and up to 55-60° C for filamentous fungi. The fungal enzyme is the only form not activated by Mg^{2+} or Mn^{2+}. Such operational diversity allows the use of microbial β-galactosidases in acidic foods (acid whey, fermented dairy foods), as well as in milk and sweet whey. B-galactosidase is subject to inhibition by product (galactose), and by

ions as Ca^{2+} e Na^{+} (Parkin, 2010).

The current study was designed to characterize partially β-galactosidase produced by *Cladosporium tenuissimum* URM 7803 in crude extract and to compare its activity with a partially purified extract by poly(ethylene glycol)-citrate (PEG-citrate) aqueous two-phase system using as parameters the temperature, pH, ions, inhibitors and detergents.

2. Materials and Methods

2.1 Microorganism

The *Cladosporium tenuissimum* strain (URM 7803) was obtained at "Micoteca - URM" of Mycology Department of the Federal University of Pernambuco (UFPE), Recife-PE, Brazil, preserved in mineral oil and maintained at 28ºC in Czapek Dox Agar.

2.2 Inoculum preparation and β-galactosidase production

Spore suspensions for inoculum were prepared in sterile saline solution 0.85% (w/v) NaCl containing 0.01% (w/v) Tween 80, and conidia density was adjusted to 10^7 conidia/mL. The fermentation medium is composed of (% w/v): lactose (2), peptone (0.4), yeast extract (0.4) and salts (KH_2PO_4 (0.2), $Na_2HPO_4.12H_2O$ (0.8) and $MgSO_4.7H_2O$ (0.025)) conducted at 28 °C, pH 6.5 and 180 rpm for 312h. An erlenmeyer (50 mL) was collected every 24 hours and β-galactosidase production was evaluated.

2.3 β-galactosidase activity

β-galactosidase activity was determined by incubating samples (50 µL), at 37 °C for 30 minutes, with 50 µL of ONPG solution (3 mM) prepared in sodium-citrate buffer (50 mM pH 4.5). The reaction was stopped by the addition of 200 µL of sodium carbonate (0.1 M) (Nagy et al., 2001). The released *O*-nitrophenol was determined spectrophotometrically at 420 nm. One unit (U) of enzyme was defined as the amount of enzyme that liberates 1 µmol of *O*-nitrophenol from ONPG per minute under the assay conditions.

2.4 β-galactosidase characterization

The crude extract and extract partially purified by PEG-citrate ATPS (composition: PEG molar mass = 8000 g/mol, 20% (w/w) PEG concentration, 15% (w/w) Citrate concentration at pH 6.0) were filtered in 0.2 µm membrane and used for characterization.

The effect of temperature on the enzyme activity was evaluated by determining the β-galactosidase activity in 50 mM sodium-citrate buffer (pH 4.5) at different temperatures (15, 25, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85 °C) using the conditions previously described (section 2.3). The effect of pH on the enzyme activity was evaluated at 37 °C using buffers with different pH (3.0, 3.5, 4.5, 5.5, 6.5, 7.5 and 8,5). The following buffers were used: 50 mM sodium-citrate buffer (pH 3.0–5.5) and 50 mM phosphate-citrate buffer (pH 6.5-8.5). For each pH, the enzymatic activity was determined using the conditions previously described (section 2.3). To study the effect of different metal ions, additives and detergents, the sodium-citrate buffer (50 mM, pH 4.5) was supplemented with each specific compound. For metal ions, the buffer was mixed with the following ions: KCl, NaCl, $BaCl_2$, $MgCl_2$, $ZnSO_4$, $MnCl_2$, $FeCl_2$, $CoCl_2$ and $CuSO_4$. Solutions with final concentration of 10 mM were obtained in each case. For the detergents, different solutions of buffer containing sodium dodecyl sulfate (SDS, 10 mM), Tween-80 (1% w/v) or TritonX-100 (1% w/v) were prepared. For the additives, buffer was supplemented with phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) or ascorbic acid to obtain solutions with final concentration of 10 mM. The effect of the metal ions, detergents and additives was evaluated by incubating 50 µL of enzyme extract with 50 µL of ONPG solutions (3 mM) prepared in the several buffer solutions supplemented with each specific compound. In all cases, the enzymatic activity was determined using the β-galactosidase assay described in section 2.3 (Cardoso et al., 2017).

3. Results and Discussion

Characterization of the crude enzyme and partially purified by ATPS was performed using the respective filtered samples. The results obtained for the effect of temperature and pH on enzyme activity (hydrolytic activity using ONPG as substrate) are shown in Figure 1 and 2, respectively. β-galactosidase from *C. tenuissimum* URM 7803 showed an ideal temperature ranging from 35 to 60 °C (crude extract) and 40 to 60 °C in ATPS extract (increasing approximately 31% in the relative enzymatic activity in this temperature range). However, for lower temperatures, a considerable reduction of the enzymatic activity in the two extracts was observed. The optimal pH for this β-galactosidase was found in the ranging from 3.0 to 4.5, and the enzymatic activity decreased significantly at higher pH values. The results presented for temperature and pH are similar to those reported by other studies using

β-galactosidase producer's filamentous fungi (Gargova et al., 1995; O'Connell and Walsh, 2008; Tonelloto et al., 2014).

According to O'Connell and Walsh, (2008), β-galactosidases of fungal origin have a higher enzymatic activity in acid pH bands, and also have higher activity at high temperatures, which corroborates with the results found for β- galactosidase from *C. tenuissimum* URM 7803.

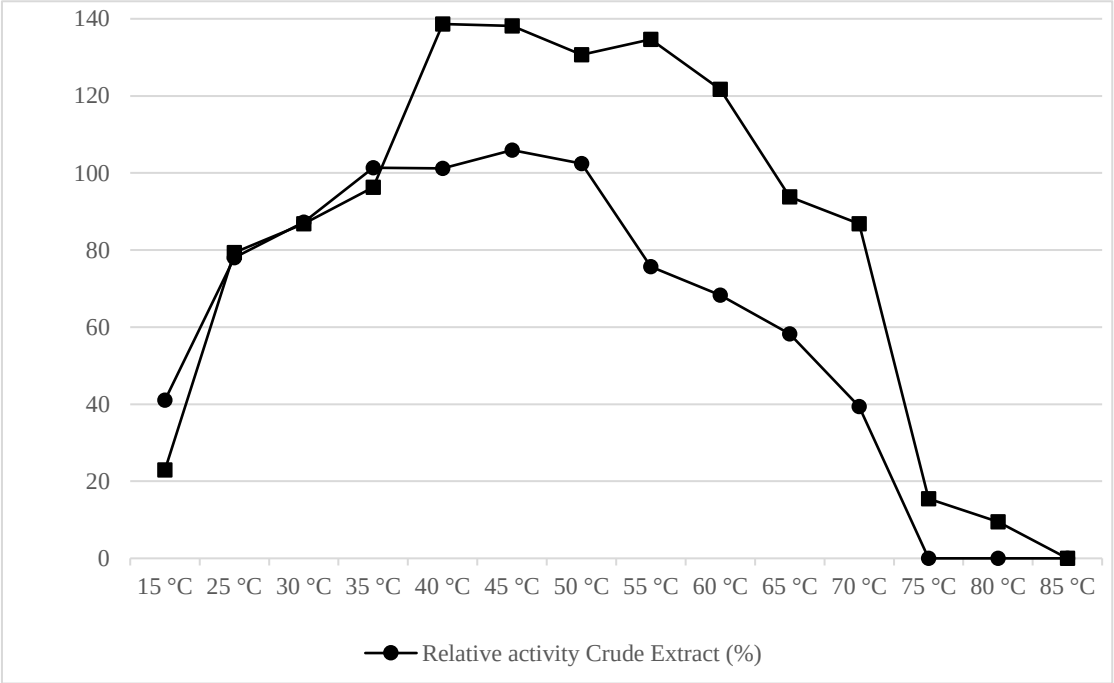

Figure 1 . Effects of temperature on the activity of □-galactosidase from Cladosporium tenuissimum URM 7803 at crude extract (sphere) and purified by ATPS (square), expressed as percentage of the maximum activity observed at pH 4.5 and 37° C.

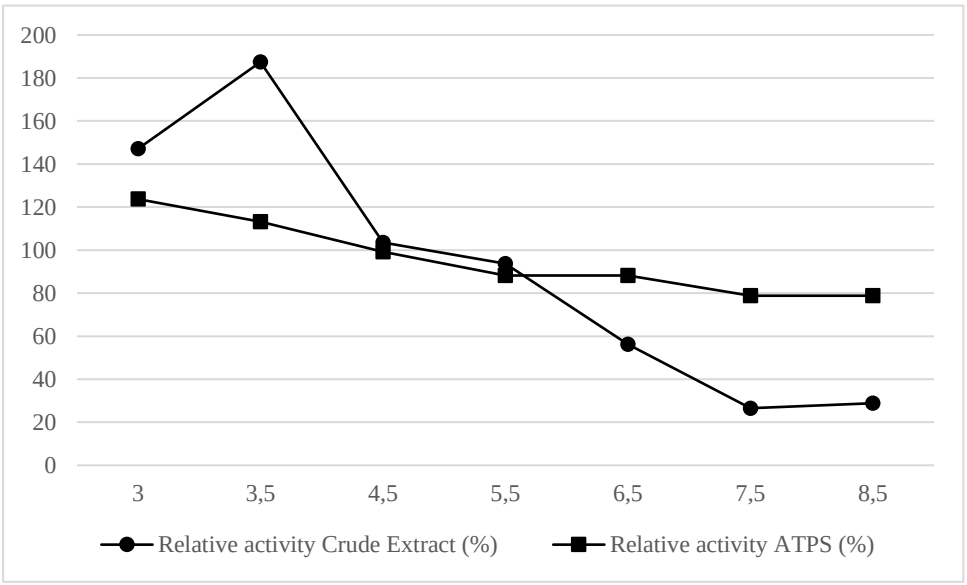

Figure 2 Effects of pH on the activity of □-galactosidase from *Cladosporium tenuissimum* URM 7803 at crude extract (sphere) and purified by ATPS (square), expressed as percentage of the maximum activity observed in different pH at 37 °C.

Like other enzymes, β-galactosidase activity may also depend on the presence of some ions in solvent, that is monovalent and divalent cations (Liu et al., 2015). However, in general, fungal β-galactosidases are reported to be less dependent on this effect (Mlichová and Rosenberg, 2006), also considering that the way the metal ions affect the activity of β-galactosidase is quite dependent on the source of the enzyme.

The mono and divalent cations displayed a significant increase in hydrolytic activity of the crude extract, however, in the ATPS extract, Fe^{2+} ion promoted a 38% increase in the hydrolytic activity, while the Zn^{2+}, Cu^{2+} and Co^{2+} ions negatively affected β-galactosidase, promoting a decrease of approximately 25% in its activity (Figure 3). The inhibitory effect of some divalent metal ions has also been reported for β-galactosidases of *Aspergillus carbonarius* (El-Gindy, 2003) and *A. alliaceus* (Sen et al., 2012).

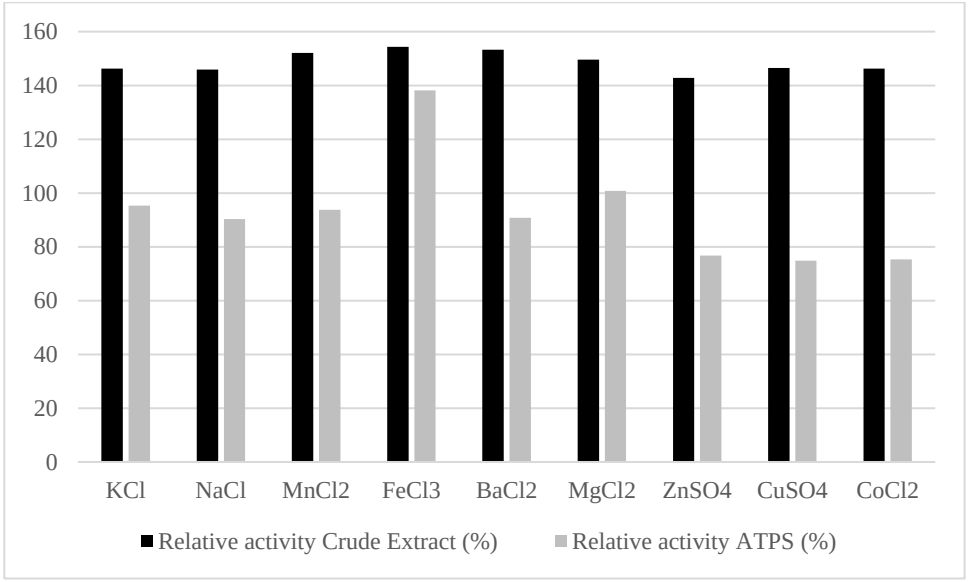

Figure 3 Effects of presence of ions on the activity of □-galactosidase from *Cladosporium tenuissimum* URM 7803 at crude extract (black) and purified by ATPS (gray), expressed as percentage of the maximum activity observed at pH 4.5 and 37 °C.

The effect of some additives and detergents on β-galactosidase activity of *C. tenuissimum* URM 7803 was also studied (Figure 4). It can be seen in Figure 4 that the enzymatic activity was improved or maintained by all the additives and detergents used, except PMSF, which is a serine protease inhibitor, having reduced by 20 and 12% the activities in the crude extract and extract ATPS, respectively. Probably, this effect may be related to the inhibition of proteolytic enzymes possibly present in the crude extract and the ATPS extract, which may promote some β-galactosidase stability during its hydrolytic activity. EDTA, a well known chelating agent, showed a positive effect on enzymatic activity (48% increase in the crude extract and 35% increase in the ATPS extract). This increase may

have occurred due to the EDTA complex with some metal ions present in the fermentation medium. In addition, the effect of EDTA may suggest that β-galactosidase of *C. tenuissimum* URM 7803 does not require metal ions for its hydrolytic activity (El-Gindy, 2003), except for Fe^{2+}, which promoted increase in enzymatic activity, mainly in the ATPS extract. This fact is justified by the results presented in Figure 4, since practically all the metal ions studied did not promote significant improvement in the enzymatic activity (except Fe2 + in the ATPS extract).

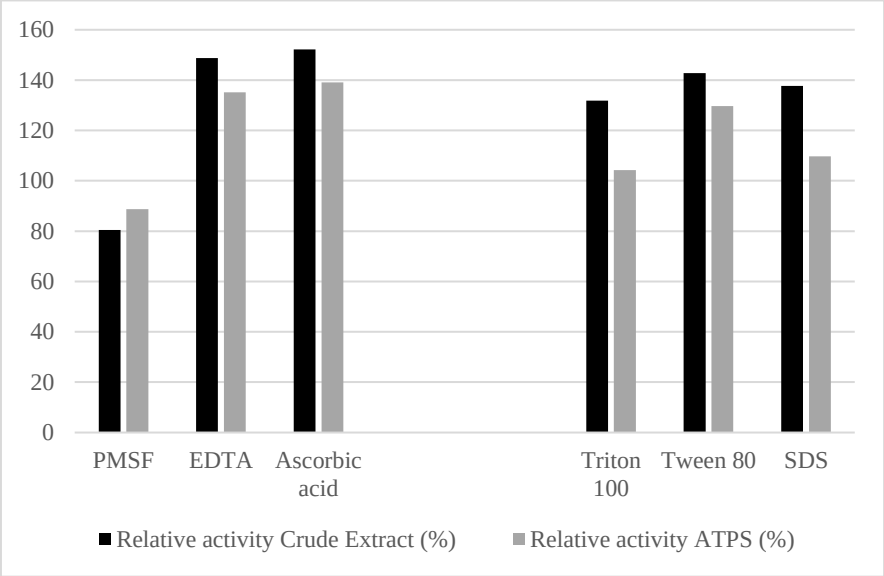

Figure 4 Effects of presence of inhibitors and detergents on the activity of □-galactosidase from *Cladosporium tenuissimum* URM 7803 at crude extract (black) and purified by ATPS (gray), expressed as percentage of the maximum activity observed at pH 4.5 and 37 °C

In relation to detergents, on the addition of SDS, a negative effect on β-galactosidase activity was expected, since this anionic detergent had already been reported as a protein denaturing agent (Bhuyan, 2010). However, the results showed an improvement in the enzymatic activity (increase of 37% in the crude extract, and 10% in the ATPS extract) indicating that possibly higher concentrations of SDS are required to induce β-galactosidase denaturation. Addition of the no anionic detergents Triton X-100 and Tween-80 to the crude extract promoted an increase of 31 and 42%, respectively; already in relation to the extract ATPS this increase was of 4 and 29%, respectively in the enzymatic activity.

4. Conclusion

The β-galactosidase of *C. tenuissimum* URM 7803 presented optimum temperature range of 35-60 ° C (crude extract) and 40-60 ° C in ATPS extract. The optimal pH for this β-galactosidase was found ranging from 3.0 to 4.5, indicating to be an acidophilic enzyme. The

ions showed a positive effect on the enzymatic activity of the crude extract, but in the ATPS extract the Fe2 + ion promoted a 38% increase in the hydrolytic activity, while the Zn2 +, Cu2 + and Co2 + ions negatively affected β-galactosidase, reducing activity in 25%. The detergents and inhibitors promoted relative increase in β-galactosidase activity, except PMSF, which reduced by 20 and 12% the enzymatic activity in crude extract and extract ATPS, respectively. These results show that β-galactosidase of *C. tenuissimum* URM 7803 presents good profile for application in the production of prebiotics such as, galactooligosaccharides, for their behavior in acidic bands of pH and higher temperatures, optimum conditions for production of prebiotics by fungal enzymes.

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Ethical Statement/Conflict of Interest

The authors are aware of the ethical responsibilities and the manuscript has no conflict of interest.

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4 CONCLUSÃO

- *C. tenuissimum* URM 7803 teve sua espécie confirmada através do sequenciamento do fator de alongamento 1-α (TEF1), confirmando que é um novo isolado produtor de β-galactosidase.
- O sistema de duas fases aquosas PEG-Citrato se mostrou eficiente e promoveu uma purificação parcial da enzima, apresentando um fator de purificação de 12.82 (MM_{PEG} 8000, C_{PEG} 20%, C_{CIT} 15%, pH 6,0) e de 12,94 (MM_{PEG} 8000, C_{PEG} 24%, C_{CIT} 15%, pH 6,0) se mostrando deste modo, uma alternativa econômica e, que pode ser aperfeiçoada para aumentar o grau de purificação da β-galactosidase.
- A β-galactosidase apresentou temperatura ótima na faixa de 35-60º C (extrato bruto) e 40-60º C no extrato parcialmente purificado por sistema de duas fases aquosas. Já o pH ótimo ficou na faixa de 3.0 a 4.5.
- Os íons mostraram um efeito positivo na atividade enzimática do extrato bruto, porém no extrato parcialmente purificado, o íon Fe^{2+} promoveu um aumento de 38% na atividade hidrolítica, enquanto os íons Zn^{2+}, Cu^{2+} e Co^{2+} afetaram negativamente a β-galactosidase reduzindo a atividade em média, 25%.
- Os detergentes e inibidores promoveram um aumento relativo da atividade de β-galactosidase, com exceção do PMSF, que reduziu em 20 e 12% a atividade enzimática no extrato bruto e no extrato parcialmente purificado, respectivamente.

5 PERSPECTIVAS

- Realizar eletroforese para demonstrar a eficiência do método de purificação utilizado.
- Realizar ajustes nas concentrações de PEG e Citrato para promover um maior particionamento da enzima, rendimento e fator de purificação no sistema de duas fases aquosas.
- Comparar a purificação por SDFA com métodos cromatográficos como o Äkta.
- Avaliar o perfil de estabilidade da β-galactosidase nas temperaturas e pH ótimos.
- Otimizar a síntese de galacto-oligossacarídeos (GOS) com a β-galactosidase de *C. tenuissimum* URM 7803 e comparar com a enzima comercial.

Realizar o sequenciamento da enzima.

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