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ESTUDOS DE CLONAGEM DA TRANSGLUTAMINASE DE *BACILLUS AMYLOLIQUEFACIENS* EM *ESCHERICHIA COLI* E SUA PRODUÇÃO EM BIORREATORES

LOVAINE SILVA DUARTE

PORTO ALEGRE

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Tese submetida ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos como requisito parcial para obtenção do título de Doutora em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Marco Antônio Záchia Ayub

Coorientador: Prof. Dr. Cristiano Valim Bizarro

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TESE

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Aprovada em: ...../...../.....

Pela Comissão Examinadora:

Prof. Dr. Marco Antônio Záchia Ayub
Orientador - UFRGS

Prof. Dr. Cristiano Valim Bizarro
Coorientador - PUCRS

Prof.ª. Dr ª. Giandra Volpato
Banca – IFRS

Prof.ª. Dr ª. Daniele M. Rossi
Banca – UFRGS

Prof. Dr. Plinho Francisco Hertz
Banca: UFRGS

Homologada em: ...../...../.....

Por:

Prof. Dr. Eliseu Rodrigues

Coordenador do Programa de Pós Graduação

em Ciência e Tecnologia de Alimentos

(PPGCTA)

Profª. Drª. Simone Hickmann Flores

Diretora do Instituto de Ciência e Tecnologia

de Alimentos - ICTA/UFRGS

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RESUMO

As transglutaminases são enzimas amplamente utilizadas em vários processos industriais devido às suas propriedades de reticulação de proteínas, principalmente na indústria alimentícia no processamento de carnes, fabricação de queijos e outros produtos lácteos, panificação e produção de filmes comestíveis. Na área farmacêutica, vem sendo utilizada em reações de PEGilação, produção de conjugados anticorpo-droga, engenharia de tecido e medicina regenerativa. As transglutaminases também podem ser uma alternativa aos tratamentos químicos tradicionais no processamento da lã e do couro. Contudo, os custos extremamente altos da obtenção de transglutaminase de fontes animais impulsionaram as pesquisas na busca de novas fontes dessa enzima. Nestes casos, os esforços têm se concentrado na produção de transglutaminase por microrganismos, que podem apresentar um escopo de uso ainda mais amplo, devido a características específicas da transglutaminase microbiana (mTGase). Esta enzima, desde sua descoberta, tem sido produzida para aplicações industriais pelo processo tradicional de fermentação usando a bactéria *Streptomyces mobaraensis*. A transglutaminase de *Bacillus* (bTGase), descoberta mais recentemente, parece ser uma alternativa promissora à produção de TGase. Estudos anteriores demonstraram que, em comparação à transglutaminase comercial de *Streptomyces mobaraense*, a bTGase é mais estável em uma ampla faixa de pH e temperatura, porém, até o momento, poucos trabalhos foram feitos para produzir ou melhorar os rendimentos de transglutaminase de *Bacillus.* Por esta razão, os objetivos deste trabalho foram clonar e expressar o gene que codifica a TGase de *Bacillus amyloliquefaciens* em *E. coli*, obtendo a proteína em sua forma solúvel e ativa e estudar sua produção em biorreator. Para esse fim, foi construído um plasmídeo bicistrônico contendo o gene da transglutaminase de *B. amyloliquefaciens* fusionado ao prodomínio de *Streptomyces caniferus* para expressar a enzima como um zimogênio inativo. Além disso, foi clonado o gene da protease 3C para ativar a enzima, evitando a necessidade de remover o prodomínio *in vitro*. A proteína foi então purificada usando um protocolo de purificação em uma única etapa e identificada por espectrometria de massa. A atividade da bTGase recombinante foi investigada por ensaios de reticulação da albumina de soro bovino e por fluorescência, demostrando uma atividade específica de 37 $mU/mg_{proteina}$ quando as células recombinantes foram cultivadas em agitador orbital. Com o intuito de melhorar a

produção de transglutaminase, foram estudadas estratégias de cultivo em biorreatores em batelada e batelada alimentada (DO-stat). Após 30 h de cultivo em batelada, foram obtidos 6 g/L de biomassa e atividade específica de 3,12 U/$mg_{proteína}$ em meio Terrific Broth (TB). Melhorias consideráveis no cultivo em batelada alimentada (DO-stat) foram observadas com 17,5 g/L de biomassa e atividade específica de 6,43 U/ $mg_{proteína}$ no mesmo meio de cultivo. Utilizando o meio M9 modificado, a atividade específica chegou a 9,14 U/ $mg_{proteína}$. Como resultado, a investigação traz uma nova visão para o desenvolvimento de transglutaminase para a indústria de alimentos e biotecnológica.

Palavras-chave: Transglutaminase microbiana; Enzimas alimentares; Biorreator; Batelada alimentada; DO-stat; Bacillus amyloliquefaciens; Plasmídeo bicistrônico.

ABSTRACT

Transglutaminases are enzymes widely used in various industrial processes due to their protein cross-linking properties, mainly in the food industry in meat processing, cheese and other dairy products, bakery and edible film production. In the pharmaceutical area, it has been used in PEGylation reactions, production of antibody-drug conjugates, tissue engineering and regenerative medicine. Transglutaminases can also be an alternative to traditional chemical treatments in the processing of wool and leather. However, the extremely high costs of obtaining transglutaminase from animal sources have boosted research in the search for new sources of this enzyme. In these cases, efforts have been focused on the production of transglutaminase by microorganisms, which may have an even wider scope of use, due to the specific characteristics of microbial transglutaminase (mTGase). This enzyme, since its discovery, has been produced for industrial applications by the traditional fermentation process using the bacterium *Streptomyces mobaraensis*. *Bacillus* transglutaminase (bTGase), discovered more recently, appears to be a promising alternative to the production of TGase. Previous studies have shown that, compared to the commercial transglutaminase of *Streptomyces mobaraense*, bTGase is more stable over a wide range of pH and temperature, however, to date, few studies have been done to produce or improve *Bacillus* transglutaminase yields. For this reason, the objectives of this work were to clone and express the gene that encodes the TGase of *Bacillus amyloliquefaciens* in *E. coli*, obtaining the protein in its soluble and active form and to study its production in a bioreactor. For this purpose, a bicistronic plasmid was constructed containing the *B. amyloliquefaciens* transglutaminase gene fused to the *Streptomyces caniferus* prodomain to express the enzyme as an inactive zymogen. In addition, the 3C protease gene was cloned to activate the enzyme, avoiding the need to remove the product *in vitro*. The protein was then purified using a single step purification protocol and identified by mass spectrometry. The activity of the recombinant bTGase was investigated by cross-linking assays of bovine serum albumin and by fluorescence, showing a specific activity of 37 mU/mg protein when the recombinant cells were cultured in an orbital shaker. In order to improve the production of transglutaminase, cultivation strategies in batch and fed batch (DO-stat) were studied. After 30 h of batch cultivation, 6 g/L of biomass and specific activity of 3.12 $U/mg_{protein}$ were obtained in Terrific Broth (TB) medium.

Considerable improvements in fed batch cultivation (DO-stat) were observed with 17.5 g/L of biomass and specific activity of 6.43 U/$mg_{protein}$ in the same culture medium. Using the modified M9 medium, the specific activity reached 9.14 U/$mg_{protein}$. As a result, the research brings a new vision for the development of transglutaminase for the food and biotechnology industry.

Keywords: Microbial Transglutaminase; Fed-batch bioreactor; DO-stat; Bacillus amyloliquefaciens; Bicistronic plasmid system.

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INTRODUÇÃO

Transglutaminase, ou proteína-glutamina γ-glutamil transferase (EC 2.3.2.13), é uma enzima que catalisa reações de acil-transferência e ligações cruzadas intra- e intermoleculares entre proteínas (Fatima e Khare, 2018), estando envolvidas em várias funções fisiológicas, como coagulação do sangue, cicatrização de feridas e queratinização epidérmica, estabilização de complexos fotossintéticos no cloroplasto e morte celular programada em plantas. Além disso, as transglutaminases são amplamente utilizadas na engenharia de tecidos, na pesquisa bioquímica e biomédica e no processamento de têxteis e couro (Griffin *et al.*, 2002; Yokoyama *et al.*, 2004; Serafini-Fracassini e Del Duca, 2008; Zhu e Tramper, 2008; Yang *et al.*, 2011; Heck *et al.*, 2013). Como agente reticulante, a transglutaminase é amplamente utilizada pela indústria alimentícia em vários processos: na fabricação de queijos e outros produtos lácteos, no processamento de carnes, na produção de filmes comestíveis e na fabricação de produtos de panificação (Rachel e Pelletier, 2013; De Góes-Favoni e Bueno, 2014; Kieliszek e Misiewicz, 2014).

Passados mais de 20 anos de sua descoberta, a transglutaminase de *Streptomyces mobaraensis* ainda é a principal fonte dessa enzima para aplicações industriais (Kieliszek e Misiewicz, 2014).

Nos últimos anos, muitos pesquisadores tentaram produzir transglutaminase em *E. coli* e outros microrganismos usando ferramentas de engenharia genética. A expressão dessa enzima tem se mostrado difícil, com baixa atividade ou produzindo proteínas insolúveis em corpos de inclusão (Washizu *et al.*, 1994; Kawai *et al.*, 1997; Liu *et al.*, 2016; Wan *et al.*, 2017).

Neste trabalho, é descrito, pela primeira vez, a expressão e produção de transglutaminase recombinante de *Bacillus amyloliquefaciens* (bTGase) em *E. coli* em sua forma solúvel e ativa. Para isso, construímos um vetor bicistrônico com o gene da protease 3C e o gene que codifica a proteína bTGase de *B. amyloliquefaciens* associado ao prodomínio de *Streptomyces caniferus* (tendo um sítio de reconhecimento de protease 3C entre eles), expressamos a proteína recombinante em *E. coli* BL 21 (DE3) PlysS, identificamos a proteína por espectrometria de massa, purificamos a proteína recombinante e escalonamos a produção em biorreator em sistema de cultivo submerso.

Este trabalho será apresentado na forma de artigos científicos de acordo com as normas estabelecidas pelo Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. No capítulo I é apresentado um artigo já publicado na revista *World Journal of Microbiology and Biotechnology* com o título **"*Transglutaminases: part I - origins, sources, and biotechnological characteristics*"**. Este artigo apresenta uma visão geral da literatura sobre as origens, tipos e caracterizações gerais desta enzima, bem como uma revisão dos principais estudos com transglutaminases recombinantes.

No capítulo II é apresentado mais um artigo publicado na mesma revista *sob* o título "***Review transglutaminases: part II - industrial applications in food, biotechnology, textiles and leather products*".** Esta revisão enfoca na versatilidade das transglutaminases nas áreas de alimentos e biotecnologia. É demonstrado, em vários grupos de alimentos, as possibilidades de aplicação da transglutaminase e os desafios para melhorar ainda mais o produto final. Além disto, são exploradas as aplicações farmacológicas da transglutaminase através da reação de PEGilação, produção de conjugados de anticorpos e medicina regenerativa, bem como as aplicações nas indústrias têxtil e de couro.

No capitulo III é apresentado um artigo publicado na *Enzyme and Microbial Technology*. O artigo foi intitulado "***Cloning and expression of the Bacillus amyloliquefaciens transglutaminase gene in E. coli using a bicistronic vector construction*".** Neste trabalho descrevemos as etapas de clonagem e expressão de TGase recombinante de *Bacillus amyloliquefaciens* em *Escherichia coli*. E para encerrar este estudo, no capítulo IV é apresentado o artigo "***Expression of Bacillus amyloliquefaciens transglutaminase in recombinant E. coli under the control of a bicistronic plasmid system in pulsed fed-batch DO-stat bioreactor cultivations*"** a ser submetido para publicação. Neste artigo, estudamos a expressão da transglutaminase recombinante no cultivo de biorreatores utilizando estratégias de batelada e batelada alimentada (DO-stat).

Para finalizar, são apresentadas considerações sobre os resultados obtidos, bem como algumas perspectivas para futuros trabalhos.

OBJETIVOS

Objetivo geral

O presente trabalho teve como objetivo clonar e expressar o gene codificador da tranglutaminase de *Bacillus amyloliquefaciens* em *E. coli* e estudar sua produção em biorreator.

Objetivos específicos

Esta tese abrange os seguintes objetivos específicos:

- construir um vetor bicistrônico utilizando o vetor comercial pBAD/HisA com os seguintes insertos:
  - o gene sintético que codifica a proteína bTGase de *B. amyloliquefaciens* associado ao prodomínio de *Streptomyces caniferus* (tendo um sítio de reconhecimento de protease 3C entre eles) juntamente com promotor e terminador T7.
  - o gene sintético de protease 3C;
- expressar a proteína recombinante em *E. coli*;
- identificar a proteína por espectrometria de massas;
- purificar a proteína recombinante pelo emprego de cromatografia líquida da proteína rápida (FPLC);
- escalonar a produção em biorreator em sistema de cultivo submerso.

REVISÃO BIBLIOGRÁFICA

A revisão bibliográfica desta tese de doutorado está estruturada no formato de dois capítulos.

No capítulo I é apresentado um artigo de revisão publicado na *World Journal of Microbiology and Biotechnology* com o título ***Transglutaminases: part I - origins, sources, and biotechnological characteristics***. Este artigo apresenta uma visão geral da literatura sobre as origens, tipos e caracterizações gerais desta enzima, bem como uma revisão dos principais estudos com transglutaminses recombinantes.

No capítulo II é apresentado um artigo de revisão publicado na World Journal of Microbiology and Biotechnology com o título "**Review transglutaminases: part II - industrial applications in food, biotechnology, textiles and leather products".** Esta revisão enfoca na versatilidade das transglutaminases nas áreas de alimentos e biotecnologia. É demonstrado, em vários grupos de alimentos, as possibilidades de aplicação da transglutaminase e os desafios para melhorar ainda mais o produto final. Além disto, são exploradas as aplicações farmacológicas da transglutaminase através da reação de PEGilação, produção de conjugados de anticorpos e medicina regenerativa, bem como as aplicações nas indústrias têxtil e de couro.

CAPITULO I - Transglutaminases: part I - origins, sources, and biotechnological characteristics

Lovaine Duarte[a], Carla Roberta Matte[a], Cristiano Valim Bizarro[b], Marco Antônio Záchia Ayub[a]*.

[a]Biotechnology, Bioprocess, and Biocatalysis Group, Food Science and Technology Institute, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, PO Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil;
[b]Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), 92A building at TECNOPUC, 4592 Bento Gonçalves avenue, Porto Alegre 90650-001, Brazil.

*Corresponding author:
Tel.: +55 51 3308 6685; fax: +55 51 3308 7048
E-mail address: mazayub@ufrgs.br (M. A. Z. Ayub)

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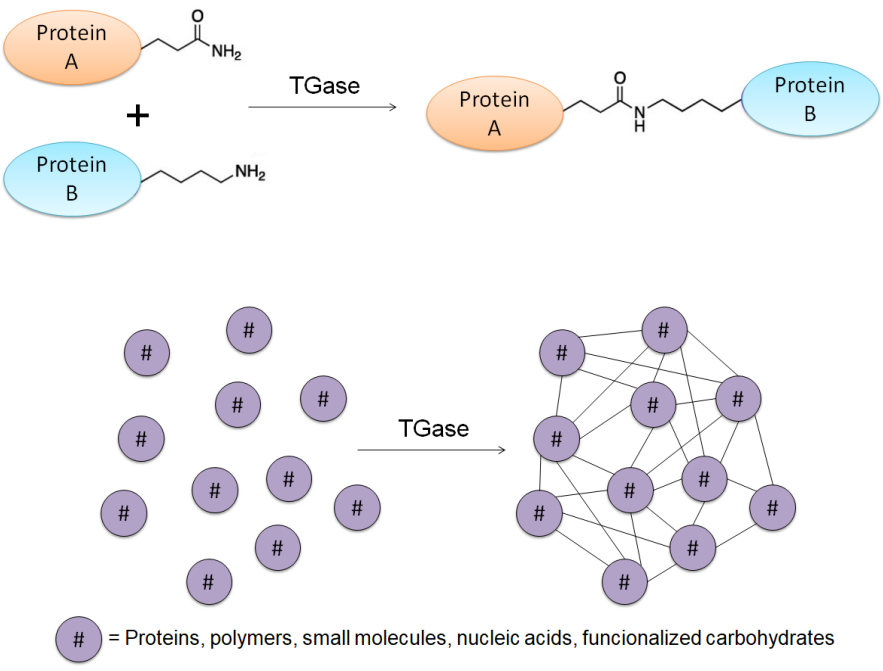

Scheme 1. Graphical abstract

ABSTRACT

The transglutaminases form a large family of intracellular and extracellular enzymes that catalyze cross-links between protein molecules. Transglutaminases crosslinking properties are widely applied to various industrial processes, to improve the firmness, viscosity, elasticity, and water-holding capacity of products in the food and pharmaceutical industries. However, the extremely high costs of obtaining transglutaminases from animal sources have prompted scientists to search for new sources of these enzymes. Therefore, research has been focused on producing transglutaminases by microorganisms, which may present wider scope of use, based on enzyme-specific characteristics. In this review, we present an overview of the literature addressing the origins, types, reactions, and general characterizations of this important enzyme family. A second review will deal with transglutaminases applications in the area of food industry, medicine, pharmaceuticals and biomaterials, as well as applications in the textile and leather industries.

Keywords: *Transglutaminase; Microbial transglutaminases; Protein cross-linking;* Bacillus subtilis; Streptomyces mobaraensis.

INTRODUCTION

Transglutaminase (EC 2.3.2.13, protein-glutamine gamma-glutamyltransferase, TGase) is a calcium-dependent enzyme, belonging to the class of transferases, which catalyzes the acyl-transfer between glutamine residues and a wide variety of primary amines (Ohtsuka et al. 2000). The reaction product results in stable, insoluble macromolecular complexes (Esposito and Caputo 2004). The formation of isopeptide bonds results in both intra- and inter-molecular cross-linking of proteins, the latter leading to protein polymerization (Griffin et al. 2002).

TGases are known to be widely distributed in nature, being found in vertebrates, invertebrates, mollusks, plants, and microorganisms (Folk 1980; Shleikin and Danilov 2011). They are involved in various physiological functions such as blood clotting, wound healing and epidermal keratinization, stabilization of photosynthetic complexes in the chloroplast, and programmed cell death in plants. Moreover, TGases are used extensively in the food industry, in tissue engineering, as well as in biochemical and biomedical research, and textile and leather processing (Griffin et al. 2002; Heck et al. 2013; Serafini-Fracassini and Del Duca 2008; Yang et al. 2011; Yokoyama et al. 2004; Zhu and Tramper 2008).

The term transglutaminase was first introduced in the literature by Clarke et al., 1959 when the authors found an enzyme showing transamidating properties in the guinea-pig liver (Clarke et al. 1959; Folk and Cole 1966). Until late 1980s, the TGase isolated from guinea-pig and from other mammalians blood were the most important sources of this enzyme (Clarke et al. 1957).

Mammalian TGases require Ca^{2+} for activation and show a red pigmentation, affecting the appearance of commercial products, thus none of these enzymes have ever been commercialized or accepted for industrial applications in food production, influencing the search for alternative, convenient commercial sources (Beninati et al. 2008; de Góes-Favoni and Bueno 2014; Jaros et al. 2006; Yokoyama et al. 2004).

In this context, the objective of part I of this review is to present a framework on transglutaminases of mammalian, non-mammalian (invertebrates, plants, fungi), and microbial origins, with special emphasis on the microbial enzymes because of their industrial importance. The properties of transglutaminases of *Streptoverticillium* and *Bacillus* – two of the most important sources of these enzymes - as well as the use of

recombinant microorganisms for their production, are presented in detail. The uses and applications of transglutaminases in the food and biotechnology industries will be presented in a second part review on this subject.

ORIGINS

The evolutionary history of TGase is not fully understood. However, given the similarities in the catalytic triad and the mechanism of transglutaminase reaction, is possible to suggests that transglutaminases have an evolutionary relationship to papain-like thiol proteases whose closest current representative is found in domain NlpC/P60. Clustering of the transglutaminase-like domains by sequence similarity identified a superfamily of proteins homologous to eukaryotic transglutaminases that are found in all archaea, some bacteria and yeast species, and the *Caenorhabditis elegans*. Sequence conservation involves the catalytic triad the transglutaminase. In 1999, Makarova et al. presented a computational analysis of this superfamily, remaining one of the most complete work comparing TGases among prokaryotes and eukaryotes. Since then, many more gene sequences of TGase have been published and a new phylogenetic tree based on the gene sequences are in preparation by our group and should be published soon (Anantharaman and Aravind 2003; Fernandes et al. 2015; Makarova et al. 1999).

In mammalian transglutaminases the catalytic mechanism is based on a triad of non-contiguous amino acids, i.e., Cys-His-Asp, and have the highly conserved active site region (GQCWVF) as can be seen in Figure 1. Microbial transglutaminases show no similarity to mammalian transglutaminases, although they have the same catalytic triad, with a different sequence order, namely Cys-Asp-His (Giordano and Facchiano 2019; Kashiwagi et al. 2002; Whitaker et al. 2002).

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GVSPMSWIGSVDILRRWKNHGCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTNYNSAHDQ   TG1
GRDPRSWNGSVEILKNWKKSGFSPVRYGQCWVFAGTLNTALRSLGIPSRVITNFNSAHDT   TG2
GTSPLHWRGSVAILQKWLKGRYKPVKYGQCWVFAGVLCTVLRCLGIATRVVSNFNSAHDT   TG3
GANPAEWTGSVAILKQWNATGCQPVRYGQCWVFAAVMCTVMRCLGIPTRVITNFDSGHDT   TG4
GVSPLEWKGSVAILQQWSARGGQPVKYGQCWVFASVMCTVMRCLGVPTRVVSNFRSAHNV   TG5
GTNPSAWVGSVEILLSYLRTG-YSVPYGQCWVFAGVTTTVLRCLGLATRTVTNFNSAHDT   TG6
GVPPSAWTGSVDILLEYRSSE-NPVRYGQCWVFAGVFNTFLRCLGIPARIVTNYFSAHDN   TG7
GTAPYKWTGSAPILQQYYNTK-QAVCFGQCWVFAGILTTVLRALGIPARSVTGFDSAHDT  FXIIIa
GALLNKRRGSVPILRQWLTGRGRPVYDGQAWVLAAVACTVLRCLGIPARVVTTFASAQGT Band 4.2
```

Figure 1. Amino acid sequences near the active site of human TGases. Band 4.2 does not show TGase activity because it carries a Cys→Ala substitution at the active site.

TRANSGLUTAMINASE CATALYZED REACTIONS

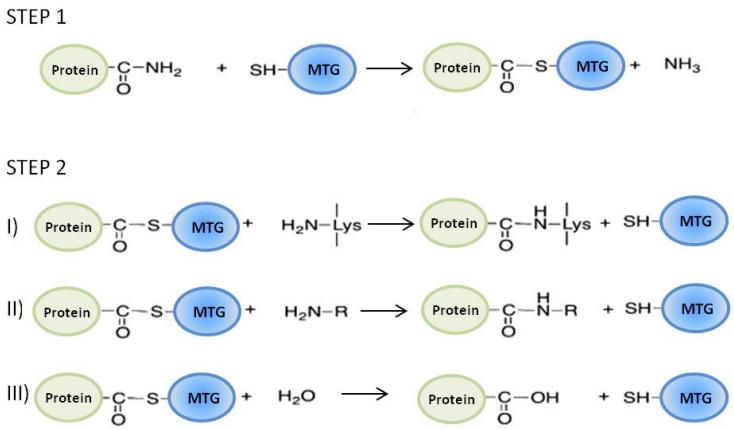
The transamidation reactions catalyzed by TGase, including crosslinking, have attracted major research interests because of the potential applications in both the food and pharmaceutical industries. Amine incorporation and deamidation reactions are also well recognized because of their importance in transglutaminase-mediated post-translational modifications of proteins (Griffin et al. 2002; Lorand and Graham 2003; Yokoyama et al. 2004). The mechanism of action of TGase is the reversion of the proteolysis reaction catalyzed by the thiol proteases (Makarova et al. 1999; Plácido et al. 2008) and consists of two steps residue (Figure 2A). In the first step the cysteine thiol group present in the active site of the enzyme attacks the side chain of the glutamine residue (acyl acceptor) on the protein substrate. In this way, the acyl-enzyme complex is formed with concomitant ammonia release. In the second step, reactions may occur in three different ways (Eckert et al. 2014; Gundemir et al. 2012; Lai et al. 2017; Lorand and Graham 2003; Yang et al. 2011):

I - Crosslinking reaction between a γ-glutamyl containing peptide substrate and either a ε-amine group from a peptide-bound Lys residue (Figure 2A, step 2, reaction I). This type of reaction is kinetically favored at pH > 7 and high substrate concentrations.

II – When transglutaminase promotes the reaction between γ-glutamyl containing peptide substrate and the available primary amine substrate (such as biogenic amines), the enzyme catalyzes the incorporation of the primary amino group and resulting of a γ-glutamyl-amine bond (Figure 2A, step 2, reaction II).

III –When water acts as the acyl acceptor and the resultant hydrolysis reaction yields a glutamic acid (E) residue (Figure 2A, step 2, reaction III).

A



B

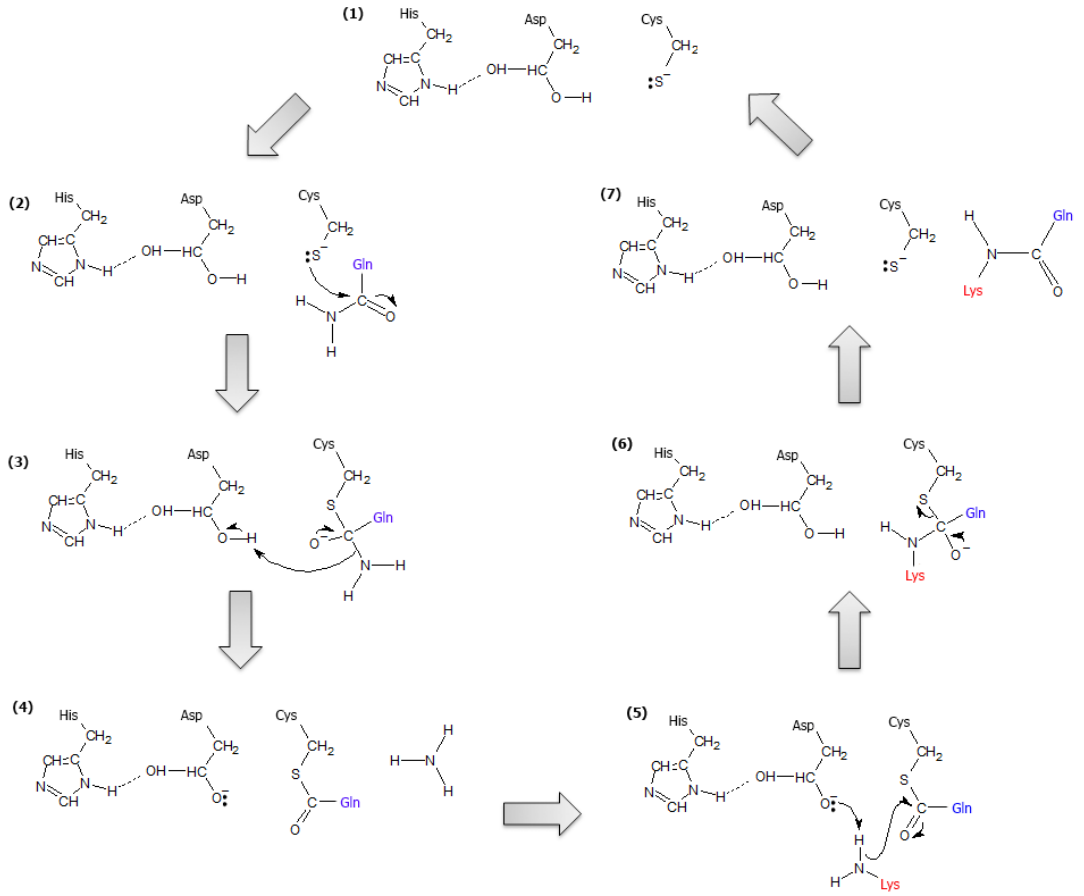


Figure 2. Reactions catalyzed by transglutaminases (TGase). A) Scheme of the reactions in two steps acyl transfer reaction, where Step I is the formation of the intermediate acyl donor-enzyme and ammonia release and Step 2 (I) crosslinking, (II) primary amine incorporation, and (III) deamination with the free enzyme release. B) A hypothetical catalytic mechanism of mTGase of *S. mobaraensis.* The residues of substrate proteins are Gln (substrate 1, blue) and Lys (substrate 2, red). Adapted from Kashiwagi et al. (2002).

All TGase may present high specificity in relation to glutamine substrates and low specify when compared to acyl-acceptor amino group. This acyl-acceptor amino group may have two types: the ε-amino group of the peptide lysine or the low-molecular primary amine (Shleikin and Danilov 2011). Figure 2B shows the possible catalytic mechanism for microbial transglutaminases (based on *S. mobaraensis*). The amino acid residues of mTGase active site are shown in step 1. In step 2, the thiolate ion of Cys producing a nucleophilic attack to an acyl donor, the side chain of the Gln residue (substrate 1). In steps 3 and 4, Asp donates a proton to the resultant oxyanion intermediate, and an ammonium is released. In step 5, an acyl acceptor, such as the side chain of the Lys residue (substrate 2), approaches the active site, and the side chain of Asp, which is now negatively charged, causing a nucleophilic attack to one proton of the acyl acceptor. Finally, in steps 6 and 7, the product is released from the resultant oxyanion intermediate, and the catalytic reaction is finished, and the catalytic site is released for a new reaction, returning to step 1 (Kashiwagi et al. 2002). In this mechanism, the Asp residue plays the role of His residue for Factor XIII transglutaminases (Pedersen et al. 1994).

METHODS FOR MEASURING TGASE ACTIVITY

There are many assay methods for measuring TGase activity described in the literature (Jeoung et al. 2010; Kobayashi et al. 1996; Sokullu et al. 2008). In one of the most used methods, mTGase activity can be determined by the formation of Z-glutamyl-hydroxamate-glycine (a detectable iron (III) colored complex at 525 nm) using Z-Gln-Gly as the amine acceptor substrate and hydroxylamine as amine donor. A calibration curve can be constructed using L-glutamic acid γ-monohydroxamate as standard. One unit of microbial transglutaminase activity is defined as the amount of enzyme causing the formation of 1.0 μmole of hydroxamate per minute, by catalyzing the reaction between Z-Gln-Gly and hydroxylamine at pH 6.0 and 37 ºC (Folk and Cole 1966; Grossowicz et al. 1950)

Additionally, several fluorescent assay procedures have been developed where it is used the increasing fluorescence intensity over time for determining enzymatic activity of transglutaminase. One of these methods consists of covalent coupling of monodansylcadaverine, catalyzed by transglutaminase to N,N-dimethylcasein using

excitation wavelength 332 nm and emission wavelength 500 nm. The increase in fluorescence is proportional to the transglutaminase activity (Lorand et al. 1971).

DIVERSITY OF TRANSGLUTAMINASES

Focus will be given to most studied and used TGases, subdivided into mammalian, non-mammalian, and microbial transglutaminases.

Mammalian Transglutaminases

The animal-like TGases form a large family of intracellular and extracellular enzymes with multiple functions. They are activated by calcium and produced in a zymogenic form, bound by inhibitory subunits, and/or negatively modulated by GTP/GDP or ATP (Esposito and Caputo 2004; Fernandes et al. 2015; Gundemir et al. 2012; Klöck and Khosla 2012; Lorand and Graham 2003).

In mammals, the transglutaminase family comprises nine enzymes: TG1 to TG7, factor XIII, and band 4.2, eight of which encode active enzymes, whereas one of them (erythrocyte membrane protein band 4.2) lacks enzymatic activity. Although the overall primary structure of TGase enzymes appears to be different, they are all encoded by a family of closely related genes. All mammalian TGase genes have been identified and their chromosomal positions have been mapped. Alignment of the gene products reveals a high degree of sequence similarity, with an identical amino acid sequence in the active site (Figure 1). The nine types of TGases of this class and some of their characteristics and functions are described in succession below.

Transglutaminase 1 (TG1), also known as keratinocyte transglutaminase, is an enzyme responsible for the formation of the cornified envelope (CE), acting as a barrier against water loss and protecting against pathogens (Aufenvenne et al. 2013; Eckert et al. 2014). Dehydration, fatal in the first weeks of life, results from autosomal recessive congenital ichthyosis caused by TG1 mutations (Cserhalmi-Friedman et al. 2002; Oji et al. 2010).

The most widely distributed and studied TGase is transglutaminase 2 (TG2), also called tissue transglutaminase. TG2 is expressed by almost all cell types in the body, being active only when bound to calcium. (Eckert et al. 2014; Grenard et al. 2001; Lorand and Graham 2003; Mehta and Eckert 2005).

The main function of TG2 is transamidation, but recent developments show that it is a multifunctional protein acting as a protein disulfide isomerase (PDI), protein kinase, scaffold protein, and even as a DNA hydrolase (Fesus and Piacentini 2002; Gundemir et al. 2012; Lorand and Graham 2003). It has been observed that expression and/or enzymatic activity is increased in several diseases, including Celiac disease, neurodegenerative diseases (e.g., Alzheimer's or Parkinson's disease), cataract formation, atherosclerosis, inflammation, fibrosis, diabetes, autoimmune diseases and in highly aggressive forms of cancer (Griffin et al. 2002; Katt et al. 2018; Lorand and Graham 2003).

Transglutaminase 3 (TG3) is also known as epidermal transglutaminase and is widely expressed in the small intestine, brain, skin, and mucosa (Eckert et al. 2014). Similar to TG1, TG3 is predominantly involved in the formation of the cornified cell envelope (critical structure for barrier function at the outermost layer of the skin epidermis) (Hitomi et al. 2001; Klöck and Khosla 2012). Studies have revealed that the down regulation of the TG3 gene is closely linked with a variety of human cancer types, including esophageal and oral squamous cell carcinoma (OSCC) (Negishi et al. 2009; Uemura et al. 2009).

Transglutaminase 4 (TG4), also known as prostate TG, is present in the prostate gland, prostatic fluids, and seminal plasma. The exact function of TG4 in humans is not well known, but some recent reports suggest a link between increased expression of TG4 and promotion of prostate cancer (Jiang and Ablin 2011; Jiang et al. 2009).

Transglutaminase 5 (TG5), also known as transglutaminase X, is a recently added member of the TGase family (Aeschlimann et al. 1998) and only its limited characterization at functional and biochemical level has been performed (Candi et al. 2004). Similarly to TG1 and TG3, TG5 is expressed in stratified squamous epithelia such as the upper layers of the epidermis, and contributes to hyperkeratosis in ichthyosis and psoriasis patients (Candi et al. 2002). TG5 inactivating mutations result in a rare pathology named Acral Peeling Skin Syndrome (APSS) in which skin peeling is strictly limited to the dorsa of the hands and feet (Cassidy et al. 2005).

Also called transglutaminase Y, Transglutaminase 6 (TG6) expression is compartmentalized in the human testes and lungs, and in the brain of mice (Eckert et al. 2014; Liu et al. 2013). Autoantibodies to TG6 were identified in immune-mediated

ataxia in patients with gluten sensitivity and human carcinoma cells with neuronal characteristics also express TG6 (Thomas et al. 2013).

Transglutaminase 7 (TG7), known as transglutaminase Z, is not fully functionally-understood and few data is known about the regulation or even the function of the TG7 gene. Like TG6, TG7 expression is restricted to testes, lungs, and brain (Eckert et al. 2014). Studying the substrate preferences of TG7, it was identified a highly reactive substrate sequence for TG7 with isozyme-specificity. The knowledge of products that are possibly cross-linked by TG7 will provide more information on the physiological significance of this enzyme and diseases that may be associated with it (Kuramoto et al. 2013).

Factor XIII-A, also known as fibrin stabilizing factor, is a zymogen and becomes active by thrombin. It is a major contributor to clot formation in the final stages of coagulation. It is also important to maintain pregnancy and wound healing. In plasma, it circulates as a tetramer composed of two subunits: a subunit A (FXIII-A) and B (FXIII-B), which requires calcium and thrombin for activation (Tahlan and Ahluwalia 2014). It is produced by the liver, although it can also be found in the extracellular space and cytoplasm of various cells throughout the body (Paragh and Törőcsik 2017). Therefore, the therapeutic potential of FXIII includes invasive bacterial infections, systemic sclerosis (scleroderma), and tissue repair (in healing of venous leg or myocardial ulcers) (Dickneite et al. 2015).

Band 4.2 plays an important role in regulating cell stability and maintaining membrane integrity. It is the only TGase that has no activity because it carries a Cys→Ala substitution at the active site, which makes the protein unable to catalyze the reaction (Figure 1). This inactive TGase is found in several tissues and cells, such as bone marrow, in erythrocytes, fetal liver, and the spleen (Eckert et al. 2014; Mariniello et al. 2008).

Non-mammalian Transglutaminases

The family of TGases has been notably enlarged due to the discovery of novel isoforms in vertebrates as well as in invertebrates, plants, fungi, and microorganisms. TGase activity was observed in different fishes, showing molecular variation among species. It was suggested that TGases may be present in eggs and skin of amphibians, in turtle shell, in epidermis, erythrocytes, and chicken gizzard (Mariniello et al. 2008;

Worratao and Yongsawatdigul 2005). Transglutaminases are also present in plant tissues of soy, fava beans, beet, and orchard apple, whose activities are related to the organization of the cell wall, in antibacterial immune reactions, and in photosynthesis (Falcone et al. 1993; Kang and Cho 1996; Kashiwagi et al. 2002; Kieliszek and Misiewicz 2014; Lilley et al. 1998). It was also confirmed that more than one transglutaminase may function in one plant, or even in one organelle (Sobieszczuk-Nowicka et al. 2009). It is presented in Table 1 some important sources of non-mammalian transglutaminases.

In addition to transglutaminases from bacterial sources (mTGase), discussed below, transglutaminase activity was also found in fungi and yeasts such as *Phytophtora sojae*, *Candida albicans*, and *Saccharomyces cerevisiae* (Brunner et al. 2002; Iranzo et al. 2002; Mazáň and Farkaš 2007; Ruiz-Herrera et al. 1995).

Phytophthora sojae is a soybean pathogen that has been shown to secrete a Ca^{2+} -dependent TGase (GP42), capable of activating defense responses in plants. GP42-related proteins are only present in plant pathogenic oomycetes belonging to the order of Peronosporales (for example, *Phytophthora*, *Hyaloperonospora*, and *Pythium* spp.), and in marine *Vibrio* bacteria. Although GP42 does not share primary sequence similarities with known mammalian or bacterial TGases, it has a central region that has significant similarity to the Group A *Streptococcus* Mac-1 cysteine protease, suggesting the lateral gene transfer between bacteria and oomycetes (Del Duca et al. 2014; Reiss et al. 2011).

In the fungus *Candida albicans*, it has been suggested that the activity of TGase plays an important role in the structural organization of the cell wall possibly through the establishment of cross-links between structural glycoproteins. Activity was detected by incorporation of radioactive putrescine and most of the activity was present in the cell wall. Inhibition of growth by incorporation of cystamine (a TGase inhibitor) was also determined in other strains, demonstrating the importance of transglutaminase in these species. Cystamine also affected cell morphology, whereas the incorporation of high molecular weight proteins covalently bound to the cell wall was inhibited (Reyna-Beltrán et al. 2018; Ruiz-Herrera et al. 1995).

Likewise, in order to determine whether cross-linking of proteins by TGase would be important for *Saccharomyces cerevisiae* growth, TGase cystamine inhibitor has been used. Addition of this compound to the growth medium reduced the growth

rate of *S. cerevisiae* proportionally to the concentration of the inhibitor by altering the cell morphology, indicating that TGase may be involved in the formation of the cell wall (Iranzo et al. 2002).

Microbial transglutaminases

Bacterial TGases, here treated as microbial transglutaminases (mTGases), are part of an extensive transglutaminase family. Although catalyzing the same reactions, mTGases have shown to possess very little sequence similarity to any mammalian TGases (Oteng-Pabi and Keillor 2013). Although the biological function of transglutaminases in microorganisms is unclear, it is known to be a cell wall-associated enzyme and it is suggested that this enzyme may be involved in cross-linking surface proteins from air hyphae and spores of some *Streptomyces*, in addition to the formation of crosslinking between cell wall proteins in *Candida albicans* and *Saccharomyces cerevisiae* and spore coat proteins in *Bacillus subtilis* (Chater et al. 2010; Kobayashi et al. 1998; Strop 2014).

The production of mTGases were first reported by Ando et al. in 1989 for the microorganism *Streptoverticillium mobaraense*, which was later classified as *Streptomyces mobaraensis* (Ando et al. 1989; Zhang et al. 2010). In contrast to many other TGases, the microbial isoforms are not regulated by calcium or guanosine-5′-triphosphate (GTP), which makes these proteins very useful in the food industry because proteins, such as milk caseins, soybean globulins, and myosins, are sensitive and easily precipitated by Ca^{2+} (Strop 2014; Yokoyama et al. 2004). In addition, they have broader substrate specificity, lower deamidation activity, and can be low-costly mass produced by traditional fermentation technologies (Kashiwagi et al. 2002; Mariniello et al. 2008; Ohtsuka et al. 2006).

Since 1998, the enzyme has been recognized as a safe substance (GRAS) for human ingestion by the FDA (Food and Drugs Administration), making mTGases very attractive for the food industry (Gaspar and de Goes-Favoni 2015; Kieliszek and Misiewicz 2014).

After an extensive search in more than 5,000 isolates of microbial origin, *Streptoverticillium* sp. strain S-8112 proved to be the first bacterium producing transglutaminase (Ando et al. 1989). From this finding, this microbial TGase has been the main source of applicable enzyme. Several studies looking for mTGase activities in

microorganisms were carried out and some are listed in the Table 2, however, these activities were mostly identified in strains of *Streptomyces* and *Bacillus* genera (Jiang et al. 2017). Microbial transglutaminases of commercial interest which had their known structures will be discussed below in more detail.

Structure of transglutaminase of Streptomyces mobaraensis

The mTGase isolated from *Streptomyces mobaraensis* is secreted through the membrane as a zymogen (pro-mTGase) and is activated by a proteolytic processing. To activate the original zymogen, *S. mobaraensis* also secretes two proteases that are responsible for the cleavage of the N-terminal pro-peptide (Zotzel et al. 2003a; Zotzel et al. 2003b). Its pro-region with 45-residue N-terminal is essential for efficient protein folding, secretion, and suppression of the enzymatic activity (Yurimoto et al. 2004). It folds into an L-shape and covers the active-site, blocking the substrates from accessing it, thus the site must be cleaved to allow mTGase to be rendered functional (Rachel and Pelletier 2013).

The *S. mobaraensis* mTGase forms a simple monomer showing overall dimensions of 65 × 59 × 41 Å, made up of ≈331 amino acids, with a molecular mass of ≈37 kDa and the isoelectric point at pH 8.9 (Ando et al. 1989; Kashiwagi et al. 2002). The tertiary structure of mTGase has a disk-like structure with a central groove having the active-center with a Cys-Asp-His triad, which is the key to the cross-linking efficiency (Griffin et al. 2002; Kashiwagi et al. 2002; Liu et al. 2006).

Structure of transglutaminase of Bacillus subtilis

A lesser-known bacterial transglutaminase from *Bacillus subtilis* was described in 1996. It has been strongly suggested that *B. subtilis* transglutaminase (bTG) form Ɛ-(Ɣ-glutamyl)lysine bonds and it is implicated in the protection of the bacterium by causing the cross-linking of coat proteins on the surface of a spore (Kobayashi et al. 1996). The coat contributes to spore protection against several physical and chemical hazards, antagonist bactericidal enzymes, and also by playing a key role in the ability of the spore to monitor its immediate environment and to activate germination (Plácido et al. 2008).

The bTG is not related to the mammalian or other microbial transglutaminases, except for their counterparts in *Bacillus* species and some other highly related spore-

formers. This enzyme functions through a catalytic dyad formed by Cys116 and Glu187 or Glu115 and the cysteine residue is required for the activity of bTG *in vitro* and *in vivo*. It also has a NlpC/P60 catalytic core, thought to represent the ancestral unit of the cysteine protease fold (Fernandes et al. 2015; Liu et al. 2014a).

In vitro, bTG is able to cross-link proteins such as BSA or α-casein (Kobayashi et al. 1998). The 20 kDa spore coat protein (GerQ) has been identified as a physiological substrate for bTG (Ragkousi and Setlow 2004; Zilhão et al. 2005). Recently, a study has allowed to screen a library of random highest affinity glutamine substrate sequences for bTG (Oteng-Pabi et al. 2018).

With a protein molecular weight of 28 kDa, bTG is ≈10 kDa smaller than other mTGases and shows little structural homology with the *S. mobaraensis* mTGase. The optimal temperature and pH for bTG activity are 60 ºC and 8.2, respectively. Additionally, bTG is expressed as a mature peptide, unlike mTGase, which exists as pro-enzyme (Oteng-Pabi et al. 2018).

RECOMBINANT MICROBIAL TRANSGLUTAMINASES

The industrial enzyme market was evaluated at around US$ 6.1 billion in 2017 and it is expected to reach US$ 8.5 billion by 2022 (Ferrer et al. 2015). Much of this production is concentrated in enzymes for the food industry. More than 55 different enzyme products are used in the food processing industry and the number is permanently increasing, related to the discovery of new food enzymes (Fernandes 2010).

Aiming to develop innovative, sustainable, and economically competitive production processes, there is an increasing need for new, more versatile and improved enzymes. Novel researches in molecular genetics and cell biology over the past four decades has reconfigured enzyme production. The majority of industrial enzymes are already coming from recombinant sources produced in bacteria, fungi and yeasts (Adrio and Demain 2014; Olempska-Beer et al. 2006). Several studies have been focused in the gene expression of mTGase in *Streptomyces lividans*, *Corynebacterium glutamicum*, *Yarrowia lipolytica*, *Streptomyces platensis*, and *Escherichia coli*, as described below (Date et al. 2004; Lin et al. 2006a; Lin et al. 2004; Liu et al. 2015; Mu et al. 2018b; Rickert et al. 2015; Salis et al. 2015; Washizu et al. 1994). Table 3 shows some of the

recombinant transglutaminases treated in this section, showing details of construction and culture information.

Streptomyces lividans

One of the earliest works on recombinant mTGase expression was presented in 1994 when the *Streptoverticillium mobaraense* gene was cloned and expressed in *Streptomyces lividans* 3131 under the control of a tyrosinase promoter, yielding an active and mature enzyme. However, the secretion level of mTGase in *S. lividans* 3131 was very low, less than 0.1 mg/L, not suitable for industrial applications (Washizu et al. 1994).

The gene mTGase from *Streptoverticillium ladakanum* B1 was cloned and expressed in *Streptomyces lividans* JT46 using an endogenous promoter. The revealed result of immunoblotting of SDS-PAGE indicated that the recombinant mTGase was not correctly processed (Lin et al. 2004). Following ahead with this study, the same group of researchers cloned and expressed the gene of mTGase of *Streptomyces platensis* M5218 in *Streptomyces lividans* JT46, with a 3.3-fold increase in enzyme activity in relation to that from the wild *S. platensis* M5218 strain (Lin et al. 2006b).

The mTGase obtained from *Streptomyces hygroscopicus* WSH03-13 was cloned into plasmid pIJ86 and has been expressed in *S. lividans* TK24. Based on deletion analysis, it was identified a negative element in the mTGase putative promoter, and the deletion of this element increased the mTGase production by up to 81.3 %. Combining optimization of the gene codons and deletion of the negative promoter element, the recombinant *S. lividans* TK24 produced mTGase activities of up to 5.73 U/mL and a maximum productivity of 0.14 U/mL/h (Liu et al. 2016).

Corynebacterium glutamicum

It has been shown that *C. glutamicum* ATCC 13869 is efficiently able to secrete the pro-mTGase from *S. mobaraense* IFO13819, when it is coupled to signal peptides derived from the cell surface proteins of *Corynebacterium*. Moreover, when a protease (SAM-P45) from *Streptomyces albogriseolus* is co-secreted by *C. glutamicum*, the prodomain is then processed, and the enzyme is converted into active-form mTGase. The maximum yield of the active form was 142 mg/L (Kikuchi et al. 2003). Replacing the pro-region of transglutaminases of *Streptomyces mobaraensis* by the pro-region of

transglutaminases of *Streptomyces cinnamoneus* for the production of mTGase in *C. glutamicum*, increased secretion of mTGase by 23 % compared to that using the native pro-region (Date et al. 2004).

Screening for the secretion of pro-mTGase, 16 strains of coryneform bacteria were tested and it was discovered that most of them secreted pro-transglutaminase. The *Corynebacterium ammoniagenes* ATCC6872 was the best producing strain, with about 2.5 g/L pro-transglutaminase over a 71 h culture in a jar fermentor (Itaya and Kikuchi 2008).

In order to improve mTGase secretion on a recombinant *Corynebacterium glutamicum* strain, it was performed a metabolic flux analysis involving ^{13}C isotope-labeling experiments (^{13}C-MFA). The strategy for enhancing mTGase secretion was developed and its effectiveness was confirmed. It was also checked that the increase in the flux to the tricarboxylic acid (TCA) cycle might result in an increase in the NADH/NAD^+ ratio, which is believed to be one of the reasons for the decrease in mTGase yields. In addition, with the aim of decreasing the NADH/NAD^+ ratio, lactate production was increased by raising the pH level in the culture, successfully increasing mTGase production (Umakoshi et al. 2011).

Further improvements on mTGase production in the heterologous host *C. glutamicum* could be achieved by the use of more powerful promoters. The mTGase secreted by *Streptomyces mobaraense*, expressed in *Corynebacterium glutamicum* ATCC, was optimized by the promoter exchange tac for tac-M, with mTGase activity of 5.2 U/mL for the first and 6.7 U/mL for the second construct (Liu et al. 2014b).

Yarrowia lipolytica

In one remarkable research, *Streptomyces hygroscopicus* pro-mTGase was efficiently expressed in *Yarrowia lipolytica*, without the need for antibiotic markers. The gene was cloned into integrative vectors monocopy and multicopy. A recombinant promoter drove the obtained expression and secretion using a XPR2 pre-sequence as a signal peptide. The highest yield of extracellular pro-mTGase was achieved by the recombinant multicopy construct, with 5.3 U/mL of mTGase. In order to improve mTGase properties, asparagines in two predicted Asn-linked glycosylation sites (Asn160 and Asn355) of pro-mTGase were mutated to glutamines. Thereby, the

mTGase yield of variant was increased to 35.3 U/mL by using a glycerol feeding strategy in a 3 L fermenter (Liu et al. 2015).

Escherichia coli

Escherichia coli has been by far the most important bacterium for cloning research. One of the earliest cloning of mTGase was performed in 1994, when mTGase from *Streptoverticillium* was chemically synthesized and inserted in the vector pIN-III with the ompA signal peptide and expressed in *E. coli*. Although the induced gene product was identical to the native enzyme, the activity was low (Takehana et al. 1994). In another attempt to overexpress mTGase, the gene from *Streptoverticillium* was chemically synthesized by fusion to a bacteriophage T7 gene 10 leader peptide (260 amino acids), using an inducible expression vector. The mTGase gene was expressed producing inclusion protein bodies in the *E. coli* cytoplasm. It was necessary to solubilize the protein with subsequent proteolytic cleavage to achieve enzyme activity of mTGase (Kawai et al. 1997).

Using the prodomain engineering, it was possible to achieve good expression levels of soluble and fully active mTGase from *Streptomyces mobarensis* in the cytoplasm of *E. coli*. Through an alanine-scan of the mTGase prodomain and the insertion of the 3C protease cleavage site, it was possible to achieve expression levels of 30 to 75 mg/L of fully active mTGase (Rickert et al. 2015).

mTGase from *Streptomyces hygroscopicus* H197 was mutated by cleaving a specific 84 bp fragment and expressed in plasmid pET32a+ in *E. coli* Rosetta cell, aiming to achieve high stabilities and activities. The purified mutant showed 0.22 U/mg and 0.69 U/mg mTGase activities before and after activated by trypsin, respectively, compared to the wild mTGase 0.16 U/mg and 0.54 U/mg activity under the same conditions (Wan et al. 2017).

The active mTGase expression of *S. mobaraensis* in *E. coli* by one constitutive system was devised without the use of a downstream proteolytic cleavage processing, obtained by constructing a synthetic operon with a prodomain encoding gene and a gene encoding the mTGase thermostable variant, both sequences paired with a previous PelB secretory sequence. The expressed products of this investigation were segregated in the periplasm, making easier the correct folding of the enzymes and reducing the formation of inclusion bodies (Javitt et al. 2017).

Developing novel designs of enzymes took two different types of synthetic components to be simultaneously incorporated into mTGase from *S. mobaraense*, through the engineering of thermostable variants and expressed in *E. coli*. The first amino acid, 3-chloro-l-tyrosine, was incorporated into mTGase in response to in-frame UAG codons to impute an increase thermostability of the enzyme. With this, the half-life was 5.1-fold longer than that of the wild-type enzyme at 60 °C. In sequence, this mTGase variant was further modified by incorporating the α-hydroxy acid analogue of NƐ-allyloxycarbonyl-l-lysine (AlocKOH), specified by the AGG codon, at the end of the N-terminal inhibitory peptide, which led to the overall stabilization of the enzyme (Ohtake et al. 2018).

Pichia pastoris

The first transglutaminase cloned in *Pichia pastoris* was the transglutaminase from *Zea mays*. This TGase was first expressed in *E. coli*, but the recombinant TGase was mainly found as inclusion bodies and the activity of the obtained protein was low (Carvajal et al. 2010). Researchers have since shift to clone *Z. mays* TGase sequences in *P. pastoris* using well-characterized yeast expression vectors, producing a soluble protein. Showing a fast growth rate, when coupled with high cell-density fermentation for secreting proteins that can be purified from the culture medium, *P. pastoris* is a promising cloning system for basic laboratory research and for industrial manufacturing (Weinacker et al. 2013). The expressing of *Z. mays* TGase in *P. pastoris* GS115, using the vector pPIC9K produced specific activities of 0.321 U/mg and mass yields of 4.4 mg/L (Li et al. 2014). Modification of codon bias of *P. pastoris* optimized TGase production and specific activities reached 0.89 U/mg (Li et al. 2013)**.** By applying the Plackett–Burman (P–B) design and the response surface methodology (RSM) using the same expression model, authors found 1.1 U/mL of TGase activity and mass yields of 7.6 mg/L of TGase (Li et al. 2017).

In a recent study, the TGase gene from *Streptomyces fradiae* was cloned and expressed in *Pichia pastoris* GS115, showing enzyme activity of approximately 0.70 U/mL, proving that mTGase can be heterogeneously expressed (Yang and Zhang 2019).

Under the control of the constitutive GAP promoter using *Pichia pastoris*, Türkanoğlu Özçelik and collaborators (2019) expressed the microbial pro transglutaminase (pro-MTGase) from *Streptomyces mobaraensis*. The obtained

enzymatic activity was calculated as 37,640 U/L for large-scale production (Türkanoğlu Özçelik et al. 2019).

Bacillus subtilis

Bacillus subtilis is a Gram-positive, non-pathogenic strain and is generally recognized as safe (GRAS). Its physiology is well investigated, and, for its genetic manipulation, a variety of tools and vectors are available. Moreover, *B. subtilis* does not produce endotoxins, which is an advantage in downstream processing (de Boer Sietske and Diderichsen 1991; Schallmey et al. 2004). Two different secretion systems were constructed for cloning and secretion of mTGase from *S. mobaraensis* in *B. subtilis*. One involves inducible expression, under the control of the promoter P lac and the other containing a constitutive expression under the control of the promoter P hpaII. With peptides signals fused to the mTGase gene, it was possible to secrete pro-mTGase into the medium. After proteolysis of the prodomain with trypsin, the concentrations of transglutaminase were: 63 mg/L for the constitutive system and 54 mg/L for the inducible system, showing enzymatic activities as high as 29 U/mg (Mu et al. 2018a).

CONCLUSION

Transglutaminases remain as one of the most important and complex family of enzymes, possessing varied structures and functions in mammalians, non-mammalian eukaryotes, and in bacteria. In recent years, several studies have been performed in relation to gene expression of transglutaminases, in order to gain versatility and to obtain more stable enzymes for broader industrial applications. Reduction of costs of production are essential aiming their application on a larger scale in industrial sectors such as in food production and biotechnological products.

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TABLES

Table 1. Non-mammalian Transglutaminases.

Organism	Common name	Species	Optima temperature and pH, molecular weight	Reference
Fishes	Alasca Pollack	NA	85 kDa	Seki et al. (1990)
	Red sea bream	*Pagrus major*	pH 9.0–9.5, 78 kDa	Yasueda et al. (1994)
	Japanese oysters	*Crassostrea gigas*	40 °C, 84 kDa; 25 °C, 90 kDa; pH 8	Kumazawa et al. (1997)
	Tropical tilápia	*Oreochromis niloticus*	37–50 °C, pH 7.5, 85 kDa	Worratao and Yongsawatdigul (2005)
	Threadfin bream	TB, *Nemipterus sp.*	pH 7.5, 66 kDa	Piyadhammaviboon and Yongsawatdigul (2009)
	Four different fish species (Bigeye snapper, Indian oil sardine, Tilapia and Common carp)	NA	Range 73-95 kDa	Binsi and Shamasundar (2012)
Invertebrates	Shrimp	*Marsupenaeus japonicus*	85 kDa	Chen et al. (2005)
	Antarctic krill	*Euphausia superba*	0–10 °C, pH 8.0–9.0	Zhang et al. (2017)
	Crayfish	*Pacifastacus leniusculus*	4–22 °C	Sirikharin et al. (2018)
	Mythimna separata larvae	*Noctuidae, Lepidoptera*	6–42 °C, pH 7.5, 3.5 KDa	Zhang et al. (2018)
Plants	Tubers of Jerusalem artichoke	*Helianthus tuberosus*	NA	Serafini-Fracassini et al. (1988)
	Maize	*Zea mays*	NA	Villalobos et al. (2004)
	Rosemary	*Rosmarinus officinalis* L.	55 °C, pH 7.0	El-Hofi et al. (2014)

NA: data not available

1 Table 2. Microorganisms producing transglutaminases.

Species	Strain	Conditions of Cultivation	Activity	Optima temperature and pH, and molecular weight	Reference
Streptoverticillium mobaraensis	S-SI12	Polypepton, glucose, K_2HPO_4, $MgSO_4$ at 30 ºC for 48 h	2.5 U/mL	50 ºC, pH 6, 40 kDa	Ando et al. (1989)
	S-SI12	Polypeptone, yeast extract, K_2HPO_4, $MgSO_4$, potato starch, glucose, pH 7.0 at 30 °C for 9 - 11 days	2.1 U/mL	50 ºC, pH 6, 40 kDa	Gerber et al. (1994) and Ando et al. (1989)
	CBS 20778	Soluble starch, peptone, $MgSO_4$, KH_2PO_4, K_2HPO_4, yeast extract and was supplemented with certain amino acids, pH 6.5 at 28 ºC for 48 h	1.8 U/mL	NA	Zhu et al. (1998)
	CBS 20778 (WSH-Z2)	Starch, peptone, yeast extract, MgSO4, K_2HPO_4, KH_2PO_4, pH7.0 at 30 ºC for 7 days	2.9 U/mL	30 ºC	Zheng et al. (2002)
	CBS 20778 (WSH-Z2)	Starch, glucose, peptone, yeast extract, $MgSO_4$, K_2HPO_4, KH_2PO_4, pH 6.8 at 30 ºC for 48 h	3.3 U/mL	NA	Yan et al. (2005)
Streptoverticillium sp.	s-8112	NA	NA	37 kDa	Kanaji et al. (1993)
Streptoverticillium cinnamoneum	CBS 683.68	Soya peptones, casein, glycerol, $MgSO_4$, KH_2PO_4, Na_2HPO_4, yeast extract, and oligoelements ($FeSO_4$, $ZnSO_4$, $MnSO_4$) at 28 °C for 120 h	0.3 U/mL	37 °C, pH 6	Junqua et al. (1997)
Streptoverticillium ladakanum	ATCC 27441	Glycerol, yeast extract, K_2HPO_4, $MgSO_4$, pH 7.0 at 28 ºC for 4 days	NA	40 °C, pH 5.5	Ho et al. (2000)
	NRRL-3191	Xylose, yeast extract, peptone, $MgSO_4$, KH_2PO_4, Na_2HPO_4 and sodium caseinate at 26 °C for 120 h	0.3 U/mL	NA	Téllez-Luis et al. (2004)
Streptomyces lydicus	NA	NA	2.2 IU/mg	37 ºC, pH 6, 37 kDa	Langston et al. (2007)
Streptomyces nigrescens	NA	NA	0.6 IU/mg	37 ºC, pH 8, 36 kDa	Langston et al. (2007)

Streptomyces hachijoensis	NA	NA	1.9 IU/mg	35 kDa	Langston et al. (2007)
Streptomyces cinnamoneus	NA	NA	0.2 IU/mg	39 kDa	Langston et al. (2007)
Streptomyces hygroscopicus	WSH03-13	Starch, glucose, glycerin, peptone, soybean powder, yeast extract, $MgSO_4$, K_2HPO_4, KH_2PO_4 and $CaCO_3$, pH 6.5 at 32 °C for 42 h	NA	37-45 °C, pH 6-7, 38 kDa	Cui et al. (2007)
Streptomyces mobaraensis	NA	NA	3.9 IU/mg	37 ºC, pH 8, 37 kDa	Langston et al. (2007)
	NRRL B-3729	Wheat bran–soybean meal mixture (9:1), KH_2PO_4, NH_4NO_3, $MgSO_4$, NaCl, $CoCl_2$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, pH 6 at 30 °C for 7 days	800 IU/mg	50 ºC, pH 7, 37 kDa	Nagy and Szakacs (2008)
Streptomyces platensis	NA	NA	1.5 IU/mg	37 ºC, pH 7-8, 38 kDa	Langston et al. (2007)
	NRRL 2364	Liver kidney bean, KH_2PO_4, NH_4NO_3, $MgSO_4$, NaCl, $CoCl_2$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, pH 6 at 30 °C for 7 days	5,100 IU/mg	45 ºC, pH 8, 37 kDa	Nagy and Szakacs (2008)
Streptomyces paucisporogenes	ATCC 12596	Liver kidney bean, KH_2PO_4, NH_4NO_3, $MgSO_4$, NaCl, $CoCl_2$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, pH 6 at 30 °C for 4 days	4,200 IU/mg	45 ºC, pH 8, 37 kDa	Nagy and Szakacs (2008)
Streptomyces sp.	CBMAI 837	Soybean flour, potato starch, glucose, peptone, KH_2PO_4, and $MgSO_4$ at 30 °C for 5 days	0.4 U/mL	35-40 ºC, pH 6-6.5, 45 kDa	Macedo et al. (2011)
Bacillus subtilis	AJ12866	Schaeffer's sporulation medium (SSM) at 37 °C for 18 h	NA	50 °C, pH 8, 23 kDa	Kobayashi et al. (1998)
	AJ1307	Schaeffer's sporulation medium (SSM) at 37 °C for 9.5 h	NA	60 °C, pH 8.2, 29 kDa	Suzuki et al. (2000)
Bacillus circulans	BL32	SLC medium (soluble starch, peptone, yeast extract, $MgSO_4$, K_2HPO_4 and KH_2PO_4), pH 7 at 30 ºC for 24 h	0.69 U/mL	NA	de Barros Soares et al. (2003a)

	BL32	SLC medium (soluble starch, peptone, yeast extract, $MgSO_4$, K_2HPO_4 and KH_2PO_4), pH 6.5 at 30 ºC for 240 h	NA	47 °C, pH 7, 45 kDa	De Barros Soares et al. (2003b)
	BL32	Optimized medium (glycerol, sucrose, peptone, tryptone, Na_2HPO_4, $MgSO_4$ and $FeSO_4$) at 30 ºC for 240 h	0.31 U/mL	NA	Souza et al. (2006)

1 NA: data not available

2

1 Table 3. Recombinants mTGases.

Recipient Strains	**TGase gene Donor**	**Expression Construction**	**Substrate Induction**	**Conditions of Cultivation**	**Activity / Yield / Specific Productivity**	**Reference**
Streptomyces lividans 3131-TS	*Streptoverticillium mobaraense* S-8112	pIJ702-derived plasmids, tyrosinase promoter	20 μg/ml thiostrepton and 40 μg/mL tyrosine	30°C for 5 day	0.1 mg/L	Washizu et al. (1994)
Streptomyces lividans JT46	*Streptomyces platensis* M5218	pIJ702 and pAE053	20 μg/mL thiostrepton and 40 μg/mL tyrosine	R2YE agar or liquid medium (glucose, trypton peptone, KH_2PO_4, $MgSO_4$, yeast extract, and glycine), 30 ºC for 3 days	2.2 U/mL	Lin et al. (2006b)
Streptomyces lividans TK24	*Streptomyces hygroscopicus* WSH03-13	pIJ86	endogenous promoter or its partially deleted (50 μg/mL apramycine)	R2YE agar or liquid medium (glycerol, peptone, yeast extract, $MgSO_4$, K_2HPO_4, KH_2PO_4, and $CaCl_2$), 30 °C and 200 rpm for 2–3 days	5.73 U/mL and 0.14 U/mL/h	Liu et al. (2016)
Corynebacterium glutamicum ATCC 13869	*Streptoverticillium mobaraense* IFO13819	Fusion gene is controlled by the *cspB* promoter	Signal peptides	MMTG medium (glucose, $MgSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, $FeSO_4$, $MnSO_4$, $CaCO_3$, thiamine, hydrochloride, biotin, and DL-methionine), pH 7.5 at 30°C for 140 h	142 mg/L	Kikuchi et al. (2003)
Corynebacterium glutamicum ATCC 13032	*Streptomyces mobaraense CICC 11018*	pXMJ19, tac-M promoter	Signal peptide ΔS0949, IPTG inducer	MMTG medium at 30 °C for 12 h, followed by an additional 40 h cultivation after induction	6.7 U/mL	Liu et al. (2014b)

Yarrowia lipolytica Po1h	*Streptomyces hygroscopicus* WSH03-13	pINA1296 (a pBR322-based mono-copy integrative vector) and pINA1297 (an auto-cloning multi-copy integrative vector)	NA	Modified PPB medium (glucose, yeast extract, NH4Cl, KH2PO4, MgSO4, and thiamine), pH 6.0, at 28 °C, 200 rpm for 5 days	5.3 U/mL	Liu et al. (2015)
Escherichia coli Rosetta (DE3)	*Streptomyces hygroscopicus* H197	pET32a+	IPTG	LB medium containing 100 μg/mL ampicillin and incubated at 37 °C or 25°C for 8 h	0.69 U/mg	Wan et al. (2017)
Escherichia coli BL21 (DE3)	*Streptomyces mobaraensis*	pET22b	NA	Terrific Broth containing 25 μg/mL kanamycin at 25 °C for 60 h	120 mg/L or 1 U/mL	Javitt et al. (2017)
Pichia pastoris GS115	*Zea mays*	pPIC9K	Methanol (5%, v/v) once every 24 h	BMMY medium (except methanol instead of glycerol) at 28 °C at 250 rpm for 96 h	4.4 mg/L and 0.889 U/mg	Li et al. (2013)
Pichia pastoris GS115	*Streptomyces fradiae*	pPIC9K	Methanol (5%, v/v) once every 24 h	BMMY medium (yeast extract, peptone, 0.1 M potassium phosphate, yeast nitrogenous base without amino acids, methanol, and biotin) pH 6.0, at 30 °C for 4 days	0.70 U/mL	Yang and Zhang (2019)

NA: data not available

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CAPÍTULO II - Review transglutaminases: part II - industrial applications in food, biotechnology, textiles and leather products

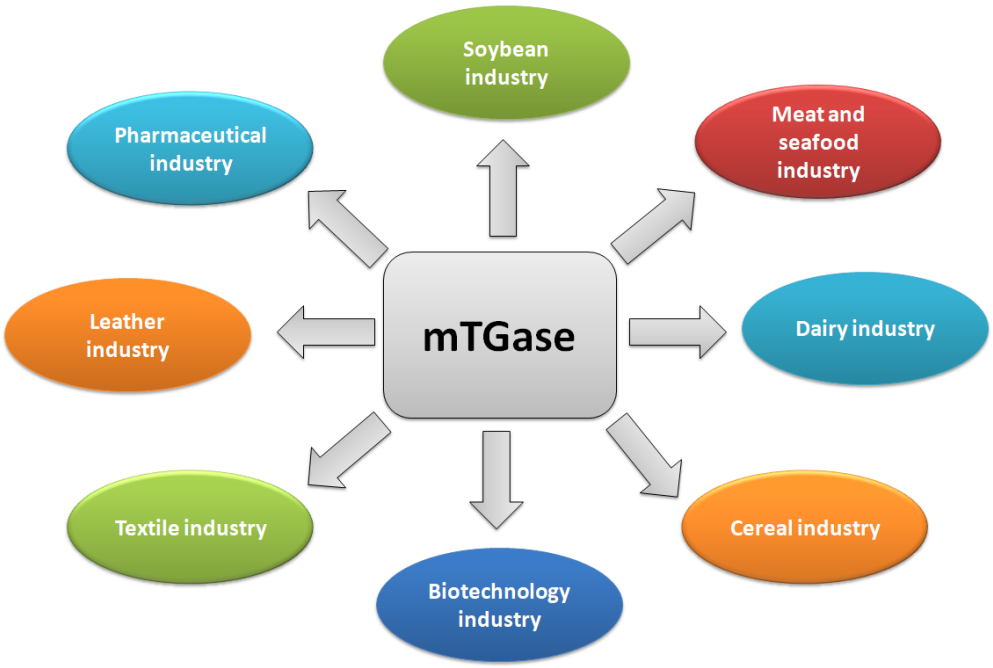
Lovaine Duarte[a], Carla Roberta Matte[a], Cristiano Valim Bizarro[b], Marco Antônio Záchia Ayub[a*]

[a]Biotechnology, Bioprocess, and Biocatalysis Group, Food Science and Technology Institute, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, PO Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil;

[b]Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Building 92A, TECNOPUC, Av. Bento Gonçalves 4592, Porto Alegre 90650-001, Brazil.

*Corresponding author:
Tel.: +55 51 3308 6685; fax: +55 51 3308 7048
E-mail address: mazayub@ufrgs.br (M. A. Z. Ayub)

https://link.springer.com/article/10.1007%2Fs11274-019-2791-x



Scheme 1. Graphical abstract

ABSTRACT

Because of their protein cross-linking properties, transglutaminases are widely used in several industrial processes, including the food and pharmaceutical industries. Transglutaminases obtained from animal tissues and organs, the first sources of this enzyme, are being replaced by microbial sources, which are cheaper and easier to produce and purify. Since the discovery of microbial transglutaminase (mTGase), the enzyme has been produced for industrial applications by traditional fermentation process using the bacterium *Streptomyces mobaraensis*. Several studies have been carried out in this field to increase the enzyme industrial productivity. Researches on gene expression encoding transglutaminase biosynthesis were performed in *Streptomyces lividans, Escherichia coli, Corynebacterium glutamicum, Yarrowia lipolytica, and Pichia pastoris*. In the first part of this review, we presented an overview of the literature on the origins, types, mediated reactions, and general characterizations of these important enzymes, as well as the studies on recombinant microbial transglutaminases. In this second part, we focus on the application versatility of mTGase in three broad areas: food, pharmacological, and biotechnological industries. The use of mTGase is presented for several food groups, showing possibilities of applications and challenges to further improve the quality of the end-products. Some applications in the textile and leather industries are also reviewed, as well as special applications in the PEGylation reaction, in the production of antibody drug conjugates, and in regenerative medicine.

Keywords: Transglutaminase; Microbial Transglutaminases; Protein Cross-Linking; Food-enhancing Enzymes; *Streptomyces mobaraensis*.

INTRODUCTION

Starting in 1989, microbial transglutaminases (mTGase) have been produced for industrial applications through traditional fermentation process using *Streptomyces mobaraensis* bacterium. mTGase is produced by this microorganism as an extracellular enzyme, having a molecular mass of approximately 38 kDa. This enzyme acts in a wide range of pH and temperatures (pH 5.0 to 8.0, and active in between 40 and 70 °C). *S. mobaraensis* mTGase is Ca^{2+} independent, and its activation requires no special cofactors (Ando, Adachi et al. 1989, Yokoyama, Nio et al. 2004).

Enzymatic modifications of proteins mediated by mTGase have been generally used as tools for improving the properties of a given target product. These enzymatic reactions provide high specificity, occurring under gentle reaction conditions, producing no toxic products (Fatima and Khare 2018). In recent years, researchers have intensified their search for application possibilities of the mTGase to obtain methods and products that can alter the technological and functional properties of final products, not only in the food industry, but also in several biochemical reactions.

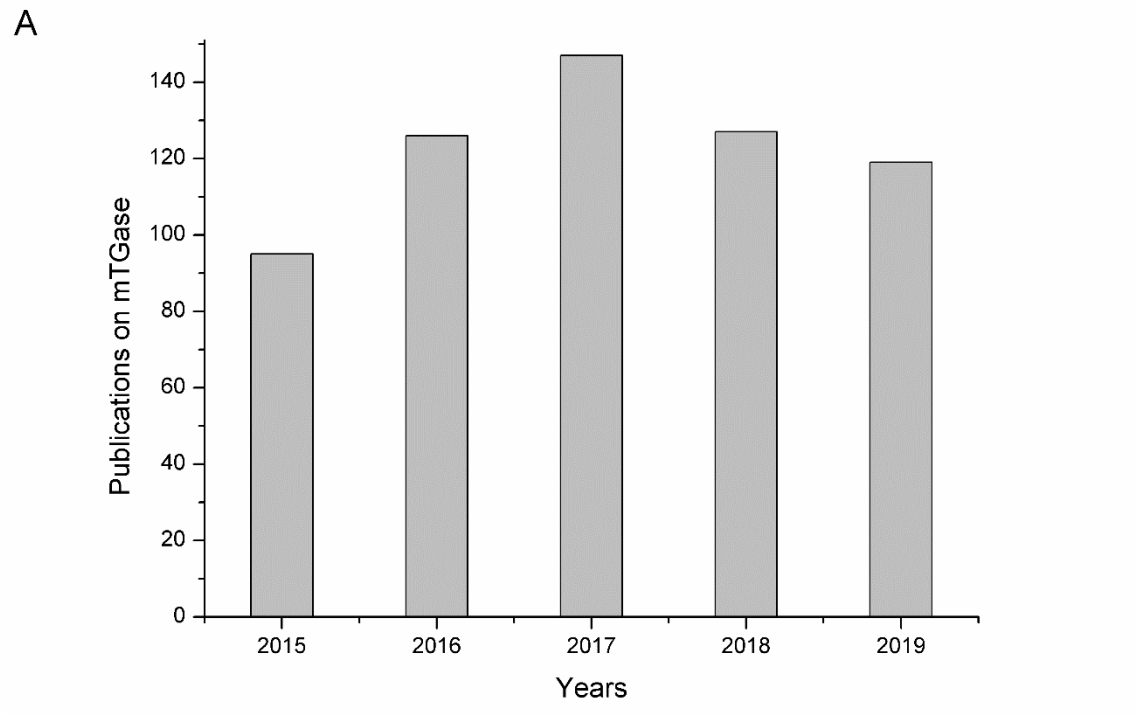
In the first part of this review, we focused on general aspects of the origins, reactions, and characteristics of transglutaminases of mammals, non-mammals, and microbial sources. Some studies on recombinant transglutaminases were also covered. In this second-part of the review, we highlight the importance of mTGase in four major research areas showing potential of applications of this enzyme: the food, biotechnology, leather, and textile industries.

FOOD APPLICATIONS

Studies for the application of transglutaminases in food technology began in the nineties, after the discovery of mTGase in microorganisms such as *Streptomyces mobaraensis (Streptoverticillium mobaraense)* (Ando, Adachi et al. 1989), *Streptomyces cinnamoneum* (Duran, Junqua et al. 1998), and *Bacillus subtilis* (Suzuki, Izawa et al. 2000). The first application of mTGase in food technology was reported by Gottmann and Sprössler in 1992 (1992), who reported that mTGase could be a cost-effective enzyme to be used in food applications. Two decades later mTGases are mainly used in the processing of meat, fish, dairy, and baking products (Strop 2014).

mTGase modifies the functional properties of food proteins by incorporation of amines, crosslinking, deamidation, and bonding surfaces of foods. However, in protein-containing food systems, the cross-linking reaction proceeds prior to other reactions (Santhi, Kalaikannan et al. 2017). In Table 1 are shown relevant aspects of some investigations on the use of mTGase for the modification of properties in different foods, according to the nature of the protein substrate, the amount of enzyme used, and the conditions of enzymatic reactions. Understanding mTGase mechanisms of action in altering protein properties is of major importance for its industrial use, which has not been completely elucidated (Gaspar and de Góes-Favoni 2015).

The first industrial scale production of mTGase was performed by the Japanese company Ajinomoto Co., in collaboration with Amano Enzyme Co. (Nagoya, Japan). The interest of the scientific community in mTGases is demonstrated by approximately 615 published papers in the last five years (Figure 1 A) investigating their applicability, structural characteristics, and substrate specificities, whereas 346 of these papers deal with applications in the field of Food Science and Technology (Figure 1 B), the area showing the greatest interest in this enzyme, as shown in Figure 1 C (Web of Science: June 2019).



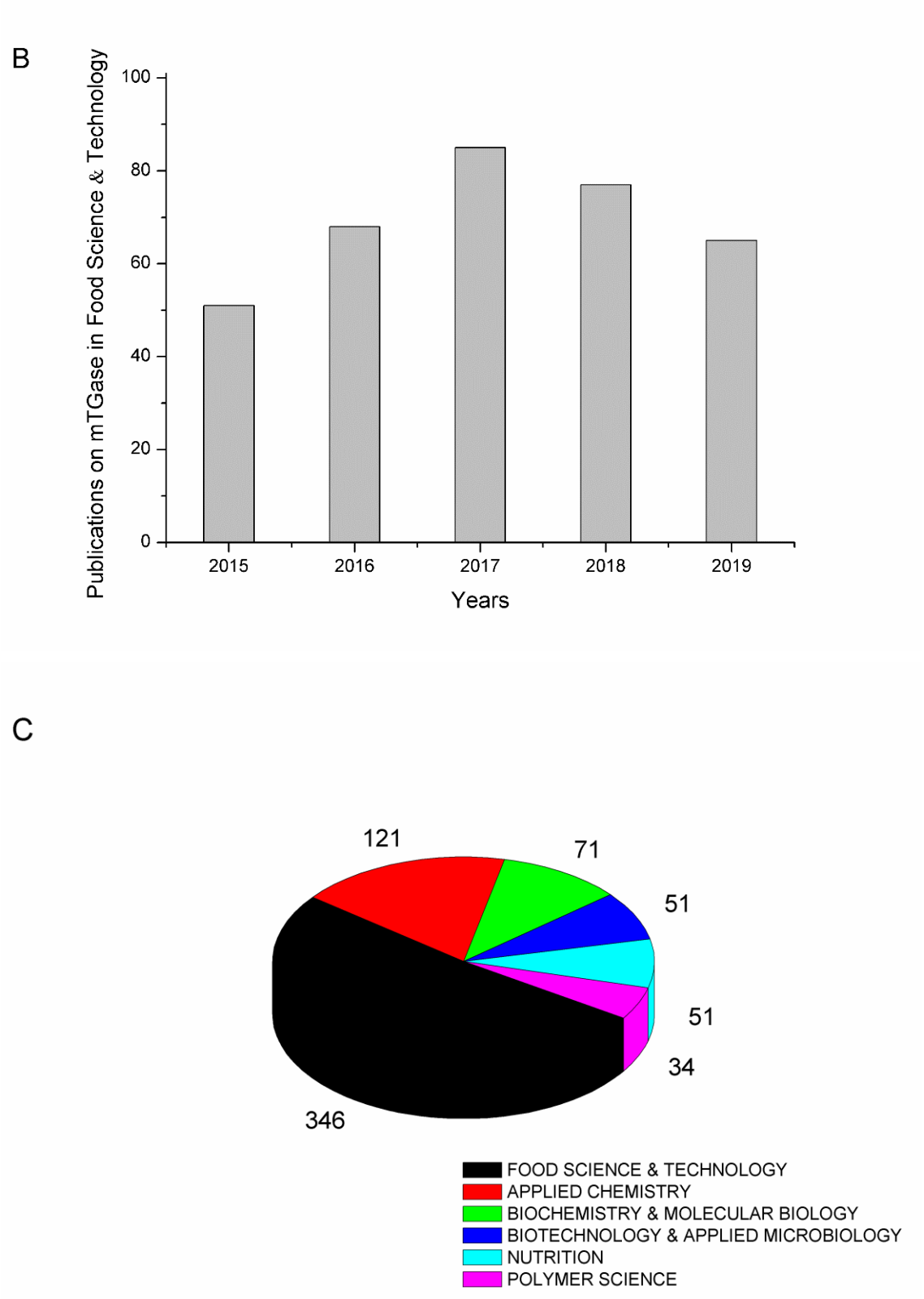


Figure 1. Publications on microbial transglutaminases in the last five years (2015-2019): (A) the general bulk of publications on mTGase; (B) publications covering applications in Food Science and Technology; (C) study areas of the web of science database that research mTGase (data as November, 2019).

Meat and seafood products

Microbial tranglutaminases attracted initial interests of the food industry due to its ability to mold minced meat into a firm steak. The restructure of meat products ensures greater firmness causing little loss of quality during cooking (Lesiow, Rentfrow et al. 2017). The cross-linking of proteins and other compounds of the gel system causes changes in the proteic fraction of food matrices, leading to improved texture and stability in terms of temperature denaturation, emulsifying properties, gelation, and increased water-binding capacity (Dondero, Figueroa et al. 2006). The mTGase yields a final product with retained organoleptic properties similar to conventional meat in terms of flavor, texture, appearance, and taste (Hong, Ndagijimana et al. 2016).

Several studies are reported on the use of mTGase in meat products. As shown in Table 1, the enzyme can be used in a wide range of temperatures, from 10 °C to 50 °C. Some of these studies also show that mTGase supplementation could increase the gel strength in meat products and cause positive effects on the development of meat proteins of pork, beef, chicken, and fish (Dondero, Figueroa et al. 2006, Ahhmed, Kuroda et al. 2009, Ahhmed, Nasu et al. 2009, Hong and Chin 2010, Hong and Xiong 2012, Canto, Lima et al. 2014, Monteiro, Marsico et al. 2015, Wu, He et al. 2016, Jira and Schwagele 2017, Feng, Cao et al. 2018, Sorapukdee and Tangwatcharin 2018).

Because meat products are highly proteic, the myofibrillar proteins have marked influence on the textural quality of these products. Actin and myosin, which constitute the majority of myofibrillar proteins, are important substrates of mTGase and can also be polymerized by its addition, thus improving the textural properties of structured meat products (URAN and YILMAZ 2018). The addition of mTGase also allows for the utilization of raw materials such as collagen and mechanically deboned meat in manufacturing meat products, with enhanced nutritive value by supplementation with amino acids otherwise deficient in these products (e.g. exogenous lysine) (Kieliszek and Misiewicz 2014).

Efforts to reduce the sodium content of meat products is an important issue concerning the health of people and to attend these demands, the meat industry is focusing on the development of techniques to reduce the use of salt in processed meat products, without impacting their quality (Atilgan and Kilic 2017). Strategies such as the use of mTGase can be applied in the manufacture of meat products with low salt content to avoid quality deterioration arising from this reduction, as suggested by

Atilgan and Kilic (2017). These authors investigated the effects of mTGase, fibrin/thrombin (fibrimex), alginate, and their combinations on the quality of reduced-salt cooked meat. Their results indicated that the fibrimex/mTGase combination improved the texture properties of minced beef with low salt content.

However, for restructured meat in which fat can interfere with meat binding, it is essential to evaluate the grading level of formulated beef trimmings to enhance product quality or, at least, to avoid the minimum detrimental impact on product quality. The research of Sorapukdee and Tangwatcharin (2018) indicated that the most suitable raw beef for producing restructured steaks without detrimental effect on product quality, was beef trimmings containing up to 17 % fat treated with 1 % (weight fraction) of mTGase Activa TG-B. At this level of enzyme addition, both the sensory quality and increased tenderness were positively affected.

Dairy products

Improving the quality and functionality of the dairy products is been considered of paramount importance for better appreciation by people in a scenario of competitive dairy market. One of the most promising strategies to promote bio-functionality properties of dairy products is the cross-linking of milk proteins with transglutaminase. The use of mTGase can be a successful strategy to improve dairy products nutritional and technological characteristics, at the same time reducing the costs production by decreasing the amount of fat and stabilizer in the final product (Taghi Gharibzahedi, Koubaa et al. 2018). This enzyme has the ability to form intra- and intermolecular covalent crosslinks between two amino-acid residues in the structure of milk proteins. Both casein and whey α-lactalbumin and β-lactoglobulin are excellent acyl donors and/or acceptors substrates for transglutaminase, although some differences between them apply in relation to the crosslinking reaction (Færgemand and Qvist 1997, Rodriguez-Nogales 2006, Oner, Karahan et al. 2008, Rossa, de Sá et al. 2011). According to a study by Chen and Hsieh (2016), in a cascade reaction, mTGase catalyzes the cross-linking of κ-casein (κ-CN) and β-casein (β-CN) before it proceeds to cross-link the serum albumin (AS), α-lactalbumin (α-LA), α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), and β-lactoglobulin (β-LG) moieties, as shown in Figure 2. In this particular case, the caseins appear to be readily cross-linked because of their flexible, random-coil structures and the absence of any disulphide bonds in the α_{s1}-CN and β-CN

(Færgemand and Qvist 1997, Rodriguez-Nogales 2006, Oner, Karahan et al. 2008, Rossa, de Sá et al. 2011). Due to their compact globular structures, whey proteins tend to cross-link less efficiently. The β-LG is more susceptible and show a higher cross-linking rate than α-LA, but the β-LG is able to cross-link with the reduction of its disulphide bonds, whereas α-LA can be cross-linked without the reduction (Rodriguez-Nogales 2006, Rossa, de Sá et al. 2011).

The benefits brought about by the application of mTGase in dairy products include increased gel strength and improved storage stability and viscosity (Domagała, Najgebauer-Lejko et al. 2016). When mTGase is added to the system, it enhances heat-resistance and firmness of gel. Yogurt, a milk gel formed by acidic fermentation mediated by lactic-acid bacteria, has the disadvantage of serum separation upon change of temperature or physical impact. The addition of mTGase to yogurt can avoid this problem because mTGase improves the water holding capacity of the gel (Yokoyama, Nio et al. 2004). Ice creams treated with mTGase result in more consistent end-products, showing better aeration and foam stability. The mTGase also makes it possible to produce ice cream and cheese with low fat contents or reduced content of non-fat solids (Yokoyama, Nio et al. 2004, Gaspar and de Góes-Favoni 2015). The addition of mTGase during cheese preparation may increase the moisture content altering the palatability and the yield of different cheese products. In relation to surface texture of curds, ice-creams, milk and cheeses, it is noticed an improvement in the creaminess, homogeneity, smoothness, and consistency after mTGase is used in the production process (Wen-qiong, Lan-wei et al. 2017).

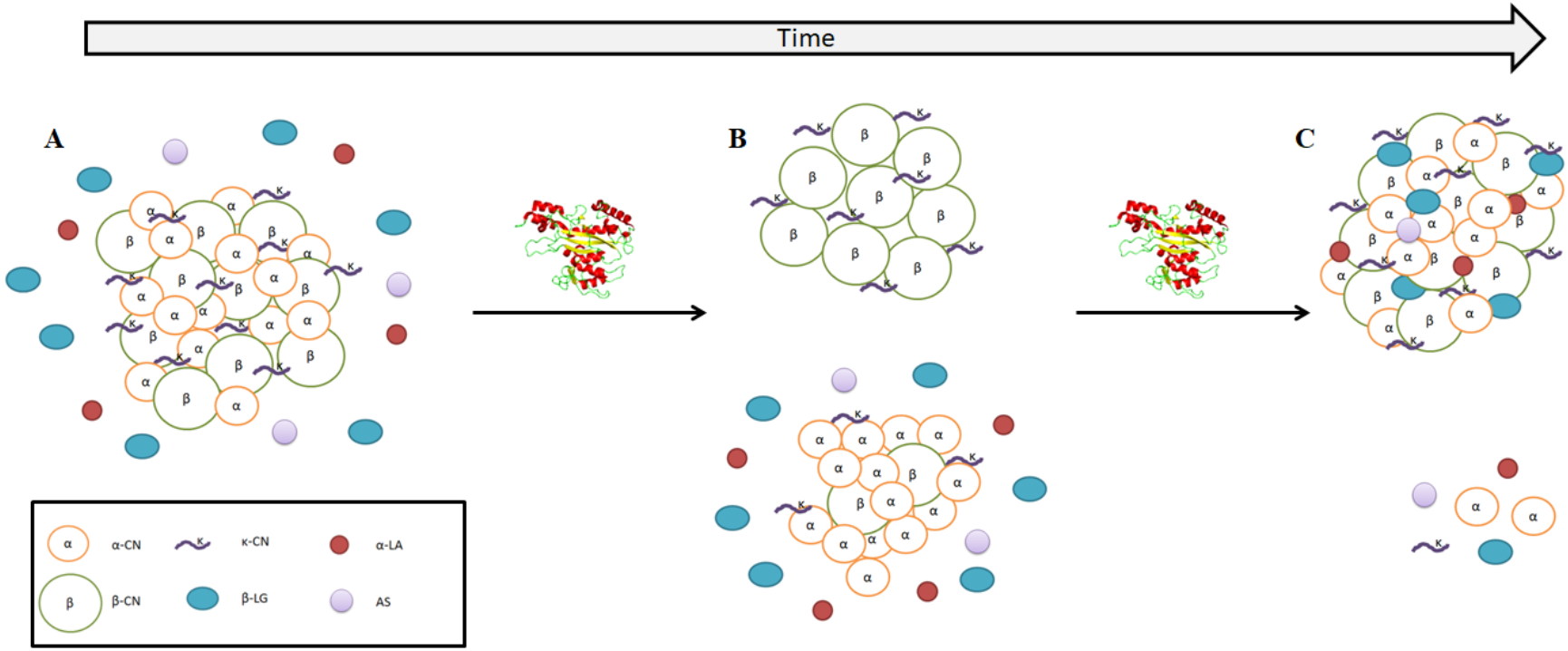


Figure 2. Representation of the cross-linking reaction of milk proteins induced by mTGase. **a** The milk proteins without mTGase action are schematized; **b** the preference of mTGase for β-CN and κ-CN; **c** cross-linking occurs with all milk proteins with time. Adapted from (Chen and Hsieh 2016).

Cereal based product

The first positive effects of mTGase application in baking was reported by Gottmann and Sprössler (1992). The applications of mTGase in cereal proteins, particularly wheat proteins (globulins, glutenins, gliadins, and prolamins), have attracted huge interest from the bakery industry (Mazzeo, Bonavita et al. 2013). The cross-links formed between the wheat proteins by the action of mTGase greatly influenced the characteristics of the products, determining the quality, functional and rheological properties of these systems, such as stability, elasticity, resilience, and water adsorption, with proper pore size along with adequate dough volume (Gerrard, Fayle et al. 1998, Gujral and Rosell 2004, Bonet, Caballero et al. 2005, Scarnato, Montanari et al. 2017). The cross-linking reaction of mTGase promotes aggregation and polymerization, leading to the formation of polypeptide networks showing differentiated viscoelastic properties (Gujral and Rosell 2004, Bonet, Caballero et al. 2005).

Gerrard et al. (1998) were the first researchers who used the mTGase in white bread. These authors suggested that the enzyme could have beneficial effects during the manufacture of bread, comparable to those produced by traditional chemical oxidant improvers. Basman et al. (2017) showed that using low amounts of mTGase, positive effects were observed on aspects of crumb and crust of bread, as well as in the rheological properties and physico-chemical properties of the dough. The release of some peptides from gluten obtained through the activity of mTGase can also influence the modulation of bread microbiota during the storage and consequently increase the final product shelf-life (Scarnato, Montanari et al. 2017).

Another application of mTGase in the bakery industry is related to the production of pasta and instant noodles. In 1996, research by Sakamoto et al. (1996) showed that the treatment of noodles and pasta with mTGase prevented the deterioration of texture upon cooking and improved the strength of the products, even when low-grade flours were used in the manufacture, reducing the costs of production (Sakamoto, Yamazaki et al. 1996, Yokoyama, Nio et al. 2004).

Soybean products

Soy protein isolate (IPS) is widely used as an important ingredient in Asiatic diets and in general processed foods due to its nutritional value and functional

properties. IPS consists of glycinin (11S) and β-conglycinin (7S), which account for approximately 70 % of its total protein content. These globulins are good substrates for mTGase activity (Song and Zhao 2014, Qin, Luo et al. 2016). Tang et al. (2005) investigated the use of mTGase on the properties and microstructures of IPS films molded with various plasticizers (glycerol, sorbitol, and 1:1 mixtures of glycerol and sorbitol). The cross-linking treatment by mTGase produced an effective method to improve the films cast properties of all tested plasticizers.

Tofu, a typical soybean curd product, is prepared by the coagulation of soybean proteins with the addition of Ca^{2+} and Mg^{2+} and/or glucono-δ-lactone. Coagulation or gelation of soymilk is the most important step in the production of tofu. A popular food in many countries, tofu shelf life is generally very short because its softness and smooth texture that prevents its sterilization. The introduction of mTGase in its processing produces an edge of texture control and enhances its quality, yielding a product with better consistency and silky texture and ability to tolerate temperature fluctuations (Chang, Shiau et al. 2011).

Finally, proteins from sources other than soy can be covalently linked to soy protein by mTGase to produce combinations showing novel functionalities. For instance, conjugation of milk caseins or soybean globulins with ovomucin (an egg white glycoprotein), has shown to improve the emulsifying activity of the combined protein when compared to both isolated proteins (Kato, Wada et al. 1991, Yokoyama, Nio et al. 2004).

Food coating and edible films

The research to produce protein films as an alternative to petroleum-based polymeric materials has been receiving a great deal of attention in the food industry. Protein films can be used as coatings on fresh fruits and vegetables to increase the shelf life of these products. These films are non-toxic, natural, health safe, biodegradable, and might be edible. Protein edible films produced by the cross-linking action of mTGase present structural homogeneity, have a smooth surface, are mechanically resistant, and are gas-permeable (Porta, Di Pierro et al. 2016). In the work to Rossi Marquez et al. (2017), apple weight losses during storage was significantly reduced, approximately 80 %, after 10 days when the samples were coated with whey protein

grafted film with pectin and transglutaminase. Similarly, this grafted film was able to prevent weight loss of potato and carrot samples until the 6th day of storage.

The research carried out by Fernandez-Bats et al. (2018) showed that it was possible to obtain mesoporous silica nanocomposite bioplastics prepared by using bitter vetch (*Vicia ervilia*) proteins crosslinked by mTGase, which showed improved gas and water vapor barrier properties. The prepared material showed antimicrobial and antifungal activities, possibly increased by nisin addition to the film-forming solutions, suggesting their potential application as an active bio-preservative packaging to improve the shelf life of a variety of different food products.

Health aspects concerning the use of transglutaminase in food industry

Because of increased applications of mTGase in food, important health concerns appeared, pressuring the need for regulations to inform people on the safety when consuming products containing this enzyme. In 1998, Motoki and Seguro (1998) showed that the only difference between food containing mTGase-modified proteins and native proteins was the number of links between glutamine and lysine residues (G-L). This chemical modification is also present when proteic foods are heated, for example, in cooking, generating the G-L bond. In this respect, humans have been ingesting foods rich in G-L residues since the discovery of fire and cooking. Although not scientifically demonstrated, the safety of the G-L modified linkage can be assumed by the long-term consumption of the G-L moiety in cooked foods (Motoki and Seguro 1998). On the other hand, Bernard et al. (1998) studied the mutagenesis and toxicity risk presented by the addition of mTGase in food preparations, tested in experimental animals. Their results suggest that the acute toxicity of the enzyme seems to be relatively low, since it was not observed any mortality, morbidity, or signs of toxicity at doses of 2 g/kg body weight.

There have been some evidences of increased nutritional properties of foods enzymatically modified by mTGase. According to studies conducted by Xing et al. (2016), the addition of mTGase to soy extract in preparation of tofu lead to modifications of proteins that increased the perception of satiety and reduced the allergenicity towards soy proteins. In another application concerning allergenicity, shrimp products processed using mTGase showed reduced allergenicity due to glycosylation of proteins catalyzed by this enzyme (Yuan, Lv et al. 2017).

Concerning bakery products, Zhou et al. (2017) have shown that mTGase can effectively transamidate gliadin peptides and gluten proteins, thus concluding that mTGase lowered the allergenicity and immunogenicity caused by wheat flours. The resulting peptides are barred to cross intestinal mucosa where they initiate the celiac immunological activity. These results demonstrate a potential strategy to prevent cereal toxicity in celiac disease (Zhou, Wu et al. 2017). However, multiple mTGase linked proteins are immunogenic in celiac disease patients. In the study conducted by Lerner and Matthias (2015), the authors indicate that the use of this enzyme can further increase antigenic load presented to the immune system and increase the risk for gluten-sensitive populations. In a recent research, Matthias et al. (Matthias, Jeremias et al. 2016) have suggested that mTGase increases immunogenicity in children with celiac disease because mTGase antibodies correlates to intestinal damage in the same degree as transglutaminase human tissue antibodies. Authors suggested that further investigation is necessary to elucidate the role of anti-mTGase antibodies in this disease.

Although scientific findings reported in the literature regarding the safety of the use of mTGase in foods can be classified as inconclusive, the FDA has approved the use of mTGase as a "Generally Recognized as Safe - GRAS" for food applications since 1998. This enzyme is considered an adjunct of technology and it is not regarded as an ingredient, and therefore does not need to be listed in the composition of ingredients of the commercial product (Romeih and Walker 2017, Taghi Gharibzahedi, Koubaa et al. 2018).

BIOTECHNOLOGY APPLICATIONS OF mTGase

The biotechnological applications of transglutaminases are one of the fastest growing areas on mTGase research. Classical applications of transglutaminases in biomedical research include PEGylation, the production of antibody-drug conjugates, tissue engineering, regenerative medicine, and the production of microparticles for enteric delivery of substances of interest in the food and pharmaceutical industry. Finally, we will be briefly reviewing the use of transglutaminases in the treatment of textiles and leather.

Enzymes Immobilization mediated by mTGase-catalyzed bioconjugation

Protein immobilization in solid supports has been used as a technique for biotechnological applications of enzymes, offering several advantages over the use of free forms, such as easing separation from reaction media and the possibility of reuse (Mateo, Palomo et al. 2007, Rodrigues, Ortiz et al. 2013, Matte, Bussamara et al. 2014, Duarte, Schöffer et al. 2017). In general, proteins bound to functional groups on supports show high stability due to reduced protein loss to the medium. The immobilization of proteins through covalent bond formation has been routinely carried out using chemically-activated supports or chemical cross-linking reagents (Mateo, Palomo et al. 2007). However, because the presence of multiple functional groups on protein surfaces, proteins are in general randomly attached onto supports, resulting in the reduction of total enzymatic activity. Several techniques have been devised to preserve the activity of biomolecules upon immobilization, among them the immobilization using mTGase as site-specific binding (Tominaga, Kamiya et al. 2004). The immobilization mediated by transglutaminase-catalyzed bioconjugations offers the advantage of improved selectivity and compatibility with sensitive biological systems relative to traditional chemical methodologies (Wang, Tang et al. 2019).

The mTGase is unique in catalyzing the acyl transfer reaction between a primary amine and the γ-carboxyamide group of glutamine (Gln) residues in peptides and proteins. When the ε-amino group of lysine (Lys) residues in protein acts as an acyl acceptor, cross-linking of proteins becomes possible through the ε-(γ-Gln)Lys bond, resulting in the formation of a new γ-glutaminyl covalent link (Motoki and Seguro 1998, Kamiya, Takazawa et al. 2003, Li, Li et al. 2018, Wang, Tang et al. 2019). The mTGase displays broad acyl-acceptor substrate specificity, enabling the use of a variety of scaffolds with primary amine groups as solid supports, such as the polysaccharide chitosan and gelatin (Li, Li et al. 2018).

The study of Tominaga, Kamiya et al. (2004) demonstrated site-specific immobilization via covalent attachment of recombinant alkaline phosphatase with a specific peptide linker by mTGase. To allow the mTGase-mediated site-specific immobilization, a solid support of casein-coated polyacrylic resin was designed to display mTGase recognition sites on its surface. It was found that this immobilization exhibited much higher specific activity, with higher stability upon repeated use than the biocatalyst prepared via chemical modification

Wang, Tang et al. (2019) reported the controlled, site-specific and covalent cross-linking of an engineered enterokinase on amine-modified magnetic nanoparticles via mTGase-catalyzed bioconjugation for the development of the oriented-immobilized enzyme. A glutamyl (Gln-donor tag) was genetically incorporated into the C-terminus of enterokinase. An amide linkage was formed between the glutamyl group of Gln tag and the primary amines of the support via the covalent immobilization catalyzed by mTGase. Upon the site-specific immobilization, approximately 90 % enterokinase activity was retained, and the biocatalyst exhibited more than 85 % of initial enzymatic activity reusable stability over a month (Wang, Tang et al. 2019).

In the work of Synowiecki and Wołosowska (2006), a β-glucosidase from *Sulfolobus shibatae* was immobilized on silica gel modified with 3-aminopropyl-triethoxysilane using transglutaminase as a cross-linking factor, and the immobilization process did not influence the optimum pH and temperature of substrate hydrolysis (Synowiecki and Wołosowska 2006). On the other hand, Bechtold et al. reported the preparation of protein G-soybean peroxidase conjugate catalyzed by mTGase with yields calculated to be only about 0.1 %, suggesting the difficulty in controlling the reaction when using native proteins (Bechtold, Otterbach et al. 2000).

PEGylation

At the end of the 1960s, Davis proposed the idea of conjugating PEG [poly (ethylene glycol)] to a protein, i.e., to "PEGylate" a protein (Davis 2002, Hoffman 2016). Since then, the PEGylation is considered one of the most successful methods to prolong the circulatory half-life and reduce the *in vivo* immunogenicity of therapeutic proteins, among many other applications in pharmacology (Pasut and Veronese 2012).

PEG is biocompatible, lacks immunogenicity and antigenicity, is soluble in water and other organic solvents, is readily cleared from the body, and has high mobility in solution and, more important, it is not toxic, making this the polymer of choice for bioconjugations. PEG use was approved by the FDA in the early 1990s (Roberts, Bentley et al. 2002, Harris and Chess 2003, Bhattarai, Matsen Frederick et al. 2005, Mariniello, Porta et al. 2014). Thus, a number of protein/PEG conjugates, are available in the market such as for the treatment of chronic hepatitis C (PEGinterferon α-2a and α-2b), for the treatment of acute lymphoblastic leukaemia (mPEG-L-Asparaginase), to treat severe combined immunodeficiency (SCID) disease (mPEG-

Adenosine Deaminase), and to treat acromegaly (PEG-visomant) (Banerjee, Aher et al. 2012).

Chemical strategies used for the PEGylation of proteins produces random derivatives of lysine (Lys) residues, leading to heterogeneity and decreased bioactivity of the products (da Silva Freitas, Mero et al. 2013). Instead, the use of transglutaminase for the covalent attachment of PEG molecules to pharmaceutical proteins shows stringent substrate specificity, and site specific modification or PEGylation of the Gln residues bound to the proteins on the substrates can be obtained. (Fontana, Spolaore et al. 2008).

Because transglutaminases have partial selectivity to the carboxamide substrate, they are interesting options for the PEGylation of proteins. However, for the reaction to occur, the carboxamide must be in the flexible part of the protein molecule. (Fontana, Spolaore et al. 2008, Dozier and Distefano 2015). Consequently, mTGase has been intensively used to site-specifically incorporate mPEG–NH_2 to the reactive Gln residue of proteins (da Silva Freitas, Mero et al. 2013). The reactive Gln residues modified by mTGase must locate at the disordered protein regions and satisfy its sequence requirement. As many target proteins lack the reactive Gln residues that can satisfy the structural and the sequence requirement of mTGase, its use is limited (Mero, Spolaore et al. 2009, da Silva Freitas, Mero et al. 2013). Several researches have been developed in this area and some of them are listed in Table 2.

So far, only a limited number of researches has been carried out on mTGase-mediated protein modification at the level of Lys residues. One of them is the work, of Zhou et al. (2016), who linked carboxybenzyl-glutaminyl-glycine (CBZ-QG) to mPEG amine to form CBZ-QG-mPEG for the PEGilation of cytochrome C.

The hydroxyethyl starch (HES), which is a biodegradable derivative of starch, can be an alternative to PEG as blood plasma volume expander and in the design of drug delivery systems (Treib, Baron et al. 1999). It has been reported the use of HES conjugation using mTGase to produce fully biodegradable polymer–drug and polymer–protein conjugates (Besheer, Hertel et al. 2009).

Antibody drug conjugates (ADCs)

Another promising technology is the use of mTGase to attach antibodies to diverse compounds in order to produce antibody-drug conjugates (ADC). ADC are

emerging therapeutic agents in the treatment of cancer, using antibodies to selectively deliver a cytotoxic compound to tumor cells, thus improving the therapeutic index of chemotherapeutic agents, and showing better safety potential than nontargeted cytotoxics (Strop, Liu et al. 2013, Anami, Xiong et al. 2017). One of the major challenges in the development of ADC is the application of suitable linkers to conjugate drugs to antibodies (Yao, Jiang et al. 2016). The ADC have been largely manufactured by using chemical conjugation methods, generally resulting in heterogeneous mixtures of ADC having different physical and pharmacokinetic properties of the proposed ones (Junutula, Raab et al. 2008, Axup, Bajjuri et al. 2012, Shen, Xu et al. 2012, Zuberbühler, Casi et al. 2012, Okeley, Toki et al. 2013, Strop, Liu et al. 2013, Xiao, Chatterjee et al. 2013, Dennler, Chiotellis et al. 2014).

An alternative strategy to the chemical modification of ADC is the use of mTGase because the enzyme will prevent the formation of these heterogeneous mixtures. Moreover, it is possible to introduce appropriate amine containing linkers making the mTGase able to conjugate structurally diverse probes and drugs (Ohtsuka, Sawa et al. 2000). Strop et al. (2013) investigated how the conjugation site influences the stability, toxicity and efficacy of ADC obtained by mTGase reaction and whether these differences could be directly attributed to the binding position. By designing a "glutamine label", 90 sites were tested to attach several compounds and 12 sites showing a high degree of conjugation were found.

A two-step chemo-enzymatic approach, where mTGase binds a spacer entity that is reactive to the antibody, and subsequently reacts with the antimitotic toxin monomethyl auristatin E (MMAE), produced the highly homogeneous trastuzumab-MMAE conjugate with DAR (Drug-Antibody Ratios) of 2 (Dennler, Chiotellis et al. 2014). Some ADC currently in use in clinical development based on target antigens using tranglutaminase are: PF-06664178, Trop-2 ADC, RN927C (Phase I, for treatment of ovarian cancers, non-small cell lung cancer and breast cancer - Site-specific transglutaminase tag, AcLys-VC-PABC linker) and PF-06647020, h6M24-vc0101, PTK7-targeted ADC (Phase I, for treatment of non-small-cell lung carcinoma, triple-negative breast cancer and ovarian cancers - Transglutaminase tag (LLQGA) located at the C-terminus of the antibody heavy chain, cleavable VC-PABC-linker) (Sachdev, Maitland et al. 2016, Strop, Tran et al. 2016, Damelin, Bankovich et al. 2017, Nejadmoghaddam, Minai-Tehrani et al. 2019).

Several other investigations have been reported on the production of monoclonal antibodies using mTGases and are well documented in recent works (Jeger, Zimmermann et al. 2010, Grünberg, Jeger et al. 2013, Strop, Liu et al. 2013, Dennler, Chiotellis et al. 2014, Farias, Strop et al. 2014, Lhospice, Brégeon et al. 2015, Siegmund, Schmelz et al. 2015, Spidel, Vaessen et al. 2017).

Tissue engineering and regenerative medicine

The term Tissue Engineering (TE) was first introduced in 1993 by Langer and Vacanti (Langer and Vacanti 1993) to describe an interdisciplinary field encompassing cell biology, material science, chemistry, molecular biology, engineering, and medicine, with the objective of developing advanced biological tissues and organs. These engineered biological materials are intended to maintain, improve, or restore functionalities of natural tissues combining scaffolds, cells and/or bioactive molecules (Langer and Vacanti 1993, Griffith and Swartz 2006, O'Brien 2011, Lee, Kasper et al. 2014). The potential applications are being investigated in the field of tissue engineering of bones, cartilage, cardiac system, pancreas, and the vascular system, among others (Zhu and Tramper 2008). The main bulk of research in this area has been focused in the development of biomaterials capable of mimicking the structure and composition of the extracellular matrix. Such biomaterials must present biocompatibility and biodegradability and should not be toxic. In addition, the production and processing of biomaterials must be easy and scalable. Because hydrogels have high plasticity and high moisture content they have been the most important biomaterials employed in tissue engineering (Polak 2010, Toh and Loh 2014). Hydrogels can be formed from gelatin, collagen, chitosan, hyaluronic acid, and sodium alginate, as well as synthetic materials such as polylactide, polylactic-co-glycolic acid copolymer, polyethylene glycol, polycaprolactone, and polyacrylamide (El-Sherbiny and Yacoub 2013). Gelatin is a protein derived from the hydrolysis of collagen with characteristics of biodegradability and cell adhesion capacity, considered as GRAS material by the FDA and it has a long history of safe use in food products, pharmaceuticals and cosmetics (Elzoghby, Samy et al. 2012). Unfortunately, owing to a lack of mechanical strength and sensitivity to *in vivo* enzymes, the biomedical applications of gelatin is limited being necessary to increase its physical performance and to strengthen its resistance against enzymes hydrolyses (Zhao, Li et al. 2016). To achieve this goal, crosslinks are usually introduced

in biomaterials such as collagen mediated by mTGase, replacing physical methods like dehydrothermal drying (DHT) and UV-irradiation, among others, and chemical crosslinking mediated by glutaraldehyde, formaldehyde, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). The physical methods produce weak bonds that have a high risk of degradation, whereas the chemical methods use often toxic compounds that must be removed from hydrogels before being applied (Stachel, Schwarzenbolz et al. 2010, Yang, Xiao et al. 2018). Therefore, the substitution of these methods by the enzymatic application of mTGases in order to generate hydrogels are among the most promising technologies to obtain biomaterials, since the mTGase-mediated process presents no risk of toxicity and eases the preparation of the materials, showing high mechanical stabilities (Milczek 2018). There is a plethora of research showing the use of transglutaminases to produce hydrogels, some of them listed in Table 3.

In 1986, Charles Hull described for the first time the technique of 3D bioprinting, which has since been used to produce a large variety of scaffolds in different tissue engineering areas. The term 3D bioprinting is used to describe the precise layering of cells, biologic scaffolds, and biologic factors with the goal of building a biological tissue (Bishop, Mostafa et al. 2017). The vascularization of 3D scaffolds is crucial for their functionalities, assuring the delivery of nutrients and oxygen to tissues, promoting cell proliferation and subsequent development of the new tissue (Castells-Sala, Alemany-Ribes et al. 2013). The main techniques used in tissue engineering by 3D bioprinting are stereolithography, extrusion-based, laser-assisted and inkjet-based printing (Derakhshanfar, Mbeleck et al. 2018), and a sample of the investigations using 3D printing for tissue engineering with the use of transglutaminase are listed in the Table 3. In this technique, a great variety of polymers, hydrogels, bioceramics, among other biomaterials, have been used. Natural biological materials, such as cells, can also be employed in 3D bioprinting (Tappa and Jammalamadaka 2018).

Transglutaminase-crosslinked microparticles for enteric delivery

The microencapsulation technique is widely used in the fields of food, pharmaceuticals, and biotechnology in order to preserve physicochemical and sensorial attributes and to produce control-released compounds. Microencapsulation is obtained

by the use of various techniques, such as spray drying, cooling, extrusion coating, fluidized bed coating, liposome retention, inclusion complexation, centrifugal extrusion, and rotational suspension separation, techniques that are chosen based on final product characteristics and costs (Desai and Park 2005). Recently, another technique has been described, the complex coacervation, which produces high encapsulation efficiencies, and has since been widely used in food and pharmaceutical industries, because it does not require harsh production conditions (temperature, pressure, pH, etc.) (Tello, Prata et al. 2016). This technique uses a combination of encapsulating agents of opposing charges to create electrostatic attraction between two molecules, and other interactions, such as hydrogen bonds and hydrophilic interactions, that also contribute to the formation of complexes (Ach, Briançon et al. 2014). As the nature of these bonds is weak, there is the need to strengthen the interactions between polymers. For this purpose, mTGase has been used as a cross-linking agent showing promising results (Sanchez and Renard 2002). In Table 4 is presented a summary the microencapsulation and complex coacervation techniques found in literature involving the use of microbial transglutaminase.

TRANSGLUTAMINASE APPLICATIONS IN TEXTILE INDUSTRY

The textile finishing industry has been the focus of considerable criticism because it uses traditional chemical treatments in wool processing, which is perceived as highly damaging to the environment. Unfortunately, the alternative enzymatic processes using proteases can cause an excessive loss of fabric weight and yarn strength. Therefore, the use of transglutaminases in treatments of wool and leather fabrics has become extensively explored in order to develop appropriate technologies based on the use of this enzyme. It has been found that mTGase is capable of recovering properties of wool and silk treated with chemicals and enzymes used at different processing stages, such as cleaning, carding, bleaching, combing, drawing, spinning, and twisting (Tesfaw and Assefa 2014). Wool fabrics treated with *Streptomyces hygroscopicus* mTGase showed recovered fiber structures that were damaged during protease treatments (Du, Cui et al. 2007). The application of Guinea pig liver transglutaminase or the mTGase isolated from *Streptoverticilium mobaraense* in wool processing resulted in the reduction of the propensity of wool yarn or fabric to shrink,

and to improve yarn resistance, suggesting that transglutaminases can remediate the negative effects of proteolytic processing of the wool (Cortez, Bonner et al. 2004). Mojsov (Mojsov 2017) showed that the characteristics of wool fabric pretreated with proteolytic enzymes and transglutaminase is comparable to untreated wool fabric. The author points to the following benefits of treating wool with mTGase: improvement in fabric softness, increased absorption characteristics, and resistance to pilling and retraction of the felting (Mojsov 2017).

Wool garments industrialized using fabrics treated with mTGase are likely to have increased resistance to domestic washing. Biological detergents containing proteases can cause irreversible damage to the fiber, leading to loss of fabric strength, shape, and color fading (Cortez, Bonner et al. 2005). However, combining the advantages of using both proteases and transglutaminases in a simultaneous enzymatic treatment of wool, resulted in the development of a bioprocess for machine washable wool with insignificant fiber damage (Gaffar Hossain, Juan et al. 2008). Casein incorporated to wool mediated by mTGase was used as a surface coating material for smoothing the texture of the wool fiber by coating or filling the damaged scales in wool yarn (Cui, Fan et al. 2011).

Finally, excellent antibacterial properties were obtained when Ɛ-Poly-L-lysine (Ɛ-PL), which is a natural biomacromolecule having a broad spectrum of antibacterial activity, was grafted onto the wool fiber via mTGase, showing 97 % bacteriostasis to *Escherichia coli* (Wang, Jin et al. 2010).

TRANSGLUTAMINASE APPLICATIONS IN LEATHER PROCESSING

The process of *filling*, which is the introduction of materials into the voids between leather fibers in order to smooth surface irregularities is considered one of the most important steps in leather processing, used to increase material quality. Common materials used as fillers are glucose, flour, and gum, as well as enzyme-modified gelatin and casein, the last two being cross-linked with leather proteins by the action of mTGase (Zhu and Tramper 2008). Experimental results showed that fillers incorporated by mTGase were firmly bound to the leather and would not be easily removed during further processing (Taylor, Bumanlag et al. 2006).

Finally, the use of gelatin-sodium caseinate modified by mTGase was investigated regarding subjective aspects of leather (visual aspects, touch, etc.), as well as for its mechanical and structural properties. The application of mTGase improved the subjective aspects, without significantly affecting the mechanical properties such as tensile strength and elongation at break (Liu, Liu et al. 2011).

CONCLUSION

We addressed the several uses of microbial transglutaminases in the food, pharmaceutical, and biotechnology industries. The applications of mTGase have important implications for the development of these industries, producing new products at low cost, improving the application and quality of food, pharmaceuticals, and other goods such as wool and leather, designed for improving human life in a more sustainable way. mTGases became crucial to produce processed meat and seafood products, dairy products, bread, noodle, soybean products, and to produce coating and edible films. In more sophisticated fields, mTGase has become relevant in PEGylation, antibody drug conjugates, tissue engineering, regenerative medicine, production of microparticles for enteric delivery, directly impacting health products and services. Due to its importance and value aggregation to final products, research on the applications of mTGases is ever growing, showing many possibilities to produce new materials and improving the quality of the existing ones. Further research should focus on the bioprocess technology to reduce production costs of mTGases and enhance their biochemical properties.

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TABLES

Table 1. Studies with mTGase applied to different protein sources.

Group of food	Protein substrate	Microorganism of TGase	Treatment conditions (enzyme concentration, temperature, and incubation time)	Ref.
Meat and seafood products	Pork myofibrillar protein	Activa® TI (*S. mobaraensis*)	0.5 % (w/w); 4 °C; 24 h	(Hong and Xiong 2012)
	Pork myofibrillar protein	Activa® TI (*S. mobaraensis*)	0.2% (w/w); 4 °C; 24 h	(Hong, Min et al. 2012)
	Pork myofibrillar protein	Activa® TI (*S. mobaraensis*)	0.6 % (w/w); 4 °C; 24 h	(Hong and Chin 2010)
	Pork leg to manufacture dry-cured ham	Activa® EB (*S. mobaraensis*)	0.1% (w/v); 7 °C; 24 h	(Romero de Ávila, Ordóñez et al. 2010)
	Beef	Activa® TG-K (*S. mobaraensis*)	0.5 % (w/w); 60 °C; 2 h	(Dondero, Figueroa et al. 2006)
	Steak - beef trimmings	Activa® TG-B (*S. mobaraensis*)	1 % (w/w); 8 °C; 4 h	(Sorapukdee and Tangwatcharin 2018)
	Chicken and beef myofibrillar proteins	Activa® (*S. mobaraensis*)	5–6.8 % (w/w); 40 °C or 78 °C, 0.5h	(Ahhmed, Kuroda et al. 2009)
	Tilapia fillets	Activa® WM (*S. mobaraensis*)	0.5 % (w/w); 4 °C; 24 h	(Monteiro, Marsico et al. 2015)
	Fish myofibrillar protein	Ns	0.1 %; 4 °C; 2 h	(Feng, Cao et al. 2018)
	White shrimp	Activa® TG-K (*S. mobaraensis*)	0.8 U/g of protein substrate; 25 °C; 2 h	(Tammatinna, Benjakul et al. 2007)

	Caiman steaks	Activa® WM (*S. mobaraensis*)	1 % (w/w); 4 °C; 18 h	(Canto, Lima et al. 2014)
Dairy products	*α*-lactalbumin concentrate	Activa® MP (*S. mobaraensis*)	10 U/g of protein substrate; 50 °C; 5 h; pH 5	(Sharma, Zakora et al. 2002)
	Na-caseinate, Ca-caseinate, skim milk powder, condensed milk, whole milk powder, whey, and milk	Activa® (*S. mobaraensis*)	1 U/g of protein substrate; 40 °C, 2 h	(Oner, Karahan et al. 2008)
	Paneer (traditional Indian milk product)	Activa® (*S. mobaraensis*)	1 U/g of protein substrate; 4 ºC; 16 h	(Prakasan, Chawla et al. 2015)
	Milk	Activa® TI (*S. mobaraensis*)	0.3 % (w/w); 84.5 °C; 1h	(Rodriguez-Nogales 2006)
	Milk	Activa® MP (*S. mobaraensis*)	3 U/g of protein substrate; 40 °C; 2 h	(Domagała, Najgebauer-Lejko et al. 2016)
	Milk	Activa® TG-B (*S. mobaraensis*)	7 U/mL of milk proteins; 30 °C; 3 h	(Chen and Hsieh 2016)
	Cheese whey protein	Ns	40 U/g of whey proteins; 40 °C; 1 h; pH 5	(Wen-qiong, Lan-wei et al. 2017)
	Ice cream	Activa® (*S. mobaraensis*)	4 U/g of protein substrate; 57 °C; 1.5 h	(Rossa, de Sá et al. 2011)
Cereal based products	Noodle	Ns	1 % (w/w); 30 °C; 0.5 h	(Wang, Huang et al. 2011)
	Rice noodle	Activa® (*S. mobaraensis*)	1 % (w/w); 40 °C; 2 h	(Kim, Kee et al. 2014)
	Rice flour	Activa® (*S. mobaraensis*)	1 % (w/w); 30 °C; 1 h	(Gujral and Rosell 2004)
	Wheat gluten hydrolysate	Activa® TI (*S. mobaraensis*)	0.05 % (w/w); 55 °C; 1 h and 5 °C; 18 h	(Agyare, Addo et al. 2009)

	Bread wheat flour	Activa® WM (*S. mobaraensis*)	8 U/g of protein substrate; 30 °C; 2 h	(Mazzeo, Bonavita et al. 2013)
	Damaged wheat flour	Activa® (*S. mobaraensis*)	1.5 U/g of protein substrate; 37 °C; 0.5 h	(Bonet, Caballero et al. 2005)
Leguminous products	Soy protein	TGase was purified from the culture medium of *Streptoverticillium cinnamoneum subsp. cinnamoneum* IFO12852	0.05 % (w/v); 55 °C; 1 h	(Babiker 2000)
	Soy protein isolate	Activa® WM (*S. mobaraensis*)	0.08 % (w/v); 50 °C; 0.4 h	(Song and Zhang 2008)
	Legume protein isolate	Ns	0.05 % (w/v); 55 °C; 1 h; pH 7.5	(Salma, Nahid et al. 2010)
	Black soybean packed tofu	Activa® (*S. mobaraensis*)	1 % (w/w); 55 °C; 0.5 h	(Chang, Shiau et al. 2011)
	Soy-based cream cheese	Ns	2.6 % (w/w); 50 °C; 24 h	(Ting-Jin, Azhar-Mat et al. 2011)
	Soy protein isolate	Activa® (*S. mobaraensis*)	0.5 % (w/v); 50 °C; 1 h	(Jin, Kim et al. 2013)
	Soybean protein	Ns	10 U/g of protein substrate; 37 °C; 3 h; pH 7.5	(Song and Zhao 2014)

* ns: not specified.

1 Table 2. Summary of PEGylation studies found in literature involving microbial transglutaminases.

Protein	Protein Size (kDa)	PEG Size (kDa)	Functional Group on PEG	Use	References
Human growth hormone (hGH)	22	20	Glutamine	Growth hormone deficiency (GHD)	(Zhao, Shaw et al. 2010, da Silva Freitas, Mero et al. 2013, Grigoletto, Mero et al. 2015, Khameneh, Jaafari et al. 2015)
Human growth hormone (hGH) and salmon calcitonin (sCT)	hGH 22 sCT 34	10 and 0.55	Glutamine	Growth hormone deficiency (GHD) and Paget disease, osteoporosis and hypercalcaemia of malignancy	(Mero, Spolaore et al. 2009)
Methionyl human granulocite colony stimulating factor (Filgrastim)	19	20	Glutamine	Neutropenia	(Scaramuzza, Tonon et al. 2012)
Cytochrome C	13.4	CBZ-QG-mPEG (5.3)	Lysine	PEGylation of protein at the level of Lys residues	(Zhou, He et al. 2016)
Fibronectina (FN)	250–270 kDa	2, 5, or 10 kDa	Lysine	Wound healing	(Chen, Raj et al. 2014)
Human glucagon-like peptide-1 (GLP-1)	20	20	Glutamine	Type 2 diabetes	(Selis, Schrepfer et al. 2012)
α-lactalbumin and granulocyte colony stimulating factor (G-CSF)	NA	20	Glutamine Immobilized mTGase on agarose beads	Enzyme was immobilized (simplifies the purification protocol) and promote the formation of homogeneous mono-conjugates	(Grigoletto, Mero et al. 2017)

Granulocyte colony-stimulating fator (G-CSF)	NA	20	Glutamine	Computational approach aimed at identifying the glutamines modified by the enzyme (to treat neutropenia)	(Maullu, Raimondo et al. 2009)
Cytochrome C	13.4	pNIPAM-mTGase (44)	Immobilized mTGase on carboxylated poly(N-isopropylacrylamide) (pNIPAM)	mTGase termo-responsivo	(Zhou, He et al. 2016)
Human granulocyte colony-stimulating factor, human growth hormone, and horse heart apomyoglobin	NA	Monodisperse Boc-PEG-NH_2	Glutamine	Direct identification of the sites of protein modification by mass spectrometry	(Mero, Spolaore et al. 2009)

NA: data not available

1 Table 3. Summary of Tissue engineering studies (Hydrogels and 3D Matrix) studies found in literature involving microbial transglutaminases.

Matrix	**3D Bioprinting**	**Uses**	**References**
Hyaluronic acid (HA) Carboxylated chitosan Recombinant human-like collagen (HLC)	Not used	Wound dressing	(Zhu, Lei et al. 2018)
Gelatin	Human mesenchymal stem cell (hMSCs) (3D cultures)	Promote myocardial lineage commitment and improve the organization and rhythmic beating of cardiomyocytes	(Ajay, Scott Alexander et al. 2018)
Gelatin	HRP to crosslink hyaluronic acid grafted with tyramine (HA-Ty)	Skin defects stemming from trauma, burns and skin infections	(Fan, Zhang et al. 2015)
Collagen peptide molecules Amino group of chitosan (CS-COP)	Schiff-base reaction CS-COP and the aldehyde of oxidized konjac glucomannan	Wound dressing	(Liu, Wen et al. 2018)
Gelatin (porcine skin)	Entrapment of Human adipose-derived stem cells (hASCs) inside the hydrogels (3D cultures)	Injectable hydrogels for the engineering of musculoskeletal and other types of connective tissues	(Alarake, Frohberg et al. 2017)

Collagen *Nano-hydroxyapatite Chondroitin sulphate*	Human mesenchymal stem cells (hMSC)	Bone grafting	(Sharma, Brand et al. 2017)
Gelatin	Adipose tissue-derived stromal cells (ADSCs) cultured on the 2D gel surface and 3D hydrogel encapsulation	Wound healing, and soft and hard tissue repair, or as a drug delivery carrier for drug screening	(Yang, Xiao et al. 2016)
Gelatin	Not used	An alternative adhesive that is analogous to the fibrin sealant for stop bleeding, seal leaks, bind tissue, and/or ease healing	(McDermott, Chen et al. 2004)
Gelatin	3T3 fibroblasts	Protein-based scaffolds for use in soft tissue regeneration	(Broderick, O'Halloran et al. 2004)
Fish gelatin	Not used	Investigates diverse types of formed networks in physical gels, chemical gels, chemical-physical gels and physical-co-chemical gels	(Bode, da Silva et al. 2011, Bode, da Silva et al. 2013)
Gelatin	MC3T3-E1 pre-osteoblastic cells (3D)	How 3D matrix and cell density regulating osteocyte differentiation and the formation of the osteocyte network *in vitro*.	(Garrigle, Mullen et al. 2016)
Gelatin	Human mammary fibroblasts (HMFs)	How 3D matrix stiffness affects breast cancer associated fibroblasts morphology and activation	(Woods, Thigpen et al. 2017)
Human-like collagen (HLC)	Not used	Injectable soft-tissue filling hydrogel	(Zhao, Li et al. 2016)
Chitosan *Tilapia fish skin gelatin*	Not used	Applying small-angle neutron scattering (SANS) was investigated the nanoscale architecture of biopolymer gels conducted in two different microenvironments: an enzymatic process and a hybrid physical-co-chemical	(da Silva, Bode et al. 2015)

		process	
Gelatin (with/without polyethylene oxide)	Human Umbilical Vein Endothelial Cells (HUVECs) and Human Embryonic Kidney Cells (HEK 293)	Optimization two gelatin bioink systems for bioengineering (2D and 3D) for cardiovascular, skin and other soft tissue bioengineering	(Irvine, Agrawal et al. 2015)
Gelatin and chitosan	Not used	Gelation kinetics and equilibrium rheological properties of mixed gels of chitosan and gelatin for tissue repair applications	(da Silva, Bode et al. 2014)
Gelatin	Mouse embryonic fibroblast cells (NIH 3T3), Adipose-derived stem cells (ADSC) and Human hepatoma Huh7 cells	Fabricating large, perfusable, macroporous and cell-laden hydrogel scaffolds	(Chen, Yang et al. 2014)
Gelatin	*E. coli* BL21/pTrcHisBGFPuv - plasmid pTrcHisB that had been modified to express a hexahistidine-tagged green fluorescent protein (GFP)	Entrapment of cells within a biopolymeric hydrogel matrix especially useful for microfluidic biosensor systems	(Chen, Small et al. 2003)

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Table 4. Summary the microencapsulation and studies of complex coacervation technique found in literature involving mTGases.

Microcapsules	**Cells**	**Results**	**References**
Sodium caseinate (Na-Cs), skim milk powder (SMP) and Extra virgin grape seed oil	*Lactobacillus paracasei* cells	Probiotic remained alive during storage time (above 8 log CFU/g). Incorporation of *L. paracasei* resulted in an incremented antioxidative activity of cheese.	(Moghaddas Kia, Alizadeh et al. 2018)
Whey proteins isolates (WPI), acacia gum and sea buckthorn (SBT) supercritical CO2 extract	Not use	A satisfactory antioxidant activity. Antifungal activity against *Penicillium expansum.*	(Mihalcea, Turturică et al. 2018)
Gelatin and gum Arabic	Not use	Effective in maintaining the integrity of the microcapsule wall under simulated gastric conditions. Dissolved under simulated intestinal conditions.	(Tello, Prata et al. 2016)
Gelatin–maltodextrin (G-MD) and oil	*Lactobacillus* spp.	Survival of *Lactobacillus* spp. in gastrointestinal tract under simulated conditions and released in the intestinal under simulated conditions.	(Nawong, Oonsivilai et al. 2016)
Soy protein isolate (SPI)	*Lactobacillus rhamnosus*	Survival the *L. rhamnosus* in the simulated gastrointestinal juice and during storage of probiotic yoghurt.	(Li, Wang et al. 2016)
Gelatin-sodium hexametaphosphate (SHMP) and tuna oil	Not use	Oil was successfully microencapsulated and the stability of omega-3 oils was more than double that of non-encapsulated oil.	(Wang, Adhikari et al. 2014)
Whey protein microcapsules (WPMs) and soy oil	*Bifidobacterium bifidum* F-35	Increased survival of the encapsulated cells at room temperature and at temperature of 4 °C.	(Zou, Liu et al. 2012)

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RESULTADOS

Os resultados deste trabalho estão apresentados na forma de dois artigos científicos.

No capítulo III é apresentado o artigo já publicado na *Enzyme and Microbial Technology* com o título "***Cloning and expression of the Bacillus amyloliquefaciens transglutaminase gene in E. coli using a bicistronic vector construction***", onde descrevemos pela primeira vez a produção de TGase recombinante de *Bacillus amyloliquefaciens* em *Escherichia coli*, obtendo a proteína em sua forma solúvel e ativa. Para isso construímos um plasmídeo bicistrônico contendo o gene inativo da TGase de *B. amyloliquefaciens* fusionado ao prodomínio de *Streptomyces caniferus*. Para tornar a enzima ativa e evitar a necessidade da remoção do prodomínio *in vitro*, também clonamos o gene da protease 3C no mesmo plasmídeo.

No capítulo IV é a presentado um artigo a ser submetido para publicação intitulado "**Expression of *Bacillus amyloliquefaciens* transglutaminase in recombinant *E. coli* under the control of a bicistronic plasmid system in DO-stat fed-batch bioreactor cultivations**", onde estudamos a expressão da transglutaminase de *Bacillus amyloliquefaciens* clonada em *Escherichia col*i BL21 (DE3) pLysS que abriga o plasmídeo pBAD/3C/bTGase, um sistema de expressão bicistrônico, no cultivo de biorreatores utilizando estratégias de batelada e batelada alimentada (DO-stat).

CAPÍTULO III - Cloning and expression of the *Bacillus amyloliquefaciens* transglutaminase gene in *E. coli* using a bicistronic vector construction

Lovaine Silva Duarte[a], Laísa Quadros Barsé[b], Pedro Ferrari Dalberto[b], William Tadeu Santos da Silva[a], Rafael Costa Rodrigues[a], Pablo Machado[b], Luiz Augusto Basso[b], Cristiano Valim Bizarro[b], Marco Antônio Záchia Ayub[a,*].

[a]Biotechnology, Bioprocess, and Biocatalysis Group, Food Science and Technology Institute, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, PO Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil;

[b]Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), 92A TECNOPUC Building, 4592 Av. Bento Gonçalves, ZC 90650-001, Porto Alegre, Brazil.

*Corresponding author:
Tel.: +55 51 3308 6685; fax: +55 51 3308 7048
E-mail address: mazayub@ufrgs.br (M. A. Z. Ayub)

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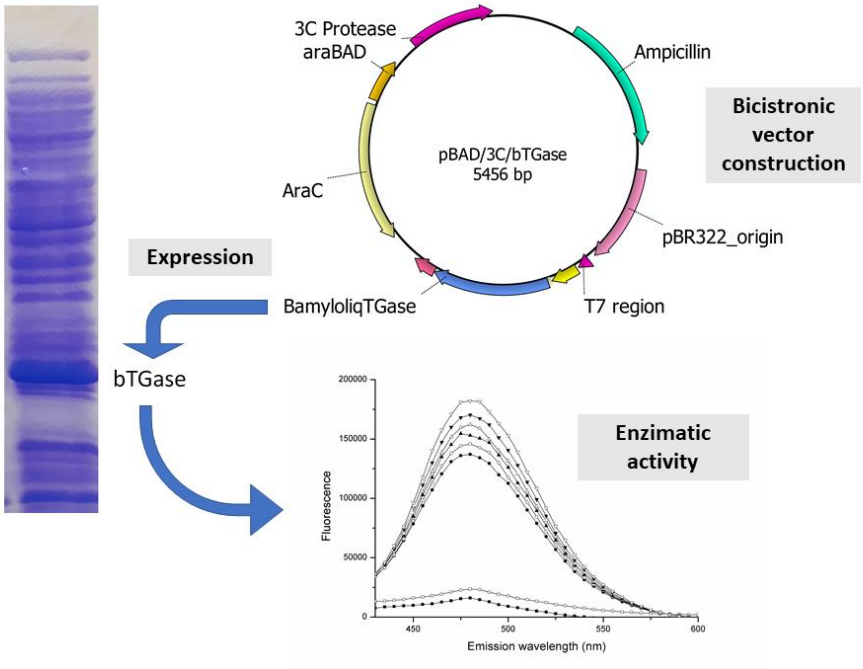


Scheme1. Graphical abstract

ABSTRACT

Transglutaminases (TGases) are a class of transferases widely used in the food and biotechnology industries. In this work, we describe the production of recombinant *Bacillus amyloliquefaciens* TGase in *Escherichia coli*, obtaining the protein in its soluble and active form. In order to reduce TGase activity inside host cells and consequently its toxicity, we constructed a bicistronic plasmid containing the *B. amyloliquefaciens* TGase gene fused to the inhibitory *Streptomyces caniferus* prodomain. To make the enzyme active and avoid the need of prodomain removal *in vitro*, we also cloned the 3C protease gene into the same plasmid. After a fast single-step purification protocol, we obtained a partially purified recombinant TGase with 37 mU/mg protein activity, that crosslinked bovine serum albumin (BSA). This is the first report on the expression of *B. amyloliquefaciens* TGase in *E. coli* in its mature and active form.

Keywords: Transglutaminase; Microbial Transglutaminase; Protein Cross-Linking; Food Enzymes; Bacillus amyloliquefaciens.

1. INTRODUCTION

The use of enzymes in industrial processes has increased steadily in the last years, particularly in the food sector. Enzymes are non-toxic biocatalysts, which are environmentally benign, and their use reduce processing time and energy consumption, increasing industrial efficiency and productivity [1, 2]. Recombinant enzymes can be produced in large quantities by genetically-modified microorganisms to attend growing demands, at low costs [3].

Enzyme applications are focused on a variety of markets, including food and beverages. This segment dominates the industrial enzyme market and it is projected to reach US$ 2.3 billion by 2020 [4, 5]. Enzyme preparations have been used in food production and processing since the early 20th century [6]. The consumer market demands high-quality products, using less chemical additives and preservatives. Consequently, there is a growing need for new, improved, and more versatile enzymes to develop innovative, sustainable and economically competitive production processes [4].

Transglutaminase (TGase, protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyzes the formation of inter- and intra-crosslinking reactions among protein molecules. Its mechanism of action is the transamidation of the carboxamide moiety of a glutamine residue (acyl donor) with a primary amine, typically the side chain of lysine residue (acyl acceptor) [7]. This reaction can modify functional characteristics such as viscosity, gelation, solubility, heat stability, and water retention capacity of a given protein system [8, 9]. TGase ability to promote reticulation is widely used by the food industry in various processes, as in the manufacture of cheese and other dairy products, in meat processing, in the production of edible films and in the manufacture of bakery products [10-12]. Moreover, TGase has a wide variety of applications in the pharmaceutical industry, such as the production of antibody-drug conjugates (ADCs), in regenerative medicine, as well as in protein PEGylation. It also finds applications in the textile and leather industries, biofilm production and enzyme immobilization [13-20].

TGase has been reported to be found in mammals and other vertebrates, invertebrates, mollusks, plants, and microorganisms [21, 22]. It is involved in various physiological functions, from blood coagulation and wound healing to stabilization of

photosynthetic complexes and programmed cell death in plants [7, 18, 23-26]. In 1989, Ando and colleagues first reported the detection of a microbial transglutaminase (mTGase), from *Streptomyces sp.* Unlike its mammalian counterparts, *Streptomyces* mTGase was found to be Ca^{+2}-independent, smaller in size and composed of a single domain [27]. mTGase is expressed extracellularly as a zymogen (inactive enzyme) containing an N-terminal prodomain that covers the active site. Along with the zymogen, extracellular proteases are expressed to cleave the prodomain in order to produce the active enzyme [28].

So far, the mTGase produced by fermentation of wild-type *S. mobaraensis*, remains the main industrial source of this enzyme [12]. Thus, there have been many attempts to produce TGase from *Streptomyces* in heterologous microbial hosts. [28-34]. The production of recombinant mTGase has been technically challenging, resulting in insoluble proteins forming inclusion bodies in the host cell, or in preparations with low activity [35-38].

Bacillus transglutaminase (bTGase), discovered in 1996, is involved in the formation of crosslinks of spore-surface coat proteins and seems to be a promising alternative to TGase production [39, 40]. bTGase is a single-domain protein, produced in its active form, and the smallest TGase characterized to date (28 kDa) [42]. Moreover, it has little structural homology to its bacterial counterparts [41]. bTGase also catalyzes the formation of inter- and intra-crosslinking reactions forming ε- (γ-glutamyl) -lysine crosslinks that are protease-resistant and stable [40]. Compared to commercial *Streptoverticillium mobaraense* transglutaminase, bTGase was more stable over a wide range of temperatures (30 to 60 °C) and pH (pH 5.0 to 9.0), with maximum enzymatic activity at 60 °C and pH 8.0 [42]. Its robust activity may be desirable for various applications as a biocatalyst [43]. Despite these important features, few works have been done to produce or improve current bTGase yields, which are too low and insufficient for large-scale and cost-effective production [44].

In this study, we cloned and expressed the gene coding for *Bacillus amyloliquefaciens* TGase for the first time in *E. coli*, and the recombinant bTGase protein was produced in its active and mature form. For this purpose, we constructed a bicistronic plasmid containing the *B. amyloliquefaciens* bTGase gene fused to the *Streptomyces caniferus* prodomain. In addition, we also cloned the 3C protease gene in order to make the enzyme active, avoiding the necessity of removing the prodomain *in*

vitro. A single-step purification protocol was developed, and the TGase activity of partially purified recombinant bTGase was investigated by both fluorescence and BSA crosslinking assays.

2. MATERIALS AND METHODS

2.1 Materials, bacterial strains, plasmids and cell maintenance

Bacterial strains *E. coli* TOP10 and *E. coli* BL21 (DE3)pLysS were obtained from Invitrogen, San Diego, CA. *E. coli* TOP10 was used as hosts for DNA manipulation, and *E. coli* BL21 (DE3)pLysS was used for recombinant protein production. Bacterial cells were frozen and kept at -20 °C.

PureLink™ Quick Plasmid Miniprep Kit and PureLink™ Quick Gel Extraction and PCR Purification Combo Kit were obtained from Invitrogen, San Diego, CA. Plasmid pBAD/HisA (Life Technologies, Gaithersburg, MD) served as a starting point to construct the bicistronic vector used for recombinant protein production.

Lysogeny broth (LB) (yeast extract 5 g/L, tryptone 10 g/L, and NaCl 10 g/L) and Terrific broth (TB) (tryptone 12 g/L, yeast extract 24 g/L, glycerol, 4 mL/L, KH_2PO_4 0.17 M, K_2HPO_4 0.72 M) were used for DNA plasmid cloning and gene expression, respectively [45].

All enzymes used for DNA manipulation were purchased from New England Biolabs (NEB). The chemicals used in this study were of analytical grade or molecular biological grade and purchased from Sigma-Aldrich, unless otherwise stated.

2.2 *Bacillus subtilis* bTGase sequence identification

The NCBI Protein BLAST web interface was used to search for similar sequences of *Bacillus subtilis* bTGase [46]. The amino acid sequence of *B. subtilis* protein–glutamine γ-glutamyltransferase (Uniprot accession number: P40746) was entered as a query. We identified a homologue protein from the strain *B. amyloliquefaciens* DSM 7 (GenBank: CBI44050.1). Alignment of *B. subtilis* and *B. amyloliquefaciens* amino acid sequences was performed with Clustal Omega version 1.2.1 [47].

2.3 Plasmid construction

Molecular cloning techniques were performed according to methods described by Sambrook et al. [48]. The genes encoding TGase and 3C protease were chemically synthesized (Biomatik®) and the gene sequence encoding for bTGase from *Bacillus amyloliquefaciens* and 3C protease from *Human rhinovirus* was codon-optimized for *E. coli* expression. The expression plasmid containing bTGase and 3C protease genes was constructed according to Rickert *et al.* [28] and Liu *et al.* [44], with some modifications as described below. Genetic sequencing was performed by the company ACTGene Análises Moleculares (actgene@ludwigbiotec.com.br) and confirmed the identity and integrity of the product.

2.3.1 First insert - Construction of the expression plasmid pBAD/3C

Expression plasmid pBAD/3C was constructed by cloning the 3C protease gene from Rhinovirus B14 (GenBank accession number NP_740524.1) into pBAD/HisA under the control of the araBAD promoter, introduced in the cloning linker site between restriction enzymes *Sac*I and *Eco*RI at the 5' and 3' ends, respectively.

2.3.2 Second insert - Construction of the plasmid pBAD/3C/bTGase

Expression plasmid pBAD/3C/bTGase was constructed by subcloning the T7 promoter and the T7 terminator of plasmid pET20b (+) into the plasmid pBAD/3C within the restriction sites *Pci*I and *Bsm*BI at the 5' and 3' ends respectively.

The fragment of bTGase gene from *Bacillus amyloliquefaciens* DSM7 (GenBank accession number CBI44050.1) and the *Streptomyces caniferus* prodomain (GenBank accession number AM746294.1) was cloned into pBAD/3C under the control of the T7 promoter. A 3C protease recognition and cleavage site was inserted between the prodomain and bTGase. To optimize bTGase expression, a codon for the amino acid lysine (AAA) was placed shortly after the initial codon of the ATG [49].

2.4 TGase prodomain mutagenesis

To suppress TGase activity and reduce toxicity to host cells, the sequence encoding the TGase prodomain region of *Streptomyces caniferus* was fused upstream to the TGase gene, producing a fused protein containing the prodomain in its N-terminal part [44].

Rickert *et al.* have suggested that the D20A mutant (*Streptomyces mobaraensis*) achieves the correct balance of prodomain interaction force. At low temperature, during protein expression, it exhibits poor self-crosslinking activity in the *E. coli* cytoplasm. However, this activity can be reestablished after dissociation of the prodomain [28]. To reduce the interaction between the prodomain and TGase, and therefore to facilitate their dissociation and achieve complete enzymatic activity after protein purification, a mutation in the *S. caniferus* prodomain was performed to replace Asp(GAC) by Ala (GCG) at position 22 (D22A), correspondent to the D20A mutation of *Streptomyces* mTGase [28].

2.5 Protein expression

E. coli BL21 (DE3) and *E. coli* BL21 (DE3)pLysS were used as hosts for recombinant protein production. Plasmids pBAD/3C/bTGase and pBAD/HisA were transformed into *E. coli* and cultured on LB agar plates containing 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol (*E. coli* BL21 (DE3)pLysS). A single colony was grown overnight in 50 mL of LB at 180 rpm at 37 °C with the same antibiotics.

A 1 mL cell culture suspension was added to 100 mL of TB medium, supplemented with 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol (*E. coli* BL21 (DE3)pLysS) and incubated at 37 °C on an orbital shaker at 180 rpm until reaching an $O.D._{600}$ of 0.4 – 0.6. The temperature was reduced to 20 °C and cells were equilibrated to the lowered temperature for 30 to 40 min under continuous shaking before induction.

The pro-bTGase gene was induced with IPTG (0.1 mM, 0.4 mM and 1.0 mM) and the 3C protease gene was induced by L-arabinose (0.002 %, 0.02 % and 0.2 %). Aliquots were taken at 0.5, 1, 2, 3, 4, 6, 9, 12, 24 and 48 h. Attempts were made for co-induction and sequential induction (using first IPTG and L-arabinose).

Cells were harvested by centrifugation at 8,000 × *g* for 30 min at 4 °C and stored at -20 °C. DNA sequencing and mass spectrometry were used to confirm the sequence identity and protein integrity.

2.6 SDS-PAGE

SDS-PAGE was performed using a BioRad Mini-PROTEAN® TGX™ system and a 12 % polyacrylamide gel as described by Laemmli [50]. The lanes of the gel were loaded with 10 µL of the samples plus 2.5 µL of SDS loading dye 5x (Tris-HCl 62.5 mM, pH 6.8; Glycerol 25 %; Sodium Dodecyl Sulfate 2 %; Bromophenol Blue 0.01 %). Then, the gel product was stained using Comassie Brilliant Blue R-250.

2.7 Mass spectrometry

The presence of 3C protease (20 kDa) and bTGase (28 kDa) in protein extracts were investigated by liquid chromatography coupled to mass spectrometry (LC-MS/MS) of sodium dodecyl sulphate 12 % polyacrylamide gels (SDS-PAGE) slices. Proteins were loaded on SDS-PAGE and sections of each protein were excised and submitted to in-gel digestion [51]. Tryptic digest was separated on an in-house made 20 cm reverse-phase column (5 µm ODSAQ C18, Yamamura Chemical Lab, Japan) using a nanoUPLC (nanoLC Ultra 1D plus, Eksigent, USA) and eluted directly to a nanospray ion source connected to a hybrid mass spectrometer (LTQ-XL and LTQ Orbitrap Discovery, Thermo, USA). The flow rate was set to 300 nL/min in a 60 min reverse-phase gradient. The mass spectrometer was operated in a data-dependent mode, with full MS1 scan collected in the Orbitrap, with m/z range of 400-1,600 at 30,000 resolution. The eight most abundant ions per scan were selected to CID MS2 in the ion trap. Mass spectra were analyzed using PatternLab platform [52]. MS2 spectra were searched with COMET [53] using a non-redundant database containing forward and reverse *E. coli* BL21 (DE3)pLysS reference proteome and the sequence of both proteins. The validity of the peptide-spectra matches (PSMs) generated by COMET was assessed using Patternlab´s module SEPro [52], with a false discovery rate of 1 % based on the number of decoys.

2.8 Purification of TGase

A one-step partial purification protocol was developed. Initially, 8 g of frozen cells were resuspended in 80 mL of 20 mM Bis Tris pH 6 (buffer A) containing 0.2 mg/mL of lysozyme (Ludwig Biotec) and gently stirred for 30 min. Cells were completely disrupted by sonication (10 pulses, 10 s each, 60 % amplitude) and centrifuged at 48,000 × *g* for 30 min. The supernatant was incubated with 1 % (w/v) of

streptomycin sulfate for nucleic acid precipitation and stirred for 30 min. The solution was centrifuged at 48,000 × *g* for 30 min and the supernatant was collected. The supernatant was dialyzed twice against 1 L of buffer A using a dialysis tubing with a molecular weight exclusion limit of 12 to 14 kDa. The sample was centrifuged at 48,000 × *g* for 30 min and the supernatant was loaded on a SP Sepharose Fast Flow cation exchange column (GE Healthcare) pre-equilibrated with buffer A. The column was washed with 5 column volumes (CV) of the buffer A and adsorbed proteins were eluted with a linear gradient (0 to 75 %) of 15 CV of 20 mM Bis-Tris pH 6 containing 1 M NaCl (buffer B) at 5 mL/min flow rate. Fractions containing the target protein were pooled and protein concentration was determined by the method of Bradford using bovine serum albumin as standard (Quick Start™ Bradford BIO-RAD). Recombinant transglutaminase protein fractions were analyzed in 12 % SDS-PAGE stained with Coomassie Brilliant Blue.

2.9 TGase activity assay

2.9.1 Fluorometric Assay

The TGase activity was measured by the incorporation of the fluorescent amine, dansylcadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide, MDC] into N, N-dimethylcasein by the method of fluorescence [44, 54, 55]. The TGase activity assay system used as substrate 12.5 μM MDC, 0.2 % N, N-dimethylcasein and 4.5 mM dithiothreitol (DTT). The fluorescence intensity was measured in a Shimadzu RF-5301 Spectrofluorometer (Shimadzu, Columbia, MD, USA) using an excitation and emission wavelength of 350 and 500 nm, respectively. One unit of TGase was defined as the amount of enzyme that incorporated 1.0 nM MDC into casein per minute at 37 °C. TGase activity could be calculated using the following formula (Equation 1):

$$[MDC]_{incorporated} = \frac{I - I_0}{13 \times I_0} \times [MDC]_{total} \quad (1)$$

where I_0 denotes the fluorescence intensity of the reaction mixture without TGase and I denotes the fluorescence intensity of the reaction mixture with TGase.

2.9.2 Crosslinking of BSA with TGase

bTGase activity was also determined as its ability to crosslink to bovine serum albumin (BSA). The reaction system consisting of 5 μL BSA (10 mg/mL in 0.1 M Tris-

HCl, pH 8.0) and 35 µL DTT solution (2 mM DTT in 0.1 M Tris-HCl, pH 8,0) was incubated at 50 °C for 12 h with or without 10 µL of bTGase. The *E. coli* BL21 (DE3)pLysS transformed with pBAD/HisA plasmid was used as a negative reaction control. The reaction products were analyzed by 12 % SDS-PAGE and the gel stained with Coomassie R-250 Bright Blue [44, 56, 57].

3. DISCUSSION AND RESULTS

3.1 Cloning, expression and purification of recombinant bTGase in *E. coli*

The main objective of the present work was the heterologous production of recombinant TGase from *Bacillus*, an enzyme with different and attractive properties for use in the food industry [42, 44, 58]. For this, *E. coli* was used as an efficient expression system. In addition to being a versatile bacterium, *E. coli* rapidly grows to a high-cell density in low carbon sources and is the host of choice for the first attempt at recombinant protein production [59].

As TGase from *Bacillus subtilis* was known, we used its amino acid sequence to search for other bacterial homologues. The TGase from *B. amyloliquefaciens*, a gram-positive spore-forming bacterium that was sequenced in 2011 [60], was among the best matches obtained. Comparison of primary structures of *B. subtilis* and *B. amyloliquefaciens* gene products showed 73 % similarity (supplementary material of Figure 1), indicating that the structure and function of these enzymes may be preserved.

However, unlike *S. mobaraensis* TGase, which is synthesized as a proenzyme, the bTGases from Bacillus species are already produced in their mature forms and the expression of bTGase as a heterologous protein in bacteria can be toxic and affect normal cell growth through protein cross-linking within host cells [39, 40, 43, 44]. Therefore, to suppress TGase activity and reduce toxicity in microbial host cells, the sequence encoding the TGase prodomain of *Streptomyces caniferus* was cloned in frame with the bTGase gene to produce a fusion protein.

In a previous work, an approach to suppress bTGase activity and reduce toxicity in microbial hosts was developed, in which TGase prodomains from seven *Streptomyces* species were fused to *Bacillus subtilis* TGase and the yield of recombinant proteins was compared. *Streptomyces caniferus* prodomain was found to have the strongest suppressive effect on bTGase activity [44]. However, to restore the enzymatic

activity of bTGase, a downstream step of prodomain removal by proteolytic cleavage after enzyme purification was required [45]. To avoid the need for *in vitro* removal of the prodomain and to directly produce active bTGase, we constructed a bicistronic plasmid containing both the fused prodomain-bTGase encoding gene and the 3C protease gene from *Rhinovirus* B14. The 3C protease gene is commonly used in molecular biology to remove high specificity protein fusion marks because it recognizes the Leu-Glu-Val-Leu-Phe-Gln-↓-Gly-Pro sequence and cleaves after the glutamine residue [28, 61]. A 3C protease recognition site was designed between the *S. caniferus* TGase prodomain and the bTGase sequence to produce bTGase in its mature and active form by proteolytic cleavage. In this construct, two different promoters were used to induce independently 3C protease and bTGase production. The 3C protease gene is controlled by the araBAD promoter and the bTGase gene is controlled by the T7 promoter. Since these promoters are responsive to different inducers (L-arabinose and IPTG for araBAD and T7 promoters, respectively), it is possible to induce production of bTGase and 3C protease simultaneously or sequentially. In this way, the timing of expression can be optimized to improve protein yields.

In TGases, prodomains also function as chaperones, assisting recombinant proteins to correctly fold within host cells [62]. The D22A mutation was introduced into the *S. caniferus* prodomain sequence in order to maintain the prodomain chaperone function, at the same time converting the interaction between prodomain and bTGase into a temperature-dependent association. In a previous work, an alanine scan mutagenesis on the *S. mobaraensis* prodomain was performed and it was concluded that the Asp20 mutation (D20A) could achieve a relatively high level of active expression of transglutaminase [28]. Thus, bTGase is expected to remain inactive during expression at 20 °C and restore its full activity when the temperature is raised to 37 °C. The alignment of the amino acids sequences of TGase prodomain of seven species of *Streptomyces* mutated at D22A introduced in the sequence of *S. caniferus* is shown in Figure 1 (marked with arrow).

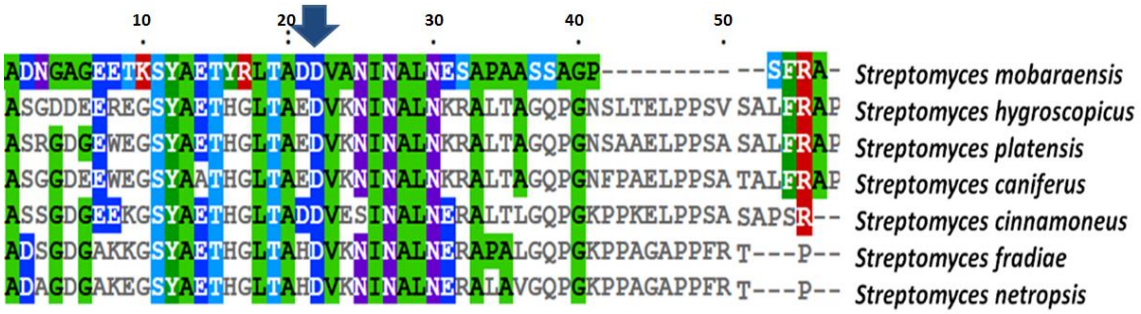


Figure 1. Alignment the amino acid sequences of transglutaminase prodomain of seven species of *Streptomyces*. In this work, a mutation at D22A was introduced in the sequence of *S. caniferus* in order to reduce the interaction between the prodomain and TGase, facilitating their dissociation and achieving complete enzymatic activity (marked with an arrow).

The construction of the pBAD/3C/bTGase vector consisted of two steps, which are schematically represented in Figure 2 and described in detail in section 2.3. Briefly, the 3C protease gene was first cloned under control of the araBAD promoter with restriction enzymes *Sac*I and *EcoR*I. (Figure 2A) and the pro-bTGase insert was introduced under the control of the T7 promoter with the restriction sites *Pci*I and *BsmB*I (Figure 2B).

After the confirmation of the correct sequence construction of pBAD/3C/bTGase, the *E. coli* host cells were used for recombinant protein production. *E. coli* BL21 (DE3) strains were successfully transformed with plasmids pBAD/3C/bTGase and pBAD/HisA. We tested different expression conditions (using first IPTG and L-arabinose) (supplementary material of Figure S2). The best results were obtained under the following conditions: induction with 0.4 mM IPTG for 24 h, followed by induction with 0.2 % L-arabinose for 4 h. In an attempt to obtain better results, we transformed *E. coli* BL21 (DE3)pLysS with plasmid pBAD/3C/bTGase. This host expression strain has been reported to improve the production of toxic proteins that contains the T7 lysozyme gene, increasing the tolerance of *E. coli* cells towards toxicity and lacking proteases that could degrade expressed proteins [63].

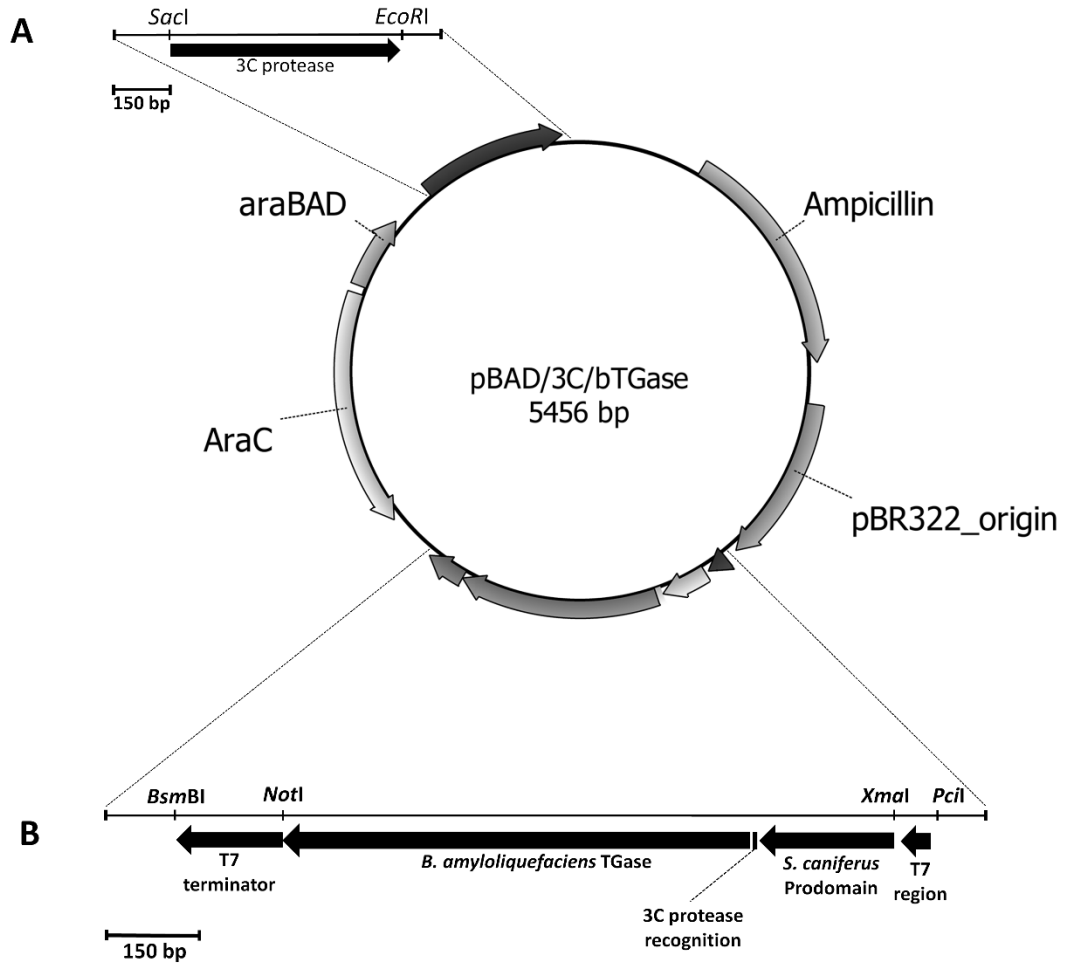


Figure 2. Structure of the pBAD/3C/bTGase expression plasmid used for transformation into *E. coli* host cells. 2A - Schematic representation of synthetic genes of the first cloning into pBAD/HisA - restriction enzyme sites and 3C protease. 2B - Schematic representation of synthetic genes of the second cloning into pBAD/3C. The construct consists of restriction enzyme sites, the *S. caniferus* prodomain gene, and the *B. amyloliquefaciens* bTGase gene. Between the prodomain and the bTGase gene is the 3C protease recognition site.

After induction with 0.4 mM IPTG and 0.2 % L-arabinose (24 h and 4 h, respectively) the whole-cell lysates were analyzed by SDS-PAGE. Total cell lysates from induced samples contained a protein band consistent with the expected size of bTGase (25 kDa), indicating that bTGase gene was successfully expressed in *E. coli* BL21(DE3)pLysS (Figure 3A, lane 5). We also detected on the same samples a protein band of ~ 20 kDa, consistent with the expected size of 3C protease (Figure 3A, lane 5).

A BSA crosslinking test was performed to further test the enzymatic activity of recombinant *B. amyloliquefaciens* bTGase, (Figure 3B). Crosslinking was verified by producing high molecular weight products on top of the separation gel, in addition to decreasing the BSA band in the gel medium (Figure 3B, lane 3). It has been reported in previous works that crosslinking significantly increases the molecular weight of proteins

[42, 64]. A negative control with pBAD/HisA in *E. coli* was performed in parallel (Figure 3B, lane 2). As expected, there was no crosslinking of BSA in the control samples. These results clearly indicated the formation of high-molecular weight polymers resulting from recombinant bTGase catalyzing the BSA cross-linking.

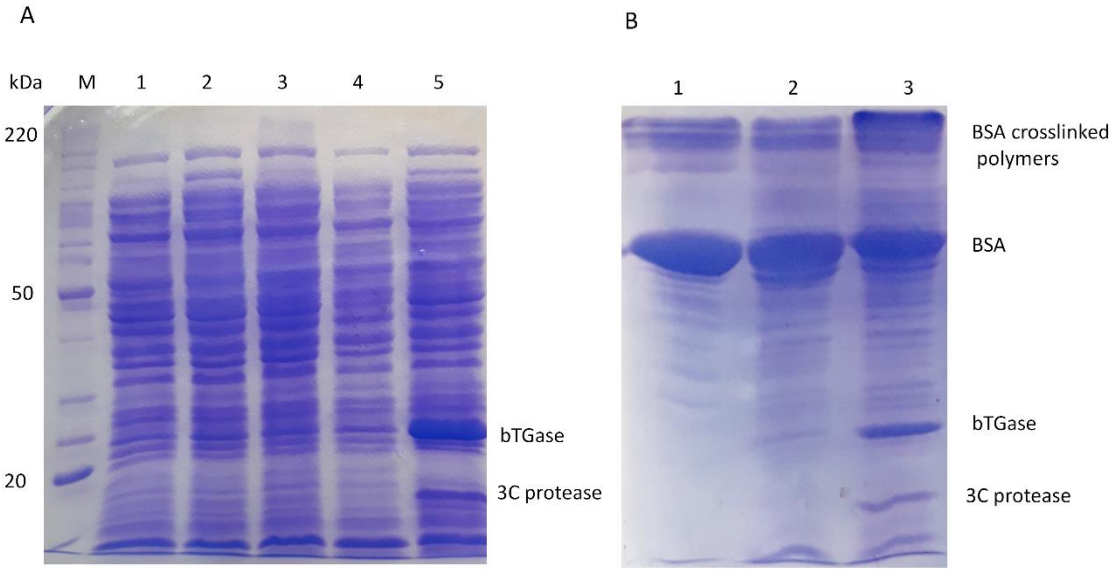


Figure 3. Gel protein expression and BSA activity of plasmid pBAD/3C/bTGase in *E. coli* BL21 (DE3)pLysS. 3A – SDS-PAGE showing expression of recombinant bTGase and 3C protease in *E. coli* BL 21 (DE3)pLysS. Lane M - Molecular weight marker; Lane 1 - 3C protease induced with L-arabinose 0.2 % at 24 h. No expression observed; Lane 2 - pBAD/His/*lac*Z induced with IPTG 0.4 mM as positive control of *lac*Z gene. The positive control yielded a 120 kDa protein corresponding to *lac*Z protein; Lane 3 – pBAD/HisA negative control after induction with L-arabinose 0.2 % for 24 h. There is no expression of 3C protease; Lane 4 – pBAD/HisA negative control after induction with IPGT 0.4 mM in 24 h and 4 h of induction with L-arabinose 0.2 %. There is no expression of bTGase and 3C protease. Lane 5 – pBAD/3C/bTGase after induction with IPTG 0.4 mM in 24 h and 4 h of induction with L-arabinose 0.2 %. There is expression of 3C protease and bTGase. 3B – Crosslinking of BSA by the recombinant bTGase. For BSA crosslinking, the crude extract of *E. coli* BL21 (DE3)pLysS transformed with pBAD/HisA as negative control and crude extract of *E. coli* BL21 (DE3)pLysS transformed with plasmid pBAD/3C/bTGase were tested. The reaction was at 50 °C for 12 h. Lane 1 - BSA control; Lane 2 - BSA polymerization by pBAD/HisA transformed into *E. coli* BL21 (DE3)pLysS (negative control). There is no polymerization of proteins; Lane 3 - BSA polymerization by pBAD/3C/bTGase transformed into *E. coli* BL21 (DE3)pLysS. Polymerization was found on the top of the gel, in addition to decreasing the BSA band in the gel medium.

With the confirmation of enzymatic activity of bTGase, cells were disrupted and bTGase was partially purified using a single-step chromatographic (cation exchange) purification procedure (described in detail in section 2.8). The crude extract and partially purified enzyme were analyzed by SDS-PAGE (Figure 4). We have decided on a partial purification because, for industrial processes, unnecessary and mandatory cost purifications, especially for the food industry, should be avoided. Additional stages of purification make the process expensive and may lead to loss of enzymatic activity. Only other enzymes and materials that may interfere with the

enzymatic catalytic process should be removed [65]. The purification result can be seen in the Table 1.

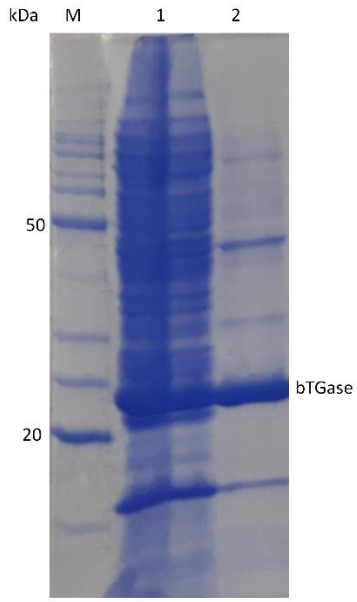


Figure 4. SDS-PAGE of recombinant bTGase protein purification. Lane M - molecular weight marker. Lane 1 – Crude protein extract loaded in SP Sepharose Fast Flow (cation exchange column); Lane 2 – Elution of bTGase protein from SP Sepharose Fast Flow. The bTGase protein band was indicated in figure.

The 25 kDa SDS-PAGE protein band was excised and subjected to trypsin digestion and the peptides analyzed by LC-MS/MS to confirm the identity of bTGase. Spectra matching from three unique peptides (YRIPASK; NPEFNPAK and TYGNTAYWRVTPEGALELK) from transglutaminase were identified, and around 15 % of coverage of the protein was identified.

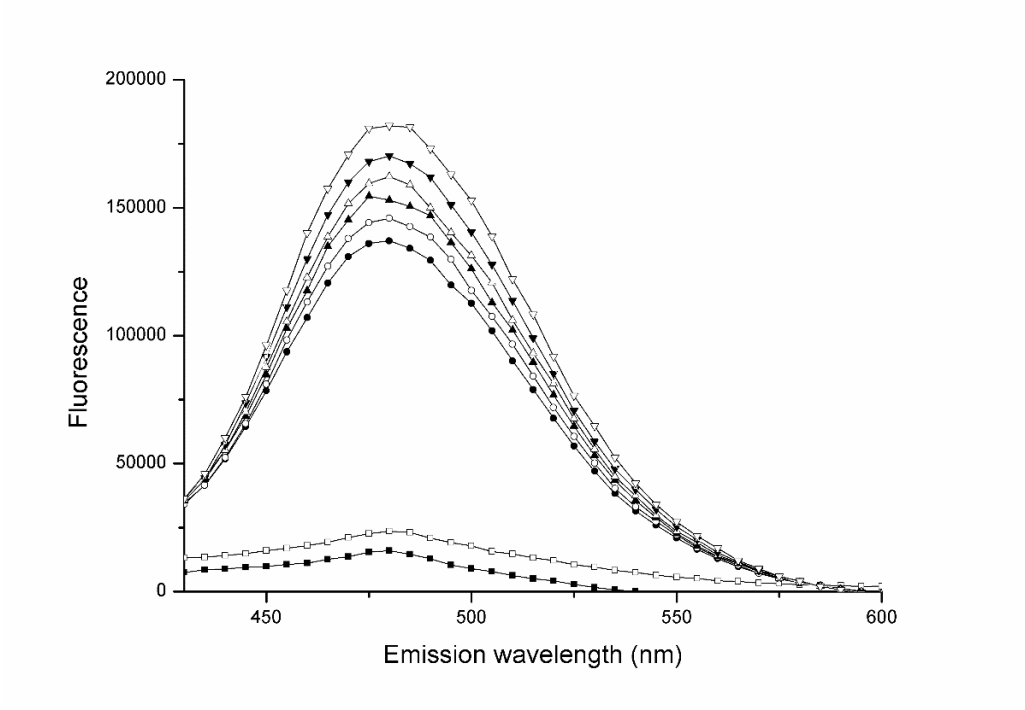
3.2 bTGase activity assay

The establishment of reliable assay methods for measuring enzyme activity has been a concern in transglutaminase research. In the last decades, several assay methods have been developed to measure and quantify bTGase activity, but many of them have shown low sensitivity [66].

In this study, we used two methods to identify the enzymatic activity of bTGase, a BSA-crosslinking method and a fluorescence-based. A quantitative fluorescence measurement method involving the TGase-dependent covalent coupling of monodansylcadaverine to N, N-dimethylcasein produces a change in the intensity and wavelength of the dansyl group fluorescence. TGase activity is measured by increasing fluorescence intensity over time [67].

The recombinant bTGase enzyme activity was determined in the crude extract (Figure 5A) and in the purified fraction (Figure 5B). Changes in the fluorescence spectrum occurred during the enzymatic reaction in both experiments. An increase in the intensity of emitted fluorescence also can be observed. In the absence of enzyme or the enzyme without the presence of substrate, no time-dependent changes in fluorescence can be seen. The specific activity of TGase in the crude extract was 13 mU/mg protein and of the partially purified recombinant protein was 37 mU/mg protein.

A



B

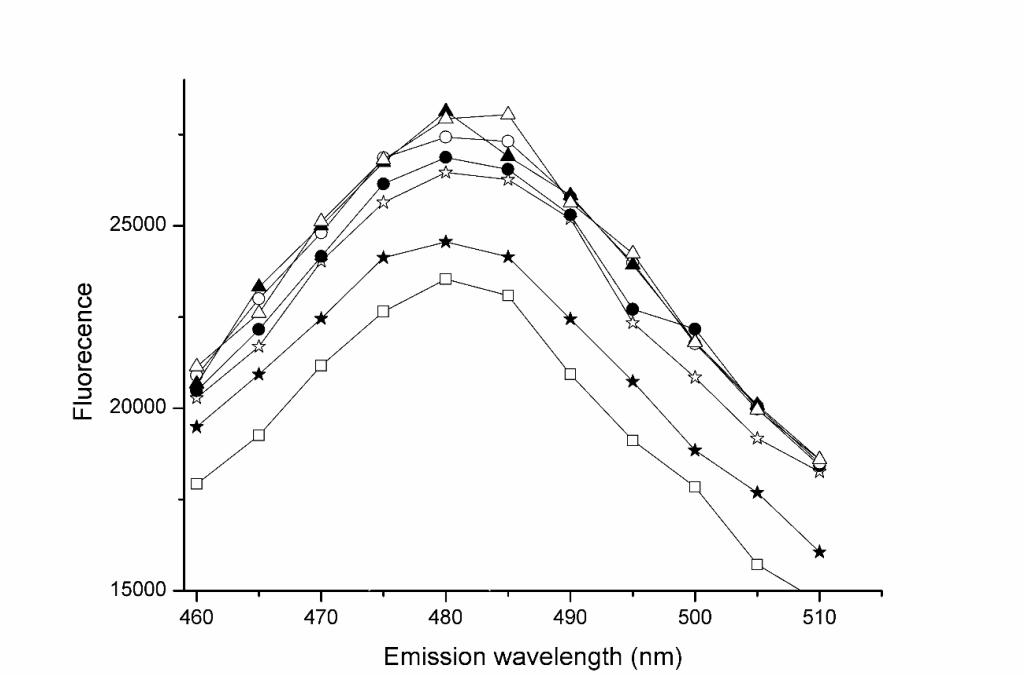


Figure 5 Determination of incorporation of monodansylcadaverine into dimethylcasein by recombinant bTGase in crude protein extract and purificated bTGase. 5A Crude protein extract of bTGase. Curve ■: only monodansylcadaverine; Curve □: only bTGase without substrate; Curve ●: 20 min of reaction; Curve ○: 30 minutes of reaction; Curve ▲: 40 minutes of reaction; Curve △: 50 minutes of reaction; Curve ▼: 70 minutes of reaction; Curve▽: 90 minutes of reaction. 5B Purificated bTGase. Curve □: only bTGase without substrate; Curve ★: 1 minute of reaction; Curve ☆: 10 minutes of reaction; Curve ●: 20 minutes of reaction; Curve ○: 30 minutes of reaction; Curve △: 40 minutes of reaction; Curve ▲: 50 minutes of reaction.

4. CONCLUSIONS

This paper describes for the first time the expression and production of *Bacillus amyloliquefaciens* transglutaminase in *E. coli* using a bicistronic vector. The recombinant protein produced was found to be active without the need of the downstream step of prodomain removal by proteolytic cleavage. The protein was purified and identified by mass spectrometry. Further improvements in protein yield can be achieved in large scale production with higher expected biomass concentrations and optimization of cultivation parameters such as temperature, pH, oxygenation, feed rate and cultivation time.

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TABLE

Table 1. Summary of bTGase purification and enzyme activity.

Step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Yield (%)	Purification Fold
Crude cell lysate	80	209.60	2.72	13	100	1
Cation exchange chromatography	110	33.02	1.21	37	44.5	2.77

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SUPPLEMENTARY MATERIAL

CBI 44050.1 *Bacillus_amyloliquefaciens*_DSM7
Sequence ID: Querry_68451 Length: 45 Number of Matches: 1

Score	Expect	Method	Identities	Positives	Gaps
388 bits(997)	3e-143	Compositional matrix adjust.	178/244(73%)	208/244(85%)	0/244(0%)

```
Query  1    MIIVSGQLLRPQDIENWQIDQNLNPLLKEMIETPVQFDYHSIAELMFELKLRMNIVAAAK  60
            MII+SGQ+LRPQDI NWQ +++L P L E+I +PVQFDY SI ELMFE +LR +IV AA+
Sbjct  1    MIISGQVLRPQDIANWQTEESLVPYLNELINSPVQFDYGSITELMFEARLRRHIVEAAR  60

Query  61   TLHKSGAKFATFLKTYGNTTYWRVSPEGALELKYRMPPSKAIRDIAENGPFYAFECATAI  120
             LH SGAKFATF KTYGNT YWRV+PEGALELKYR+P SKAIRDI ENG FYAFECATAI
Sbjct  61   ELHGSGAKFATFAKTYGNTAYWRVTPEGALELKYRIPASKAIRDIIENGAFYAFECATAI  120

Query  121  VIIYYLALIDTIGEDKFNASFDRIILYDWHYEKLPIYTETGHHFFLGDCLYFKNPEFDPQ  180
            V+IYYLA++ TIGE++F+  F  I LYDWHYE LPIYTETG HF  GDCLYFKNPEF+P
Sbjct  121  VVIYYLAVLKTIGEERFDRRFRDITLYDWHYEHLPIYTETGRHFLRGDCLYFKNPEFNPA  180

Query  181  KAQWRGENVILLGEDKYFAHGLGILNGKQIIDKLNSFRKKGALQSAYLLSQATRLDVPSL  240
            KAQWRGENVI++G D+YFAHGLGIL  +QII +LNS R+K A+QSAYLLSQATRLD P+L
Sbjct  181  KAQWRGENVIVMGNDQYFAHGLGILTAEQIIQRLNSLRRKNAVQSAYLLSQATRLDAPAL  240

Query  241  FRIV  244
            ++I+
Sbjct  241  YQIM  244
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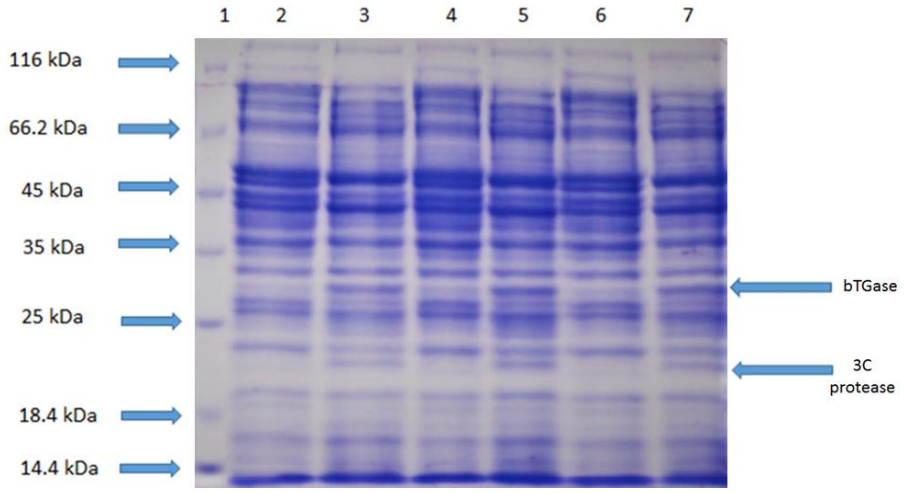
Supplementary material for Figure 1. Sequence alignment of bTGase from Bacillus subtilis and *Bacillus amyloliquefaciens*. Overall identity between *B. subtilis* and *B. amyloliquefaciens* is 73 %.



Supplementary material for Figure 2. SDS-PAGE analysis of expression of recombinant proteins in *E. coli* BL21 (DE3): Lane 1 - Molecular weight marker; Lane 3 shows the induction of bTGase and 3C protease with 0.1 mM IPTG and 0.2 % L-Arabinose. Lane 5 shows the induction of the bTGase and the 3C protease with 0.4 mM IPTG and 0.2 % L-Arabinose. Lane 7 shows the induction of the bTGase and the 3C protease with 1 mM IPTG and 0.2 % L-Arabinose. All inductions were performed for 24 hours with IPTG and more 4 hours of induction with L-Arabinose, totalizing 28 h of induction. Lanes 2, 4 and 6 represent the plasmid pBAD/HisA transformed into *E. coli* BL21 (DE3) at the same inducer concentrations as their pairs. The arrows mark the positions for bTGase and protease 3C protease.

CAPÍTULO IV - Expression of *Bacillus amyloliquefaciens* transglutaminase in recombinant *E. coli* under the control of a bicistronic plasmid system in DO-stat fed-batch bioreactor cultivations

Lovaine Silva Duarte[a], Carla Roberta Matte[a], Paulo Roberto Dalcortivo[a], José Eduardo Sacconi Nunes[b], Laisa Quadros Barsé[b], Cristiano Valim Bizarro[b], Marco Antônio Záchia Ayub[a,*].

[a]Biotechnology, Bioprocess, and Biocatalysis Group, Food Science and Technology Institute, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, PO Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil;

[b]Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), 92A TECNOPUC Building, 4592 Av. Bento Gonçalves, ZC 90650-001, Porto Alegre, Brazil.

*Corresponding author:
Tel.: +55 51 3308 6685; fax: +55 51 3308 7048
E-mail address: mazayub@ufrgs.br (M. A. Z. Ayub)

ABSTRACT

We studied the expression of *Bacillus amyloliquefaciens* transglutaminase cloned in *Escherichia coli* BL21(DE3)pLysS harboring the plasmid pBAD/3C/bTGase, a bicistronic expression system, in bioreactor cultivation. Batch and fed-batch controlled as DO-stat strategies were employed for the production of the recombinant enzyme. In 30 h-batch cultivations using Terrific Broth, 6 g/L of biomass and 3.12 $U/mg_{protein}$ of transglutaminase activity were obtained. DO-stat fed-batch cultivations under the control of oxygen concentration (DO-stat) under the same of conditions produced considerable improvements in biomass (17.5 g/L) and enzyme activity (6.43 $U/mg_{protein}$). An improved mineral medium (M9) used in cultivations under same conditions produced even higher enzymatic activity (9.14 $U/mg_{protein}$). The pH effect was investigated, and the best enzymatic activity could be observed at pH 8. In all cultivations, the bicistronic system remained stable, with 100 % of plasmid-bearing cells. These results show that the bioreactor cultivations under DO-stat fed-batch in M9 medium is a promising strategy for the production of recombinant transglutaminase in *E. coli*.

Keywords: Microbial Transglutaminase; Food enzymes; Fed-batch bioreactor; DO-stat; *Bacillus amyloliquefaciens*; bicistronic plasmid system.

1. INTRODUCTION

Transglutaminase (TGase, protein-glutamine gamma-glutamyltransferase, EC 2.3.2.13) is a multifunctional enzyme that catalyzes the acyl transfer reaction between a γ-carboxamide group of glutamine residue and the ε-amino group of a lysine residue from a variety of primary amines, resulting in the formation of inter- or intramolecular bonds, highly resistant to proteolysis [1-3]. TGases can be found in animal tissues and body fluids and are involved in several biological processes such as blood coagulation, epidermal keratinization and stiffening of the erythrocyte membrane [4, 5]. It has been suggested that TGases may also be present in eggs and skin of amphibians, in turtle shell, in vegetable tissues of soy, broad beans, and orchard apples. They are also present in fungi and yeasts such as *Phytophthora* sp., *Candida albicans* and *Saccharomyces cerevisiae*. However, the largest body of studies refer to microbial transglutaminases (mTGase) of bacteria such as *Streptomyces* and *Bacillus* [6].

The crosslinking properties of transglutaminases are widely used in industrial processes, especially in the food and pharmaceutical industries. The mTGase is used in several types of food to further improve the quality of final products such as viscosity, firmness, water holding capacity and elasticity. Important applications in the textile and leather industries are also observed, as well as in regenerative medicine, PEGylation reaction and in the production of antibody conjugates [7].

In general, the most common techniques applied for the production of TGases for industrial uses are extracting and purifying the enzyme from the tissues or body fluids of animals (pigs, fish, cattle); screening for TGase-producing microorganisms (enzyme production by traditional fermentation technologies); and genetic manipulation using host microorganisms (*Escherichia coli*, *Aspergillus*, *Bacillus*) [8-11]. Because of the generally low titer of enzyme concentration in fermentation broths, many researches have been made in the areas of genetic engineering and bioprocess engineering aiming at improving the production of this enzyme on a large scale [11, 12].

E. coli has proved to be the preferred platform for the production of various enzymes and biological products of commercial interest because of its rapid cell growth, reaching high cell densities through simple cultivation procedures and low production costs in addition to the ability to express high concentrations of recombinant proteins [13, 14]. However, this laboratory workhorse bacterium present several limitations for

heterologous protein expression, such as codon bias, formation of inclusion bodies, lack of post-translation modification, and the efficient growth of *E. coli* has been a challenge for the industry since the early 1970s [15]. Therefore, the optimization of the production bioprocess, such as the modification of media composition, and genetic techniques, such as expression at lower temperatures, co-expression of molecular chaperones, development of new strains, vectors, and markers, have been reported as ways to overcome these limitations [15-18].

High cell density cultivation strategies are necessary to increase microbial biomass and the productivity in bioprocess, and many techniques have been developed for this purpose [19], including optimization of glucose feed rate profile, control of low acetate excretion, and high dry cell weight (DCW), techniques to improve plasmid stability [20-22].

Fed-batch cultures of *E. coli* strains that host gene-products are often reported in literature, including simple methods of indirect feedback such as DO-stat, pH-stat as well substrate feeding based on glucose uptake rate or demand, predetermined feeding strategies (exponential feeding), among others [23-25]. The DO-stat is a simple scheme that involves the application of glucose feeding pulses and the control of the dissolved oxygen concentration (DO) response to those pulses, a technique that requires only an DO sensor and the control of oxygen concentration and the glucose feeding pump [26].

However, *E. coli* cultures produces acetate that can inhibit cell growth and product formation, specially under anaerobic or oxygen-limiting conditions, or when carbon flux exceeds biosynthetic demands and the power generation capacity within the cell [27]. As the metabolic flow through glycolysis is closely related to respiratory activity by reducing NAD^+ to $NADH + H^+$, the glucose feeding rate is directly linked to oxygen consumption [28]. An increase in acetate (above 5 g/L) leads to a reduction in the growth rate with a reduction in biomass. There are reports in the literature, in which the accumulation of acetate in cultures of recombinant cells may be even greater than in cultures of the wild strain, under the same conditions and therefore must be controlled by special strategies during the fed-batch phase [27].

In a previous work, we constructed a bicistronic plasmid containing the TGase gene fused to the inhibitory *Streptomyces caniferus* prodomain. We also cloned the 3C protease gene in the same plasmid, in order to make the enzyme active and avoid the need for removal of the prodomain *in vitro* [8]. The activity of recombinant bTGase was

investigated by cross-linking assays of bovine serum albumin (BSA) and by fluorescence, showing a specific activity of 37 mU/mg in shaker cultivations [8].

Based on these considerations, in the present work, we describe the production the mTGase cloned in *E. coli* bearing the plasmid pBAD/3C/bTGase, comparing the enzyme expression in batch bioreactors and cultures operated using a DO-stat fed-batch controlled as strategy controlled by oxygen concentration (DO-stat).

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, cell maintenance, and materials

The strain used in this work was *E. coli* BL21(DE3)pLysS (Invitrogen,) harboring the plasmid pBAD/3C/bTGase, which contains the fragment of bTGase gene from *Bacillus amyloliquefaciens* DSM7 and the *Streptomyces caniferus* prodomain beyond 3C protease gene. The construction of this expression vector, cell transformation, and sequencing were described in detail in a previous work [8].

Unless otherwise stated, the chemicals used in this study were of analytical grade or molecular biological grade and purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2 Cultivation media and inoculum preparation

Terrific broth (TB) (tryptone 12 g/L, yeast extract 24 g/L, glycerol, 4 mL/L, KH_2PO_4 0.17 M, K_2HPO_4 0.72 M) [29] and modified M9 medium (Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, NH_4Cl 1 g/L, NaCl 0.5 g/L , yeast extract 20 g/L, $MgSO_4.7H_2O$ 0,12 g/L, glucose 5 g/L, trace elements solution 1 mL ($FeSO_4.7H_2O$ 2.8 g/L, $MnCl_2.4H_2O$ 2 g/L, $CaCl_2.6H_2O$ 2 g/L, $CuCl_2.2H_2O$ 0.26 g/L, $ZnSO_4.7H_2O$ 0.3 g/L)) [30], both media containing 100 μg/mL carbenicillin and 34 μg/mL chloramphenicol were used for cell growth and recombinant enzyme production. The feed solution used in the fed-batch cultivations were controlled as DO-stat and it was composed of glucose 300 g/L, yeast extract 30 g/L $MgSO_4$ 0,5 g/L and antibiotics (carbenicillin and chloramphenicol). The inoculum were prepared by transferring a single colony of *E. coli* BL21(DE3)pLysS transformed with plasmid pBAD/3C/bTGase into 500 mL flasks containing 100 mL of Lysogeny broth - LB (yeast extract 5 g/L, tryptone 10 g/L, and NaCl 10 g/L) containing 100 μg/mL carbenicillin and 34 μg/mL chloramphenicol and incubated at 37 °C in a

rotatory shaker operating at 180 rpm, and allowing cell growth until an OD_{600} of 1.0 was reached.

2.3 Batch bioreactor cultivations

Batch experiments were performed in bioreactor BIOSTAT® B plus (Sartorius Stedim, Goettingen, Germany), containing 1 L of TB medium. The bioreactor was equipped with two Rushton turbines and with aeration, temperature, agitation, and pH controllers. A polarographic electrode (Ingold, Germany) was used to measure the dissolved oxygen concentration in the culture. The pO_2, pH, stirrer speed (STIR), base and acid consumption and aeration rate were measured online and recorded by an external data acquisition and control system (Sartorius Stedim, Germany). The initial pH of the culture was adjusted to 7.2. In order to control the temperature at 20 ºC during the expression (induction) of the recombinant proteins, the bioreactor was coupled to a cooling bath (Frigomix® B – B.Braun Biotech International, Germany). The best growth conditions and protein expression were applied to the batch of bioreactors according to conditions previously described in Duarte and collaborators [8]. The culture medium was supplemented with 100 μg/mL carbenicillin and 34 μg/mL of chloramphenicol, a cell suspension of 100 mL with concentration of OD_{600} of 1.0 was used as inoculum in all cultivations. The cultivation followed with constant air flow rate of 1 vvm (air volume per volume of culture medium per minute), 37 °C, and 300 rpm until reaching an OD_{600} of 0.4 - 0.6 (approximately 2 h). At this point in cultivation, the temperature was reduced to 20 °C and continued for another 40 min before induction. Then, the pro-bTGase gene was induced by the addition of 0.4 mM IPTG, followed by 20 h; after this period of time, the 3C protease gene was induced by the addition of L-arabinose 0.2 %, and culture followed for another 6 h. Cells were then harvested by centrifugation at 4000 × g for 30 min at 4 °C and stored at -20 °C. All experiments were performed in duplicate.

2.4 DO-stat fed-batch bioreactor cultivations

DO-stat fed-batch bioreactor experiments were performed in the same bioreactor system and its assembly and culture conditions were the same as described for the batch cultivations. All media were supplemented with antibiotics, as described in section 2.2. The pH was controlled at 7.2 using either 5M NaOH or 5M H_3PO_4.

Feeding was implemented using an internal peristaltic pump after the first 5 h of operation as batch cultures.

The DO-stat fed-batch process was designed to operate as follows:

I – Start as batch cultivation until depletion of the carbon source (glucose), with air supply at a constant rate of 1 vvm and the DO kept at 30 % saturation, varying the agitation rate from 300 to 1000 rpm (cascade of agitation);

II - After 5 h of feeding cultivation (DO-stat) was started. Air supply continued at a constant rate of 1 vvm and agitation was fixed to 800 rpm. Using this approach, whenever the DO rose above the set value (30 %), the pump controlling the feeding supply turned on automatically. As a result, the metabolic activity of *E. coli* increases again and there is a decline in DO. Likewise, when the DO was lower than the set value, the pump that controlled the feeding supply automatically shut down.

Samples were taken at regular intervals to determine biomass (dry weight), residual sugar and acetate.

At the end of the exponential growth phase, the temperature was reduced to 20 °C and the cultivation continued for another 40 min before induction. Then, the pro-bTGase gene was induced by the addition of 0.4 mM IPTG for 4 h; after this time, the 3C protease gene was induced by the addition of 0.2 % L-arabinose, and the culture continued for another 10 h. At the end of this time, the cells were harvested by centrifugation at 4,000 × g for 30 min at 4 °C and stored at -20 °C. All experiments were performed in duplicate.

2.5 Effects of pH on the enzymatic activity of TGase

To evaluate the best buffer to be used in cell disruption, the effects of pH on the enzymatic activity of TGase were studied. Thus, 2 g of frozen cells were resuspended in 20 mL of the following buffers: pH 5 (0.1 M sodium acetate), pH 6 (0.1 M sodium phosphate), pH 7 (0.1 M sodium phosphate), pH 8 (0.1 M This-HCl), and pH 9 (0.1 M This-HCl). Cell suspensions were completely disrupted by sonication (10 pulses, 10 s each, 60 % amplitude) and centrifuged at 13,000 × g for 30 min and the supernatant was collected for analysis of protein and enzyme activity. All experiments were performed in duplicate.

2.6 Analytical methods

2.6.1 Determination of protein and enzymatic activity

The determination of protein concentration was carried out following the Bradford method [31] using bovine serum albumin as a standard (Quick Start ™ Bradford BIO-RAD).

TGase activity was determined by the colorimetric hydroxamate procedure using N-carbobenzoxy-L-glutaminyl-glycine and hydroxylamine [32]. A calibration curve was prepared using L-glutamic acid γ-monohydroxamate. One enzymatic unit of TGase (U) generates 1 μmol hydroxamic acid per minute at 37 °C.

2.6.2 Off-line measurements of cultivation substrates and biomass

The concentrations of glucose and acetate were determined by high performance liquid chromatography - HPLC (Shimadzu, Japan) equipped with a refractive index detector (RID-10A, Shimadzu) and Bio-Rad HPX-87H column (300×7.8 mm) in isocratic mode using as mobile phase a 5 mM H_2SO_4 solution as eluent with a flow rate of 0.6 mL/min and oven temperature of 45 °C. Biomass was measured as DCW, 10 mL of the culture were collected and centrifuged (3,000 × g, 15 min) and oven dried at 80 °C to constant weight using an analytical balance.

2.6.3 Determination of plasmid stability

Because the plasmid pBAD/3C/bTGase was constructed by us in a previous work [8] and had never been tested in bioreactor cultivations, we decided to determine its stability. As the *E. coli* BL21(DE3)pLysS is resistant to chloramphenicol and, when hosting the plasmid pBAD/3C/bTGase, also resistant to carbenicillin, samples from bioreactor cultivations were appropriately diluted and spread in culturing plates with LB medium containing 100 μg/mL carbenicillin and 34 μg/mL chloramphenicol (selection of cells holding the plasmid) and LB medium containing only 34 μg/mL chloramphenicol (cells that have lost the plasmid), according to methodology described in the work of Thomas and collaborators [33] and compared as colony forming unit (CFU).

3 RESULTS AND DISCUSSION

3.1 **Effects of pH on the enzymatic activity of TGase**

Initially, to determine the ideal pH of the expressed enzyme, the cells were disrupted at pH 5, 6, 7, 8 and 9 in the buffers described in section 2.5. TGase activity was determined in the supernatant of samples by the colorimetric hydroxamate procedure using N-carbobenzoxy-L-glutaminyl-glycine and hydroxylamine (section 2.5) at 37 °C. The graph of the enzymatic activity can be seen in Figure 1. The best enzymatic activity was observed at pH 8, which is in accordance with the literature. In previous works, it has been reported that the ideal pH for *Bacillus* transglutaminase is around 8, whereas for *Streptomyces mobaraensis*, the ideal pH is around 6 to 7 [34-36]. With this result, all further tests were performed at the pH 8.

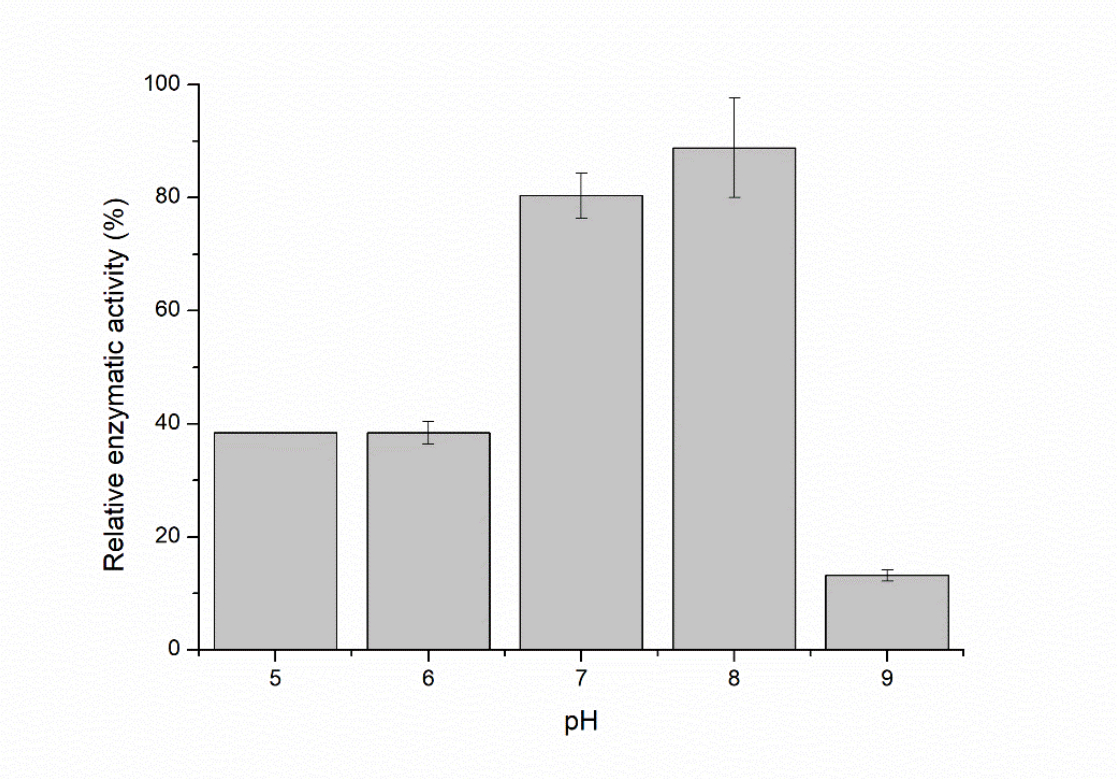


Figure 1. Optimal relative pH activity of the expressed TGase. The cells of *E. coli* were disrupted using the following buffers: pH 5 (0.1 M sodium acetate), pH 6 (0.1 M sodium phosphate), pH 7 (0.1 M sodium phosphate), pH 8 (0.1 M This-HCl), and pH 9 (0.1 M This-HCl). The results represent the mean of duplicates, considering the highest activity detected as 100 %.
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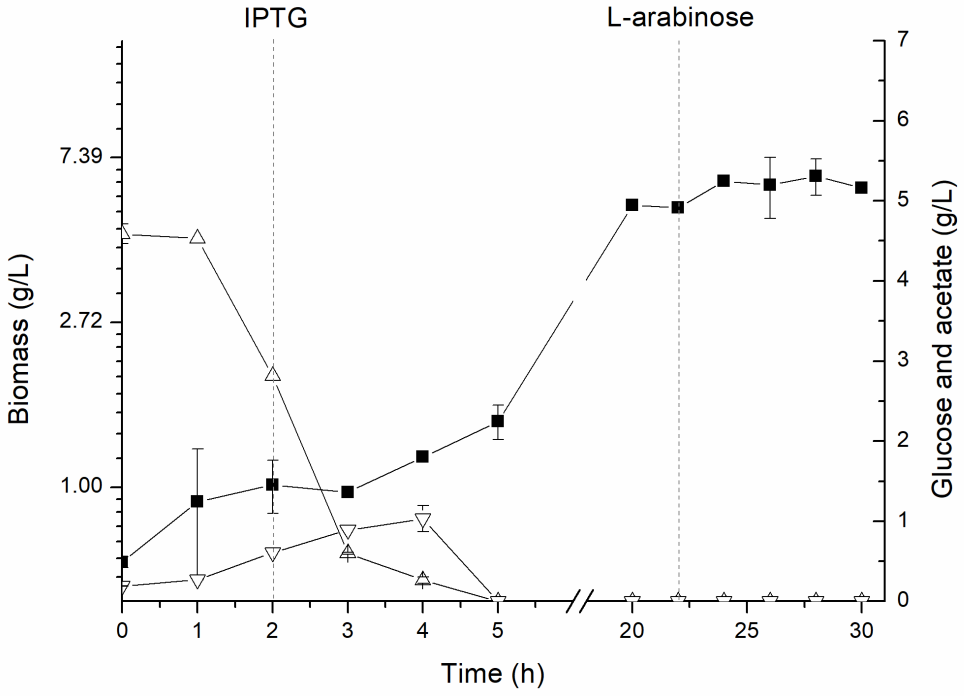
3.2 **Batch bioreactor cultivations**

The batch bioreactor cultivations using the TB medium, were run under the same induction conditions that were previously used in shaker flask cultivations, which are described in Duarte and collaborators [8]. In that work, cultures in TB medium supplemented with carbenicillin and chloramphenicol were incubated at 37 °C on

rotatory shaker at 180 rpm until reaching OD_{600} of 0.4-0.6. At this point, the temperature was reduced to 20 °C for induction of recombinant pro-bTGase gene, induced with IPTG, and the 3C protease gene was induced by L-arabinose. The results of batch cultures are depicted in Figure 2A and 2B, and Table 1, comparing with results from the DO-stat fed-batch experiments. Cell concentration reached 5.5 g/L until IPTG was added. After that there was a small increase in cell concentration, up to 6 g/L in 30 h of cultivation, possibly due to the limitation of nutrients and also the necessary energy for the expression of proteins [37]. With 30 h of culture, the specific enzyme activity was 3.12 $U/mg_{protein}$.

An important factor in cultures of recombinant microorganisms in industrial processes is plasmid stability, which can be affected by culture conditions such as temperature, aeration, pH, induction factors, among others. A decrease in the concentration of DO in the culture medium as well as elevated temperatures can reduce plasmids stability [38]. In our study, as shown in Figure 2 B, a high stability of plasmid was observed, with practically 100 % of cells bearing the plasmid at the end of cultivation. These results demonstrate the viability of using this recombinant strain-plasmid system in order to scale up this process for future industrial applications of TGase cloning and production methodology.

A



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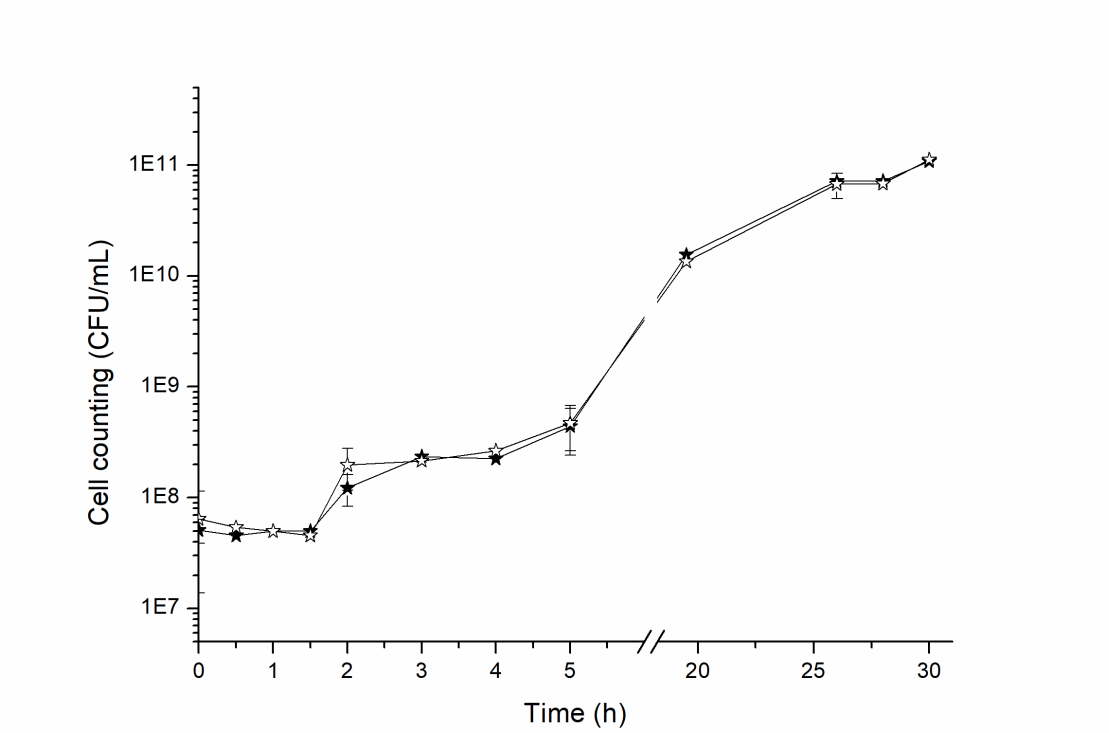


Figure 2. Batch cultures of *E. coli* BL21(DE3)pLysS (pBAD/3C/bTGase) in TB media. At 2 h, the culture temperature was reduced to 20 °C, the pro-bTGase gene was induced by the addition of 0.4 mM IPTG and cultivations proceeded for 20 h; gene 3C protease was induced by the addition of 0.2 % L-arabinose, followed by 6 h of cultivation. 2A) (■) biomass DCW (g/L); (△) glucose (g/L); (▽) acetate (g/L). The vertical lines mark the time of induction of pro-bTGase and 3C protease. 2B) Cell plasmid stability test: (★) Cell count in LB medium containing 34 μg/mL Chloramphenicol; (☆) Cell count in LB medium containing 100 μg/mL Carbenicillin and 34 μg/mL Chloramphenicol. All experimental runs were performed in duplicates.

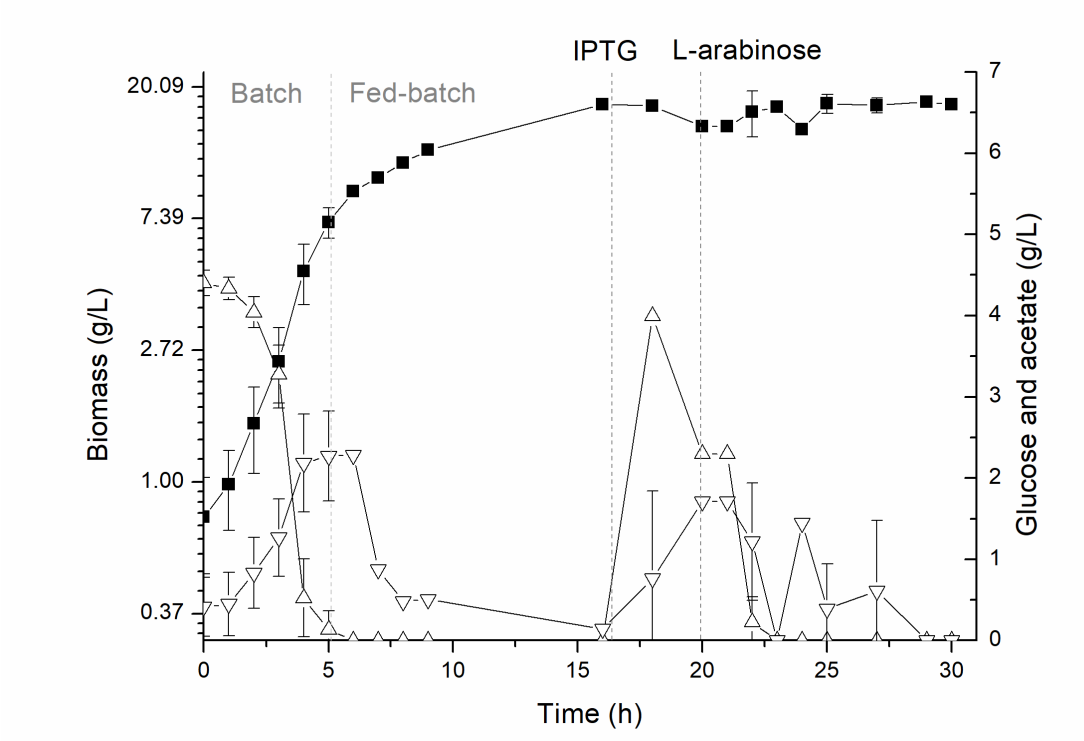
3.3 DO-stat fed-batch cultivations

In order to improve the production of the recombinant TGase and achieve high cell densities, it is necessary to adequately control the feeding of glucose and the transfer of oxygen to the culture medium. This control of cellular metabolism can be obtained by adding nutrients in fed-batch mode and appears to be the technology of choice in the bioindustry [27]. Thus, we decided to test a DO-stat fed-batch controlled strategy using an indirect feedback method.

The TB medium was used in the culture medium reservoir and feeding was controlled by a DO-stat feeding strategy. Preliminary bioreactor experiments of cell growth were run to define the best moment to start the feeding, taking into considerations of the conditions used in this research. The DO was kept at 30 % saturation by adjusting the agitation rate, starting from 300 rpm. The results showed that in about 5 h of cultivation, glucose was depleted, indicating the need to start the

feeding. With these data, we set the DO-stat fed-batch cultivations, whose results are depicted in Figure 3A and 3B, and in Table 1, in comparison with the batch experiments. As previous studies have shown that the induction of plasmids with gene inductions control by IPTG can cause a toxic effect on cells, decreasing their plasmid stability and cell growth [38], leading to reduced production of interest proteins. Transglutaminase, due to its own ability to cross-link proteins, will be toxic to host cells [39], unless its expression is tightly controlled. Therefore, induction of the pro-transglutaminase was done after 16 h of cell growth, at the end of the exponential growth phase and, after 4 h after TGase induction, the 3C protease gene was induced with L-arabinose injection and culture proceeded for another 10 h. In Figure 3 A, it can be seen that the growth of recombinant *E. coli* was prolonged without accumulation of the carbon source, with control of acetate formation. An increase in the concentration of glucose can also be seen in the same figure in approximately 16 h of culture. This increase may be related to the induction of IPTG, because at that moment there is a change in the metabolism of cell growth by protein induction.

A



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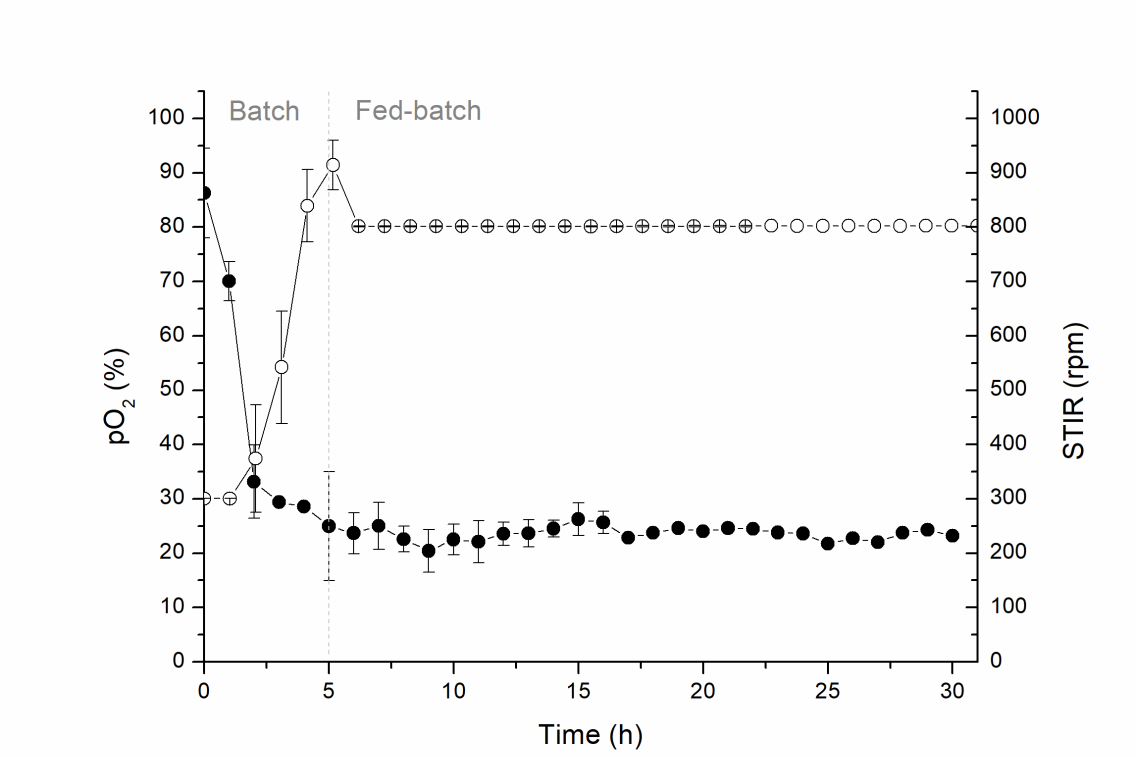
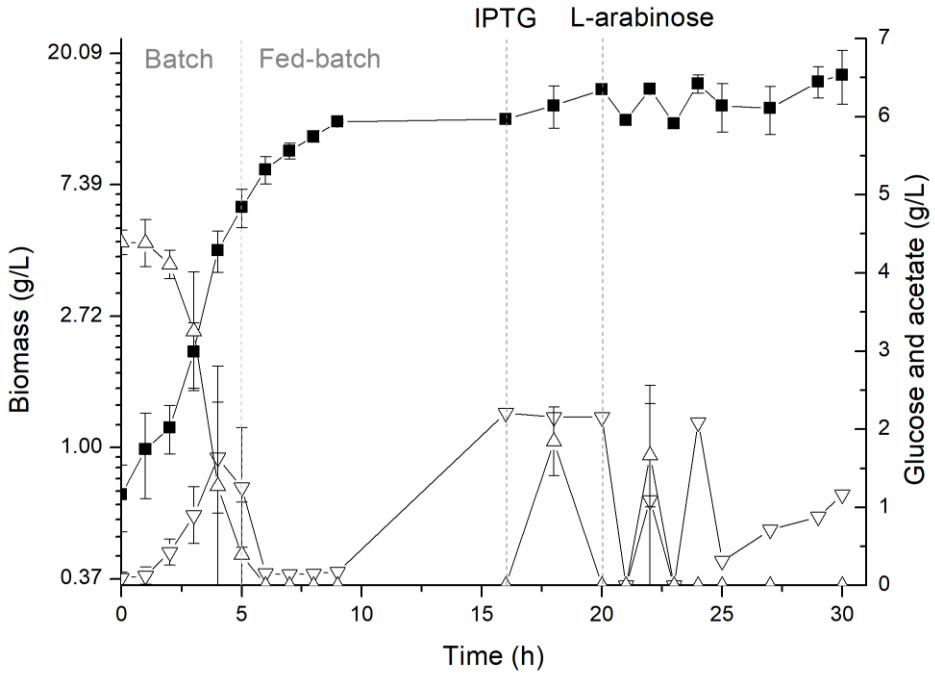


Figure 3. Fed-batch culture by *E. coli* BL21(DE3)pLysS (pBAD/3C/bTGase) with DO-stat feeding using Terrific broth (TB). In approximately 16 h, the culture temperature was reduced to 20 °C, the pro-bTGase gene was induced by the addition of 0.4 mM IPTG and cultivations proceeded for 4 h; gene 3C protease was induced by the addition of 0.2 % L-arabinose, followed by 10 h of cultivation. 3A) (■) biomass DCW (g/L); (△) glucose (g/L); (▽) acetate (g/L). 3B) (○) STIR (rpm); (●) pO_2 (%). All experimental runs were performed in duplicates. The vertical grey lines mark the batch and fed-batch strategy and the vertical black lines mark the time of induction of pro-bTGase and 3C protease.

The DO-stat fed-batch cultures produced 17.56 g/L of DCW in 30 h much higher than in batch experiments using the same culture medium. The specific enzymatic activity reached 6.43 U/mg protein, approximately 2 times greater than in batch culture.

To further improve the production of enzymes, the modified M9 medium was used, because in addition to be a richer medium in nutrients, a higher level of recombinant proteins was produced in the presence of $MgSO_4$ [40]. Conditions of feeding and controls were the same as for the experiments using TB. In Figure 4A and 4B, and Table 1 are shown the kinetics of the DO-stat fed-batch using this medium.

A



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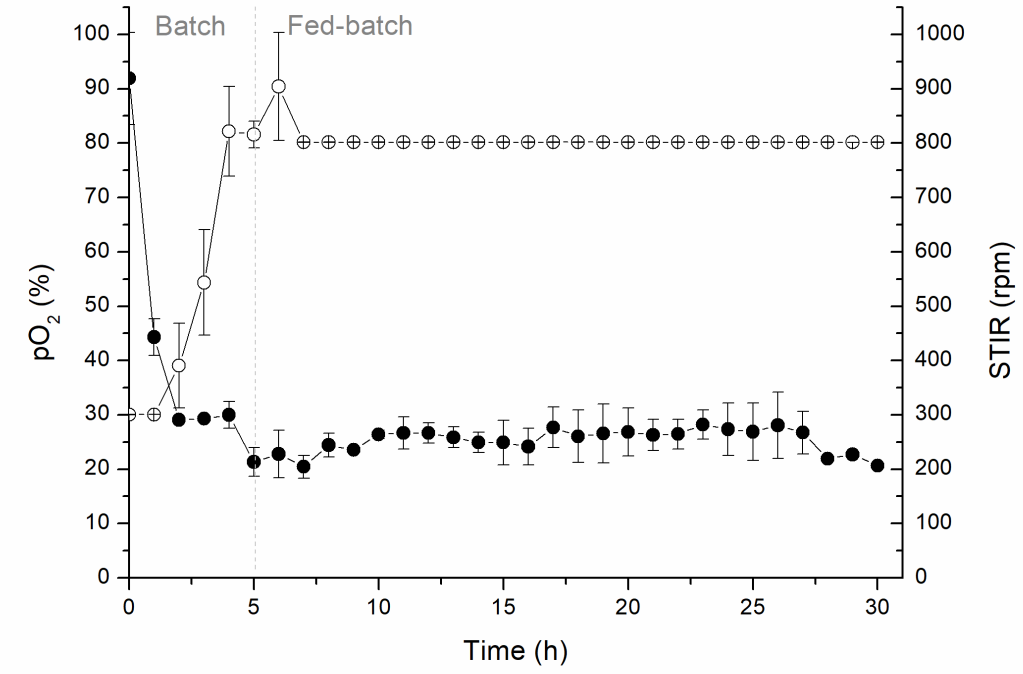

Figure 4. Fed-batch culture by *E. coli* BL21(DE3)pLysS (pBAD/3C/bTGase) with DO-stat feeding using M9 medium. In approximately 16 h, the culture temperature was reduced to 20 °C, the pro-bTGase gene was induced by the addition of 0.4 mM IPTG and cultivations proceeded for 4 h; gene 3C protease was induced by the addition of 0.2 % L-arabinose, followed by 10 h of cultivation. 4A) (■) biomass DCW (g/L); (△) glucose (g/L); (▽) acetate (g/L). 4B) (○) STIR (rpm); (●) pO_2 (%). All experimental runs

were performed in duplicates. The vertical grey lines mark the batch and fed-batch strategy and the vertical black lines mark the time of induction of pro-bTGase and 3C protease.

Results showed a biomass formation of 17.07 g/L in 30 h, the same as for TB cultivations. However, the specific TGase activity increased to 9.14 U/mg protein, 1.43 times higher than for medium TB, this being the highest enzyme title achieved in our experiments. The SDS-PAGE showing the expression of recombinant bTGase and 3C protease in *E. coli* BL21(DE3)pLysS with plasmid pBAD/3C/bTGase with fed-batch culture using feedback-controlled DO-stat in modified M9 medium can be seen in figure 5.

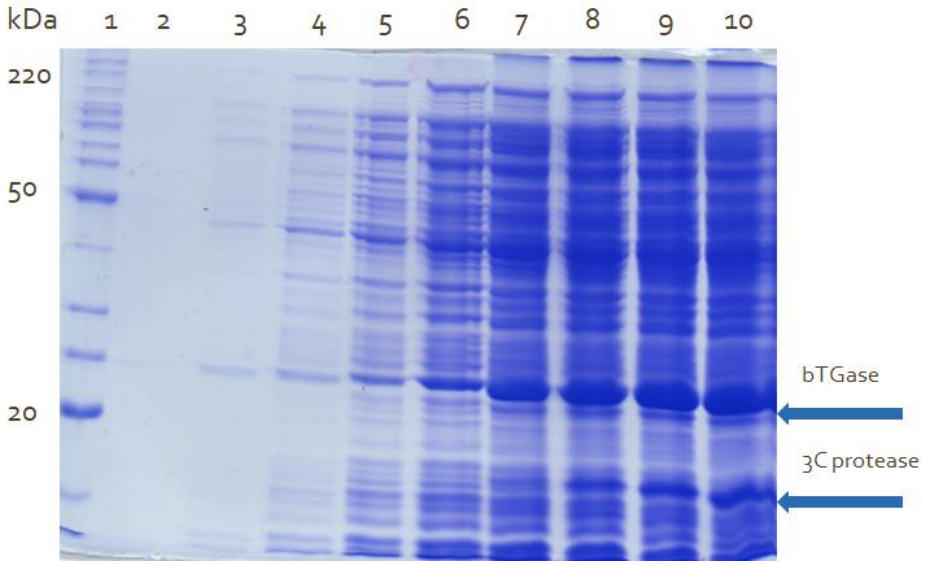

Figure 5. SDS-PAGE analysis of transglutaminase and 3C protease expression in fed-batch controlled as DO-stat cultures. The recombinant *E. coli* BL21(DE3)pLysS (pBAD/3C/bTGase) cells were cultured in modified M9 medium for 30 h. Feeding started 5 h after the start of batch cultivation and pro-bTGase expression was induced after 16 h of culture by adding 0.4 mM IPTG to the cultures and 3C protease expression was induced after 20 h of culture by adding 0.2 % L-arabinose. Lane 1: molecular weight marker, lane 2: pre-inoculum addition, lane 3: 3 h cultivation, lane 4: 5 h cultivation, lane 5: sample collected immediately after induction of IPTG (16 h of culture); lanes 6: 18 h cultivation, lane 7: sample collected immediately after induction of L-arabinose (20 h of cultivation), lane 8: 24 h cultivation, lane 9: 27 h cultivation and line 10: 30 h cultivation.

Since 1989, when Ando *et al.*, first reported the production of microbial transglutaminase by *Streptoverticillium mobaraense*, bioengineering has been striving to improve the production of this enzyme [41]. Efforts to improve the yields of microbial transglutaminase have been made through metabolic optimization, substrate

optimization and environmental control strategies (temperature, pH, agitation and dissolved oxygen) in cultures of different microorganisms, including *S. mobaraense*, *S. cinnamoneum*, *S. ladakanum*, *Streptomyces nigrescens*, *S. hygroscopicus*, *S. platensis*, *Bacillus circulans* and *B. subtilis* [12]. However, after several studies, the activity of transglutaminase in the fermentation broth increased from 2.0 to 6.0 U/mL, the *Streptomyces* spp. showing higher yields and, therefore, being currently used industrially as a producer of microbial transglutaminase [11].

Presently, the focus for the optimization of transglutaminase production has been the genetic engineering, using the transglutaminase gene from *Streptomyces* or *Bacillus* for exogenous expression of the enzyme [12]. In comparison with concentrations of *S. mobaraensis* native transglutaminase (22.6 $U/mg_{protein}$), 23 $U/mg_{protein}$ was observed for recombinant *S. mobaraensis* transglutaminase expressed in *Corynebacterium ammoniageneses*; and 26 $U/mg_{protein}$ for recombinant *S. mobaraensis* transglutaminase expressed in *Corynebacterium glutamicum* [41-44]. Some studies described improved strategies for expressing mTGase, comparing constitutive versus thermo-inducible expression systems, instead of expressing the protein by fusion or wild type protein, for production in *E. coli* of a recombinant transglutaminase from *Streptomyces mobaraensis*. The results showed enzymatic activity of the purified mTGase of 24 U/mg in the thermo-inductive expression system, compared to 15 U/mg in the constitutive system [45].

Several authors have reported the production of recombinant enzymes expressed in *E. coli* with bioreactors using the fed-batch culture approach under DO-stat. Comparison of production of recombinant β-galactosidase in bioreactors by fed-batch culture using feedback-controlled DO-stat and ascendant linear pump feeding, showed an increase of approximately 2.5 times in the enzymatic activity using this strategy [46]. The DO-stat strategy proved to be effective in achieving high cellular density and suppressing the accumulation of ethanol in the culture medium, improving the production of interferon-α (pIFN-α) by *Pichia pastoris* [47]. Higher concentrations and productivities of poly-γ-glutamic acid (γ-PGA) by *B. subtilis* were achieved after application of the strategy of DO-stat feeding compared with a glucose-feedback feeding strategy [48].

4 CONCLUSIONS

This study presents for the first time the production of recombinant *Bacillus amyloliquefaciens* transglutaminase expressed in *Escherichia coli* under the control of a bicistronic plasmid pBAD/3C/bTGase using batch and fed-batch bioreactor cultivations under the control of DO-stat strategies for the production of the recombinant enzyme. Using an improved mineral medium (M9) in the DO-stat fed-batch controlled cultivations it was possible to obtain mTGase with high enzymatic activity, showing to be a promising strategy for the industrial production of this important enzyme.

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TABLE

Table 1. Biomass, as DCW, and the maximum specific activity of TGase in batch and DO-stat fed-batch bioreactor cultures under the different conditions and media tested in this work. Results are the mean of duplicates.

Cultivation strategy	Culture medium	Dry Cell Weight (g/L)	Specific activity ($U/mg_{protein}$)
Batch	TB	6.13 ± 0.10	3.12 ± 0.08
Fed-batch controlled as DO-stat	TB	17.56 ± 0.96	6.43 ± 0.66
Fed-batch controlled as DO-stat	M9	17.07 ± 3.44	9.14 ± 0.42

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DISCUSSÃO GERAL

Este trabalho teve como objetivo o desenvolvimento e aprimoramento de técnicas de produção de uma nova transglutaminase que possa ser utilizada na indústria de alimentos para reticulação de proteínas. O desenvolvimento do projeto visou identificar, clonar, expressar e escalonar a produção em biorreator de uma nova transglutaminase de *Bacillus amyloliquefaciens*.

Em um primeiro momento, um levantamento bibliográfico a fim de proporcionar embasamento teórico do assunto foi realizado. Desta extensa revisão foram publicados dois artigos. A primeira revisão apresenta uma visão geral da literatura sobre as origens, tipos, reações e caracterizações gerais das transglutaminases, bem como os estudos feitos até o momento sobre transglutaminases microbianas recombinantes. A segunda revisão mostra a versatilidade de aplicação do mTGase em três grandes áreas: alimentícia, farmacológica e biotecnológica.

O trabalho experimental iniciou por um *screening* de *Bacillus* produtores de esporos da coleção do banco de microrganismos do BiotecLab, possíveis produtores de transglutaminase. Dos microrganismos analisados, *Bacillus amyloliquefaciens* mostrou ser um potencial produtor da enzima (Apêndice A). O sequenciamento por 16 S confirmou a linhagem bacteriana (Anexo 1). A fim de diminuir o tempo e aumentar a produção da enzima, este trabalho foi direcionado para a produção de transglutaminase recombinante de *Bacillus amyloliquefaciens* (bTGase) em *E. coli.*

Uma análise *in silico* da possível transglutaminase codificada pelo genoma de *Bacillus amyloliquefaciens* foi feita. Como esta análise mostrou a presença de polipeptídeos contendo domínios de transglutaminase, a sequência peptídica obtida no banco de dados do NCBI foi submetida a análises de domínios conservados e de predição de similaridade. Os resultados indicaram que a transglutaminase codificada pelo genoma de *Bacillus amyloliquefaciens* possuía similaridade de 73 % com transglutaminase de *Bacillus subtilis* (GenBank: E13095.1), microrganismo conhecido como produtor da enzima (Apêndice B e Figura suplementar 1 do Capítulo III).

Como a expressão de transglutaminase, como uma proteína heteróloga, pode ser tóxica e afetar o crescimento normal das células, foi necessário que a sequência codificadora da transglutaminase (bTGase) de *Bacillus amyloliquefaciens* fosse clonada

com o prodomínio da transglutaminase de *Streptomyces caniferus* para produzir uma proteína de fusão inativa. A construção do plasmídeo bicistrônico partiu do plamídeo comercial pBAD/HisA (Anexo 2) e consistiu de duas inserções. Para a primeira inserção, o plasmídeo de expressão foi construído por clonagem do gene sintético da protease 3C (GenBank NP_740524.1) no vetor comercial (Apêndice C). Para a segunda inserção o plasmídeo de expressão contendo a protease 3C (obtido da primeira inserção – pBAD/3C) foi construído por subclonagem do promotor T7 e do terminador T7 oriundos do plasmídeo pET20b (+) (Anexo 3) com o gene que codifica para bTGase de *Bacillus amyloliquefacines* DSM7 e o prodomínio de *Streptomyces caniferus* (GenBank AM746294.1) (Apêndice E). Para permitir a clivagem proteolítica do prodomínio da transglutaminase e tornar a enzima ativa, um sítio de reconhecimento da protease 3C foi desenhado entre o prodomínio de *S. caniferus* e a sequência bTGase. A fim de otimizar a expressão de bTGase, um códon para o aminoácido lisina (AAA) foi colocado logo após o códon inicial ATG.

Com base no alinhamento das sequências do prodomínio das transglutaminases de *Streptomyces* (Figura 1 Capítulo III), e a para reduzir a interação entre o prodomínio e a transglutaminase e facilitar a sua dissociação alcançando a atividade enzimática completa, uma mutação no prodomínio de *Streptomyces caniferus* com a troca de Asp (GAC) por Ala (GCG) posição D22 foi efetuada.

O plasmídeo, com os dois insertos, foi transformado em *E. coli* TOP10. Colônias transformadas com a reação de ligação foram selecionadas ao acaso e tiveram seus plasmídeos extraídos para verificação da clonagem (Apêndice D). Foram desenhados primers (Apêndice G) para a confirmação das clonagens do plasmídeo pBAD/3C/bTGase, e esta confirmação foi feita pela empresa ACTGene no Centro de Biotecnologia da UFRGS.

Para o estudo da expressão da proteína recombinante, primeiramente o plasmídeo pBAD/HisA (controle negativo) e o pBAD/3C/bTGase foram transformados em *E. coli* BL21(DE3). O gene pro-bTGase foi induzido com IPTG (0,1 mM, 0,4 mM e 1,0 mM) e o gene da protease 3C foi induzido por L-arabinose (0,002 %, 0,02% e 0,2 %). As alíquotas foram coletadas em pontos de 0 a 48 h. Foram feitas tentativas de co-indução e indução sequencial (usando primeiro IPTG e após L-arabinose), com temperaturas de indução de 20 e 37 °C. Os melhores resultados foram obtidos nas

seguintes condições: indução com IPTG 0,4 mM por 24 h, seguida por indução com L-arabinose a 0,2 % por 4 h, a 20 °C (Figura suplementar 2, Capítulo III). A banda referente ao tamanho esperado da proteína foi excisada do gel SDS-PAGE e analisados por espectrometria de massas, mas não houve confirmação. Optou-se, então, por transformar o plasmídeo pBAD/3C/bTGase em *E. coli* BL21 (DE3)pLysS, linhagem com maior tolerância para expressar proteínas tóxicas. Os mesmos estudos de expressão foram feitos e os lisados celulares analisados por SDS-PAGE mostraram uma banda de proteínas consistente com o tamanho esperado de bTGase (25 kDa), indicando que o gene da bTGase tinha sido expresso com sucesso. Também foi detectado, na mesma amostra, uma banda proteica de aproximadamente 20 kDa, consistente com o tamanho esperado da protease 3C (Figura 3A, Capítulo III).

Confirmamos a atividade da enzima por entrecruzamento com BSA (fig. 3B, artigo III) e seguimos para a purificação da enzima em sistema AKTA. Vários experimentos foram feitos a fim de obter uma enzima purificada, ativa, bem como torná-lo um procedimento simples para a utilização na indústria. Optou-se por um procedimento de purificação cromatográfico em uma única etapa, com uma coluna de troca catiônica (SP Sepharose Fast Flow). A banda de 25 kDa do SDS-PAGE (Figura 4, Capítulo III) obtida após a purificação, foi excisada, submetida a digestão com tripsina e analisados por LC-MS/MS com confirmação de peptídeos únicos de transglutaminase.

A atividade da enzima bTGase recombinante foi determinada por um método de medição quantitativa de fluorescência com utilização de monodansilcadaverina e dimetilcaseína. As análises foram feitas no extrato bruto (Figura 5A, Capítulo III) e na fração parcialmente purificada (Figura 5B, Capítulo III), obtendo-se uma atividade enzimática de 37 mU/mg $_{\text{proteína}}$.Com o objetivo de obter um processo para maximizar a produção de transglutaminase, foram realizados cultivos em biorreator em regime batelada e batelada alimentada por pulsos (DO-stat) com a *E. coli* BL21 (DE3)pLysS contendo o plasmídeo bicistrônico pBAD/3C/bTGase, utilizando Terrific broth e meio mineral modificado (M9). Para isso, foi utilizado o biorreator BIOSTAT® B plus (Sartorius Stedim, Goettingen, Germany) (Apêndice H) acoplado a um banho de resfriamento para induzir a proteína recombinante a 20 °C. O pO_2, pH, velocidade do agitador (STIR), consumo de base e ácido e taxa de aeração foram medidos on-line e registrados por um sistema externo de aquisição e controle de dados (Sartorius Stedim,

Alemanha). As concentrações de glicose e acetato foram determinadas por HPLC- RID. A atividade da enzimática da transglutaminase foi determinada pelo método colorimétrico do hidroxamato. Melhorias consideráveis na biomassa foram obtidos com a utilização do regime de batelada alimentada por pulsos (DO-stat). Além disso, utilizando o meio mineral melhorado (M9) sob as mesmas condições, foi possível obter uma atividade enzimática de 9,14 $U/mg_{proteína}$ um resultado promissor para a produção industrial desta enzima se comparado aos resultados de atividade específica da transglutaminase nativa de *S. mobaraensis* de 22,6 U/mg, até hoje a única transglutaminase comercial disponível.

CONCLUSÃO

Neste estudo, produzimos, pela primeira vez, a transglutaminase recombinante de *Bacillus amyloliquefaciens em E. coli*. Construímos um plasmídeo bicistrônico, que foi capaz de expressar duas proteínas: transglutaminase e protease 3C. A enzima foi purificada em uma única etapa utilizando o sistema AKTA com uma coluna de troca catiônica e identificada por espectroscopia de massas. Ensaios de atividade enzimática por entrecruzamento de BSA e por fluorescência confirmaram a atividade da enzima. Foram realizados experimentos de biorreator em escala piloto comparando o sistema em batelada com batelada alimentada pulsada (DO-stat) para investigar o crescimento celular e a produção de transglutaminase recombinante em dois meios de cultivo. A utilização de um sistema de batelada alimentada pulsada (DO-stat) em meio mínimo modificado (M9), suplementado com oligoelementos, mostrou ser um promissor método para a produção da enzima.

Este trabalho traz uma nova visão para o desenvolvimento de transglutaminase para a indústria de alimentos e biotecnológica. Desta forma, abrem-se perspectivas para trabalhos futuros, buscando a inovação e melhorias.

PERSPECTIVAS

O presente trabalho demonstrou o potencial tecnológico para a produção de transglutaminase recombinante de *Bacillus amyloliquefaciens*. Desta forma, o avanço nos estudos pode ser realizado a partir das seguintes sugestões:

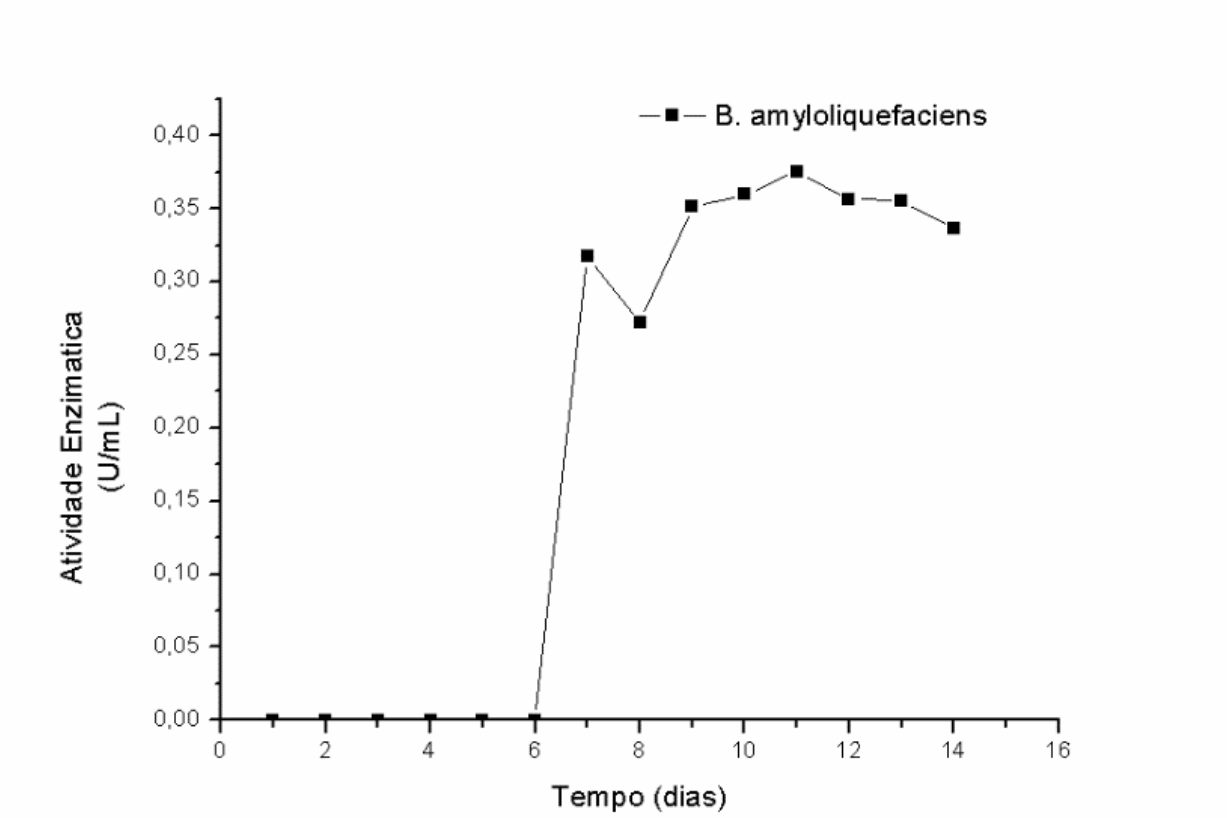
A utilização de um sistema de expressão capaz de produzir a enzima de forma extracelular. Isso evitaria a etapa de rompimento celular, diminuindo o tempo e os custos para a produção;

Novas abordagens para a produção da enzima em biorreator, bem como estudos para a otimização dos meios de cultivo, do tempo de indução (de ambas as proteínas), da concentração dos indutores e com expressão auto induzível utilizando lactose para substituir IPTG a fim de melhorar a eficiência dos processos em escala industrial.

Testes toxicológicos, visando a segurança alimentar e reconhecimento da enzima pela Anvisa são necessários, já que a Resolução ANVISA/DC Nº 53 de 07/10/2014, que traz a lista de enzimas com suas respectivas fontes de obtenção, aditivos alimentares e veículos autorizados em preparações enzimáticas para uso como coadjuvante de tecnologia na produção de alimentos em geral, até agora, somente autoriza a utilização de transglutaminase de *Streptoverticillium mobaraense*/*Streptomyces mobaraense.*

APÊNDICES

Apêndice A. Gráfico da atividade enzimática de transglutaminase de *Bacillus amyloliquefaciens.*



Apêndice B. Identificação *in silico* da sequência da transglutaminase. 1) Sequência nucleotídica de *Bacillus amyloliquefaciens* GenBank: CBI44050.1. 2) Peptídeo codificado *Bacillus amyloliquefaciens*. 3) Sequência nucleotídica de *Bacillus subtilis* GenBank: E13095.1. 4) Peptídeo codificado *Bacillus subtilis*. 5) Região do prodomínio de transglutaminase de *Streptomyces caniferus* (em amarelo) 6) 3C protease codificado por *Rhinovirus* B14

1)CBI44050.1 Gene *Bacillus amyloliquefaciens* para transglutaminase

ATTATCATATCCGGCCAAGTGCTTCGGCCCCAGGACATCGCGAACTGGCAGACGGAAGAAAGCCTTGTTC
CGTATTTAAATGAACTGATCAATTCTCCCGTACAATTCGATTACGGGTCAATTACTGAACTGATGTTTGA
AGCACGGCTGCGGCGCCATATCGTGGAGGCTGCAAGAGAACTGCACGGATCAGGCGCAAAATTTGCAACT
TTCGCCAAAACATACGGAAATACGGCTTATTGGAGAGTGACGCCGGAAGGAGCTCTGGAGCTGAAGTACA
GAATCCCCGCTTCCAAAGCGATACGGGATATTATTGAAAACGGGGCGTTTTATGCATTTGAATGCGCTAC
CGCTATTGTTGTGATTTATTATCTGGCCGTCTTAAAGACGATCGGTGAAGAACGGTTTGACCGCCGTTTC
CGGGATATTACTTTGTATGACTGGCACTATGAGCACCTGCCGATCTACACGGAAACCGGCAGACACTTTT
TACGGGGAGATTGTTTATATTTTAAAAACCCTGAGTTTAATCCCGCAAAGGCCCAGTGGCGGGGAGAAAA
TGTCATTGTGATGGGGAATGATCAATATTTTGCCCACGGTCTCGGGATTCTGACCGCTGAACAAATCATC
CAGAGATTGAATTCTTTAAGAAGAAAAAATGCCGTGCAGTCAGCTTATCTGCTTTCTCAGGCGACAAGGC
TTGATGCGCCCGCGCTTTATCAGATCATGCATTAA

2)CBI44050.1 Proteína-glutamina gama-glutamiltransferase (transglutaminase) [*Bacillus amyloliquefaciens* DSM 7]

MIIISGQVLRPQDIANWQTEESLVPYLNELINSPVQFDYGSITELMFEARLRRHIVEAARELHGSGAKFA
TFAKTYGNTAYWRVTPEGALELKYRIPASKAIRDIIENGAFYAFECATAIVVIYYLAVLKTIGEERFDRR
FRDITLYDWHYEHLPIYTETGRHFLRGDCLYFKNPEFNPAKAQWRGENVIVMGNDQYFAHGLGILTAEQI
IQRLNSLRRKNAVQSAYLLSQATRLDAPALYQIMH

3)E13095.1 Gene *Bacillus subtilis* para transglutaminase, cds completos

CTGCTTAAAAAGTTTTAAAATAAAAAATGGAAGAAGTTCTTTTTGGCAGTCTTCTGTCTTTTTAGCTTTC
ATTGCCCAAGCTCTTTGCATATCTTATATAAACAAGGGGGGCTAAACATGATTATTGTATCAGGACAATT
GCTCCGTCCCCAGGATATTGAAAATTGGCAGATTGATCAAAATCTGAATCCGCTGTTAAAAGAGATGATT
GAGACGCCTGTTCAGTTTGATTATCATTCAATTGCTGAACTGATGTTTGAGCTTAAACTGCGGATGAATA
TTGTAGCAGCGGCAAAGACGCTGCACAAAAGCGGGGCGAAGTTTGCCACTTTTTTAAAAACATACGGGAA
TACAACGTATTGGAGGGTTTCACCGGAGGGCGCCTTGGAGCTGAAATACAGAATGCCGCCTTCAAAAGCG
ATTCGGGACATTGCAGAGAACGGCCCGTTTTATGCGTTTGAATGCGCAACCGCAATCGTTATCATTTATT
ACTTGGCCTTAATCGATACAATCGGTGAAGATAAATTCAATGCCAGCTTTGACAGAATTATTTTATATGA
CTGGCATTATGAGAAATTGCCGATCTATACGGAAACAGGACACCACTTTTTCCTTGGAGATTGTTTGTAT
TTTAAGAATCCTGAATTTGATCCGCAAAAGGCGCAATGGAGAGGCGAAAATGTGATTTTACTGGGGGAAG
ATAAATATTTTGCCCATGGTCTTGGAATCTTAAACGGAAAGCAAATTATAGATAAGCTGAATTCTTTGGA
AAAAAGGAGCCTTACAGTCAGCCTACCTTCTGTCTCAGGCGACCAGACTGGATGTTCCGTCTCTTTTCCG
CATCGTCCGCTAAAAAGCCCCATCGCCTATTTTCGGGACGATGGGGTTTCAAATGCCTTTCGTTTTCGAT
AGAAGGGGGCTGTGCCGAAATATTGGTTCGCAGCCCACTCCATTTTTTCAAGGTCATTTCTTGTCACGAT
TGGATCCTGGCTGCTCCATTTGATAAAGCGGACAAAATAGTAGCCTTTGATAGGAACCAT

4) E13095.1 Proteína-glutamina gama-glutamiltransferase (transglutaminase) [*Bacillus subtilis*]

MIIVSGQLLRPQDIENWQIDQNLNPLLKEMIETPVQFDYHSIAELMFELKLRMNIVAAAKTLHKSGAKFA
TFLKTYGNTTYWRVSPEGALELKYRMPPSKAIRDIAENGPFYAFECATAIVIIYYLALIDTIGEDKFNAS
FDRIILYDWHYEKLPIYTETGHHFFLGDCLYFKNPEFDPQKAQWRGENVILLGEDKYFAHGLGILNGKQI
IDKLNSFRKKGALQSAYLLSQATRLDVPSLFRIVR

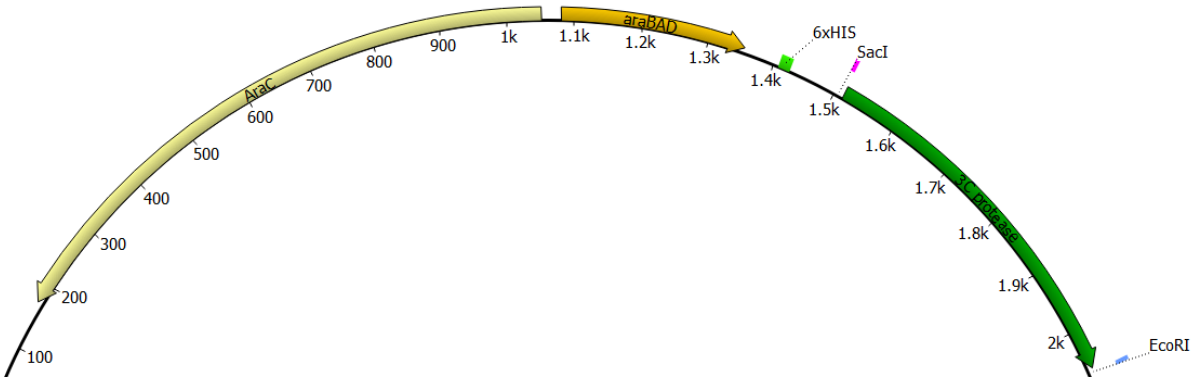
5) AM746294.1 *Streptomyces caniferus* TGase gene for transglutaminase, strain TUB B-402 – em amarelo o prodomínio

MFKRRSLLAIATMGAVICASGVMPSVGHAASGGDEEWEGSYAATHGLTAEDVKNINALNKRALTAGQPGN
FPAELPPSATALFRAPDAADDRVTPPAEPLSRMPDAYRANGGRATTVVNNYIRKWQQVYSQRGGSQQQMT
EEQREQLSYGCVGVTWVNTGPYPTNKLAFAFFDENKYKNDLENSRPRPNETQAEFEGRIAKDSFDEAKGF
KRAREVASVMNKALENAHDEGTYIDHLKTELTNKNDALLYEDSRSNFYSALRNTPSFKERDGGNYDPSKM
KAVVYSKHFWSGQDQRDSSDKRKYGDPDAFRPDQGTGLVDMSRDRNIPRSPAKPGESWVNFDYGWFGAQA
EADADKTIWTHANHYHAPNGMGPMNVYESKFRNWSAGYADFDRGTYVITFIPKSWNTAPAEVKQGWP

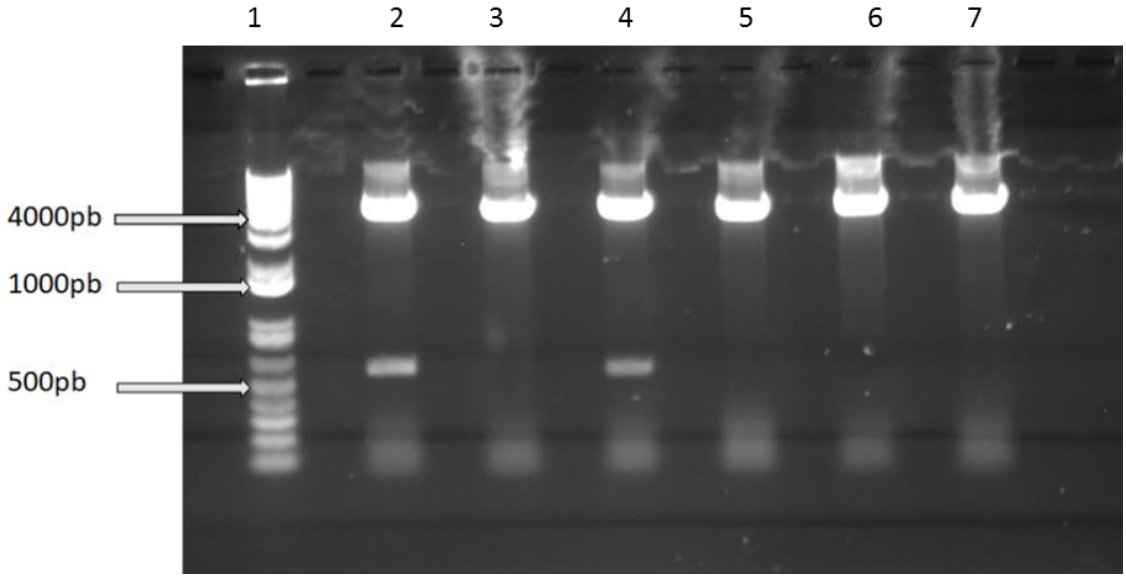
6) NP_740524.1 3C (protease) [*Rhinovirus* B14]

GPNTEFALSLLRKNIMTITTSKGEFTGLGIHDRVCVIPTHAQPGDDVLVNGQKIRVKDKYKLVDPENINL
ELTVLTLDRNEKFRDIRGFISEDLEGVDATLVVHSNNFTNTILEVGPVTMAGLINLSSTPTNRMIRYDYA
TKTGQCGGVLCATGKIFGIHVGGNGRQGFSAQLKKQYFVEK**Q**

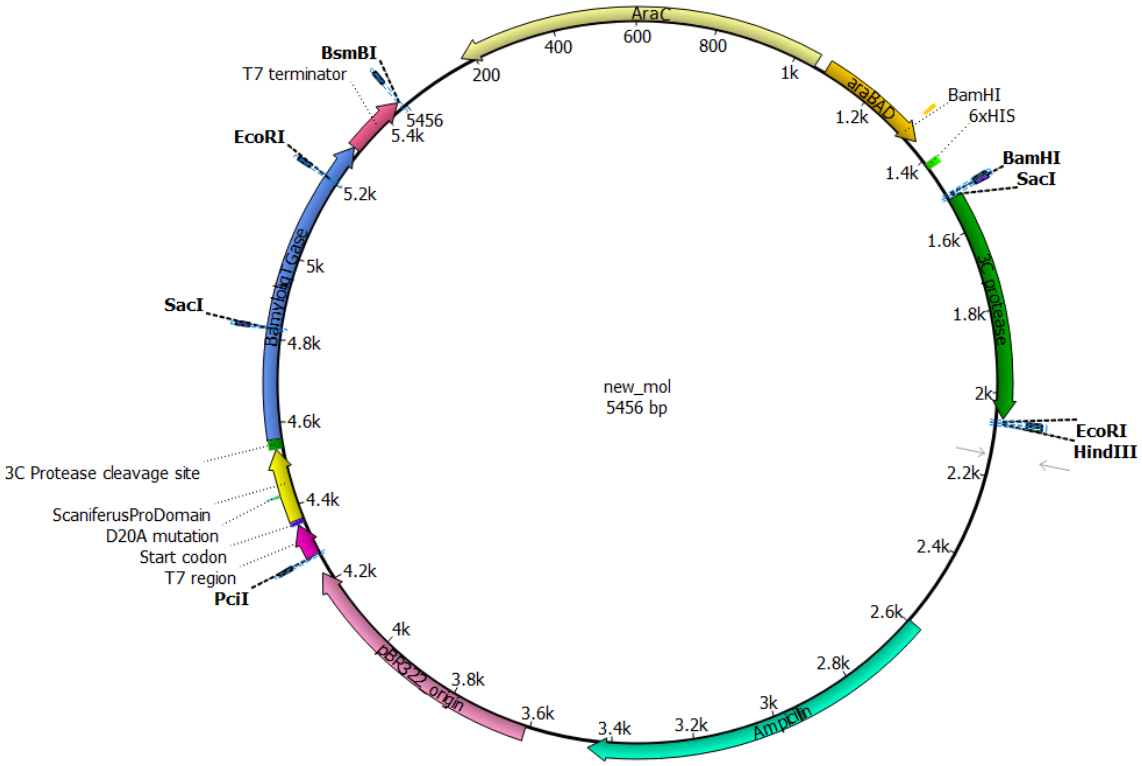
Apêndice C. Visão parcial do plasmídeo pBAD/HisA com o gene da protease 3C sob o controle do promotor araBAD e com as enzimas de restrição *SacI* e *EcoRI* nas extremidades 5' e 3', respectivamente.



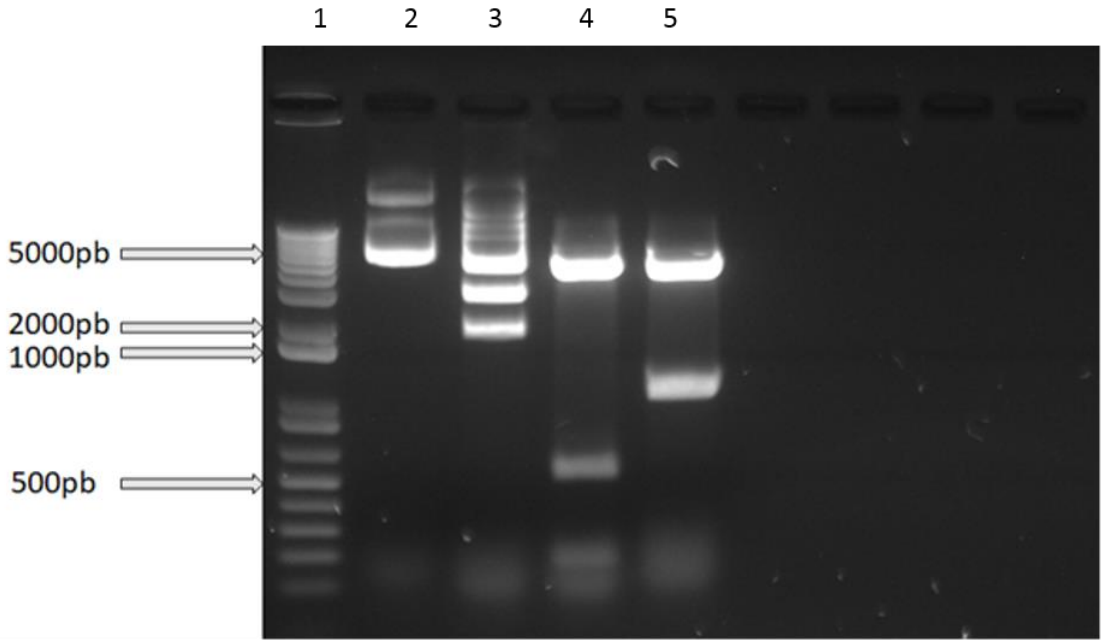
Apêndice D. Teste de clonagem da protease 3C. Análise feita em gel de agarose 2 %. Canaletas 1 mostra o marcador de peso molecular, 2-7 mostram os possíveis construtos pBAD/3C clivados com as enzimas *SacI* e *EcoRI*, sendo que apenas aqueles das canaletas 1 e 3 apresentaram a banda do inserto em 564 pb, indicando que haviam sido ligados com a protease 3C.



Apêndice E. Plasmídeo pBAD/3C/bTGase com o gene da protease 3C com as enzimas de restrição *SacI* e *EcoRI*, o gene da transglutaminase de *Bacillus amyloliquefaciens* e o prodomínio da transglutaminase de *Streptomyces caniferus* com as enzimas de restrição *PciI* e *BsmBI*.



Apêndice F. Teste de clonagem da protease 3C e bTGase. Análise feita em gel de agarose 12 %. Canaleta 1 mostra o marcador de peso molecular, a canaleta 2 mostra o plasmídeo pBAD/3C/bTGase com 5456 pb. Canaleta 3 o plasmídeo parcialmente digerido com a enzima de restrição *SacI* (tamanho esperado das bandas: 3328 pb e 2128 pb). Uma banda em torno de 5400 pb é visível pois não houve digestão completa do plasmídeo. Na canaleta 4 o plasmídeo foi digerido com *BamHI e HindIII* (tamanho das bandas esperadas: 4886 pb e 570 pb) para confirmação da protease 3C. Na canaleta 5 o plasmídeo digerido com *PciI* e *BsmBI* (tamanho das bandas esperado: 4252 pb e 1204 pb) para confirmação do segundo inserto clonado.



Apêndice G. Primers para sequenciamento do plasmídeo pBAD/3C/bTGase. Para isso foram projetados um par de primers (R e F) externos para amplificar a sequência da protease 3C. Dois pares de primers (R e F) internos e externos foram projetados para amplificar a sequência da T7/bTGase

Primer	Sequência
Protease 3C F	5’CTGTACGACGATGACGATAAGG-3’
Protease 3C R	5’AATCTTCTCTCATCCGCCAAA-3’
mTGase F externo	5’TTTACGGTTCCTGGCCTTT-3’
mTGase F interno	5’TTCCAAAGCGATACGGGATATT-3’
mTGase R interno	5’GCTCATAGTGCCAGTCATACA-3’
mTGase R externo	5’TTCGGTGATGACGGTGAAA-3’

Apêndice H. Biorreator BIOSTAT® B plus (Sartorius Stedim, Goettingen, Germany), acoplado um banho de resfriamento (Frigomix® B - B. Braun Biotech International, Alemanha).

ANEXOS

Anexo 1. Relatório de identificação de microrganismo por sequenciamento de rDNA

DSMA Desenvolvimento Sustentável e Monitoramento Ambiental

REM 2016.018.037

1 OBJETIVO

Realizar a identificação taxonômica dos micro-organismos enviados pelo solicitante com o sequenciamento da região ribossomal do DNA.

2 AMOSTRAS

As amostras foram enviadas pelo solicitante em placa de Petri contendo o micro-organismo isolado em três réplicas.

Tabela 1. Descrição das amostras recebidas:

Nº DSMA	Amostra	Tipo	Data
2016.018.037.005	*B. circulans*	Bactéria isolada	03/06/2016

3 METODOLOGIA

Procedimento	Método	Observações
Extração de DNA	Kit de Extração Comercial	GenomicWizard - Promega
Amplificação de DNA	*Polymerase Chain Reaction*	27F: 5' – AGAGTTTGATCMTGGCTCAG- 3' 1387R: 5' - CGGTGTGTACAAGGCCCGGGAACG- 3'
Sequenciamento	Sanger	Applied Biosystems, modelo 3730XL
Alinhamento	Construção do Consenso	CodonCodeAligner V4.2.7 (LI-COR®)
Identificação	Comparação no Banco de Dados	NCBI, RDP.
Árvore Fenética	Cladograma	Software MEGA 6

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4 RESULTADOS

Tabela 2. Identificação de microrganismo por sequenciamento em comparação com o GenBank NCBI:

Amostra	Identificação Classificação Taxonômica (NCBI)
2016.018.037.005	***Bacillus amyloliquefaciens*** Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae

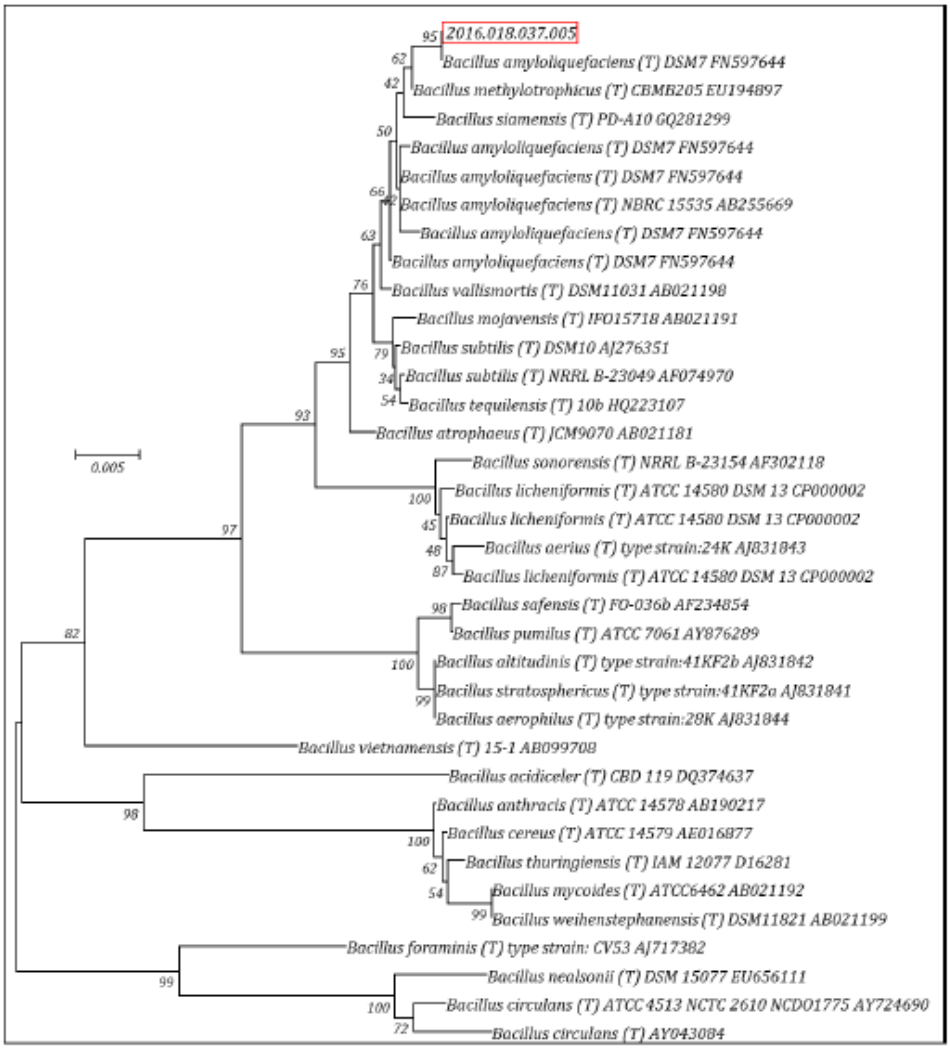

Figura 1. Árvore Fenética apresentando distância filogenética entre bactérias, a partir do 16S rDNA. Construída com o auxílio do software MEGA 6.0 pelo método de Neighbor-Joining com parâmetro Jukes-Cantor e Bootstrap de 1000 réplicas.

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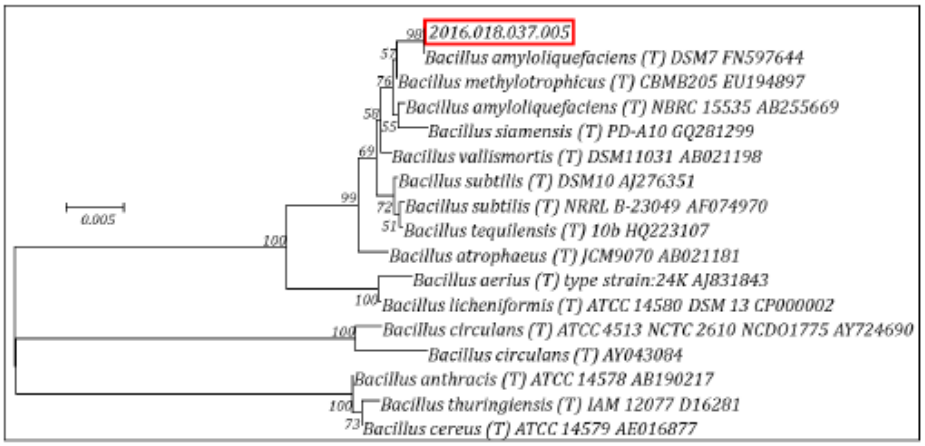

Figura 2. Árvore Fenética apresentando distância filogenética entre bactérias, a partir do 16S rDNA. Construída com o auxílio do software MEGA 6.0 pelo método de Neighbor-Joining com parâmetro Jukes-Cantor e Bootstrap de 1000 réplicas.

Obs: Os resultados se aplicam somente às amostras recebidas.

Mogi das Cruzes, 06 de julho de 2015.

MSc. Marília Bixilia Sanchez
CRBio 1ª Região: 074487/01-D

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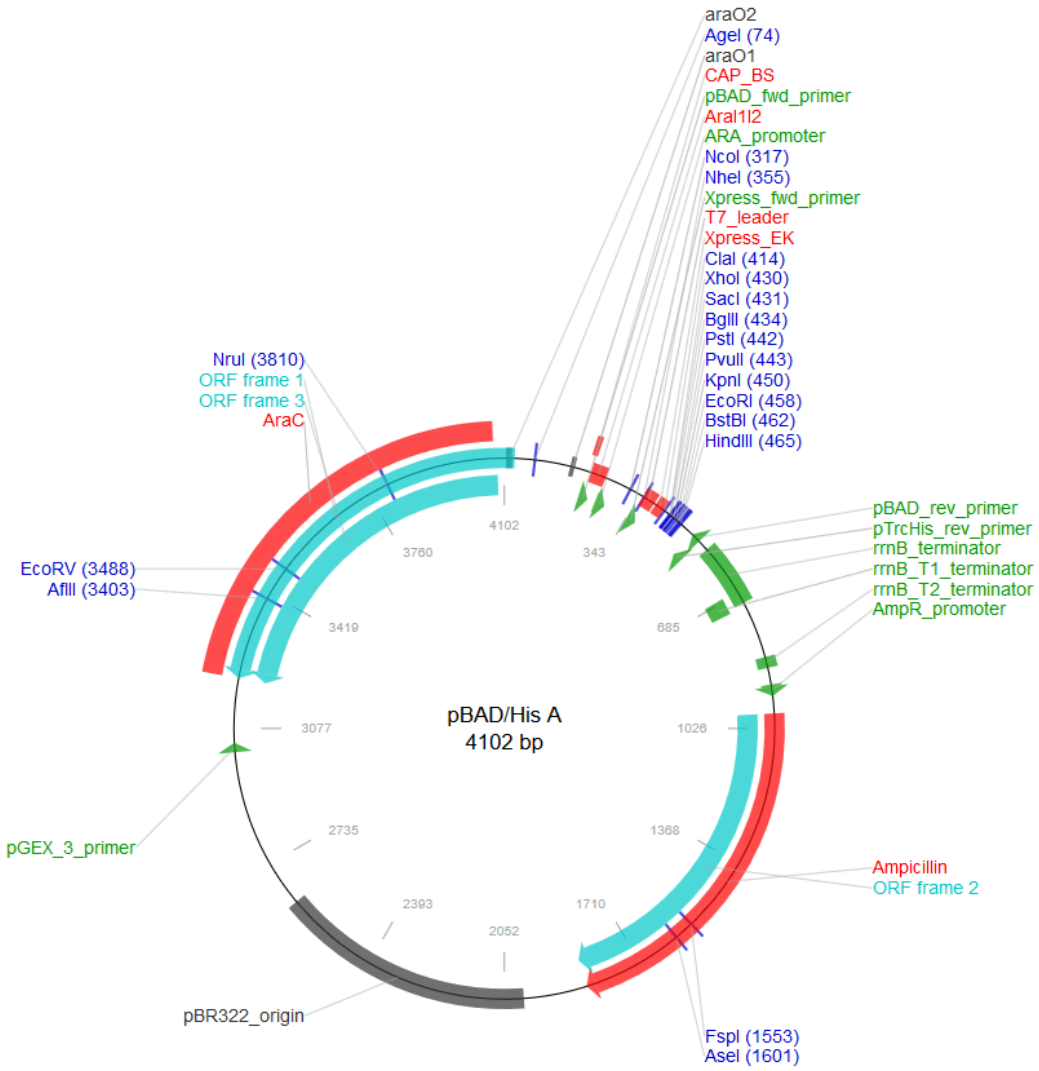
Anexo 2. Mapa de plasmídeo pBAD/HisA - Invitrogen (www.addgene.org/)



Anexo 3. Mapa do plasmídeo pET20b (+) - Novagen (EMD Millipore) (www.addgene.org/)