UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA DO ESTADO DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

PRODUÇÃO EM CULTIVO SUBMERSO E NO ESTADO SÓLIDO E CARACTERIZAÇÃO DA TRANSGLUTAMINASE (EC 2.3.2.13) DO ISOLADO AMAZÔNICO *Bacillus circulans*

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como um dos requisitos à obtenção do grau de Doutor em Ciências.

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Porto Alegre (RS), Brasil

Agosto de 2008.

Este trabalho foi desenvolvido no Instituto de Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul e contou com recursos de financiamento e bolsas da CAPES, CNPq e FAPERGS.

AGRADECIMENTOS

Ao professor Marco Antônio Záchia Ayub, que me acompanha deste os tempos da iniciação científica e me ensinou a estudar pelo prazer de aprender. Muito obrigada por ter acreditado em mim e sempre me incentivado!

À professora Célia Carlini e ao professor Jeverson Frazzon, minha comissão de acompanhamento do PPGBCM, pelo exemplo de profissionalismo. Obrigada pelo estímulo à pesquisa!

Ao professor Plinho Francisco Hertz e a professora Erna Vogt de Jong por estarem sempre prontos para ajudar e ensinar, a qualquer hora, em qualquer momento. Obrigada por tudo!

À Débora Faccin, Giandra Volpato, Janaina Venzke, Júlio Heck, Omar Mertins, Rafael Rodrigues, Renato Rosa, ao professor Argimiro Resende Secchi e as professoras Eliane Dallegrave, Nádya Pesce da Silveira e Simone Hickmann Flôres pelo apoio nas diferentes etapas desse trabalho, seja na parte experimental ou na análise dos resultados, e por possibilitarem discussões científicas valiosas. Aprendi muito com todos vocês!

Aos meus bolsistas Carine, Priscila, Hemilliano, Mateus, Leonardo e Carolina pelo empenho e dedicação durante a execução dos experimentos. Muito obrigada pela valiosa cooperação na concretização desse trabalho!

À professora Eniz Conceição Oliveira e ao professor Daniel Lehn, colegas de trabalho, mas principalmente grandes amigos, que foram meus braços direito e esquerdo, na Univates, durante esses anos que compartilhei atividades de docência e de doutorado.

A todos os colegas de laboratório com os quais convivi durante esses anos, pelo apoio durante o desenvolvimento desse trabalho. Obrigada pelos momentos que vivemos juntos!

À Silvia e ao Luciano, funcionários do PPGBCM, por sua eficiência, dedicação e atenção sempre que precisei.

Ao meu marido Léo, com quem compartilhei todos os momentos durante esses anos de doutorado. Seu apoio incondicional e seu companherismo incansável foram fundamentais para a realização desse trabalho. Obrigada por ser essa pessoa maravilhosa, por todo o carinho, compreensão e por estar ao meu lado nos momentos alegres e também nos difíceis! Essa vitória é nossa!

RESUMO

PRODUÇÃO EM CULTIVO SUBMERSO E NO ESTADO SÓLIDO E CARACTERIZAÇÃO DA TRANSGLUTAMINASE (EC 2.3.2.13) DO ISOLADO AMAZÔNICO *Bacillus circulans* [1]

A Transglutaminase (TGase; proteína-glutamina γ-glutamil-transferase; EC 2.3.2.13) é uma enzima que catalisa reações de acil-transferência introduzindo ligações cruzadas entre cadeias protéicas. Em função de suas características, as TGases microbianas têm ampla e crescente aplicação na indústria alimentícia e em outras áreas. Portanto, o objetivo desse trabalho foi aumentar o conhecimento existente sobre a produção e as aplicações dessas enzimas. A composição do meio de cultura para a produção da enzima em cultivo submerso (CSm) pelo *Bacillus circulans* BL32, um isolado Amazônico, foi otimizada através de uma estratégia em três etapas. A otimização do meio resultou numa atividade de TGase que é 60 % maior que a máxima obtida utilizando um meio de cultura previamente citado na literatura para a produção dessa enzima, além da redução dos custos dos constituintes do mesmo. Metodologias de planejamento experimental foram utilizadas para otimizar a temperatura de incubação e o pH do meio de cultura. As melhores condições de cultivo para a produção da TGase pelo *B. circulans* BL32 para a produção da enzima em CSm foram 30 °C e pH 8.5, sendo que a máxima produção foi obtida no final da fase estacionária de multiplicação. Os efeitos da agitação e aeração sobre a produção de TGase e esporulação do *B. circulans* BL32 em sistema de CSm também foram estudados. Os resultados demonstraram que as condições ótimas de processo para a formação de biomassa e esporulação são diferentes. Portanto, foi adotada uma estratégia de controle da taxa de aeração em dois estágios, com formação de biomassa, no primeiro estágio, nas condições ótimas de crescimento seguido por um segundo estágio sob as condições de esporulação. Também estudou-se a produção de TGase pelo *B. circulans* BL32 em cultivo no estado sólido (CES). Vários resíduos agroindustriais foram usados como substrato para crescimento do microrganismo e produção da enzima. As melhores condições de cultivo foram 0,6 L/min de ar, 33 °C e 10 $\log_{10}$ UFC/g de substrato para a concentração celular do inóculo, em resíduo fibroso de soja como substrato. A determinação das condições de extração para a efetiva recuperação da TGase produzida em CES foi realizada através do emprego de ferramentas de planejamento experimental. A melhor condição de extração da enzima foi quando utilizou-se água a 7 ºC como solvente, por 5 minutos, 250 rpm e uma relação de sólidos/líquidos de 1:6. Caseína, proteína isolada de soja e proteína hidrolisada de carne foram tratadas com essa TGase microbiana. A redução do número de grupos aminos livres após o tratamento com a enzima, principalmente, na caseína, demonstrou a formação de ligações cruzadas catalisadas por essa TGase. As propriedades emulsificantes dessas proteínas foram melhoradas após o tratamento com a TGase do *B. circulans* BL32. Além disso, a fim de investigar o mecanismo de inativação térmica, incubou-se a enzima por diferentes períodos de tempo em temperaturas entre 30 e 70 °C. As cinéticas de termoinativação desta TGase seguiram o modelo de Lumry-Eyring e a enzima mostrou-se estável até 50 °C, sendo que após 12 h nessa temperatura a mesma ainda mantém 50 % da sua atividade enzimática. Os resultados sugerem que esta TGase microbiana apresenta um grande potencial de uso em aplicações alimentícias e não alimentícias.

[1]/Tese de Doutorado em Biologia Celular e Molecular (área de concentração Biotecnologia), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (256p.), agosto de 2008.

ABSTRACT

PRODUCTION ON SUBMERGED AND SOLID-STATE CULTIVATIONS AND CHARACTERIZATION OF TRANSGLUTAMINASE (EC 2.3.2.13) FROM AMAZON ISOLATED *Bacillus circulans* [1]

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) is an enzyme capable of catalyzing acyl transfer reactions by introducing covalent cross-links between proteins. Microbial TGases have found widespread and growing applications in the food and non-food industry. In this work, an effort has been made in order to increase available knowledge about microbial TGases. Medium composition for TGase production on submerged cultivations by *Bacillus circulans* BL32, a recently isolated strain from the Amazon basin, was optimized using a stepwise strategy. The optimization of the medium resulted not only in a 60 % higher TGase activity than the obtained in a media previously cited in the literature but also in a reduction of constituents costs. Statistical experimental methods also were used to optimize the temperature and pH parameters. The best culture conditions for TGase production by *B. circulans* BL32 were 30 °C, pH 8.5 and the highest production was obtained in late-stationary culture phase. And besides, the effects of agitation and aeration on TGase production and cell sporulation on submerged cultivations were studied. The results demonstrated that the optimal process conditions are different for biomass and spore production. It was adopted a two-stage aeration rate control strategy with biomass production under the growth conditions in the first stage followed by the second stage under the conditions for sporulation. The present work also dealt with the TGase production in solid state cultivations. Several agro-industrial residues were used as substrates for microbial growth and enzyme production. The best culture conditions were determined as being 0.6 L air min^{-1}, 33 °C and 10 $\log_{10}$ CFU g^{-1} of dried substrate to the inoculum cell concentration, on industrial fibrous soy residue as substrate. The optimization of downstream processing parameters for the effective enzyme recovery of the cultivated solids was carried out. The optimal conditions for the extraction were: water as solvent at 7 ºC; 5 min of extraction time; agitation speed of 250 rpm; and 1:6 solid:liquid ratio. Casein, soy protein isolated, and hydrolysed animal protein were treated with this microbial TGase. The decrease in the amount of free amino groups after TGase treatment, mainly in the casein, demonstrated the cross-linking catalyzed by this enzyme. The emulsifying properties of these proteins were improved after treatment with *B. circulans* BL32 TGase. Furthermore, in order to investigate the mechanism of thermal inactivation, the enzyme was incubated at temperatures ranging from 30 to 70 °C. The thermoinactivation kinetics of this microbial TGase followed a Lumry-Eyring model. The enzyme presented good stability until 50 °C. About 50 % of the activity still remained after heating for 12 h in this temperature. Results presented in this work suggest that this microbial TGase exhibit some interesting properties for food and non-food industrial applications.

[1]/ Doctor of Science Thesis on Cellular and Molecular Biology (Area of Biotechnology), Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre (256p.), august, 2008.

SUMÁRIO

1. INTRODUÇÃO

O uso das enzimas microbianas pelo homem remonta há muitos séculos. Nessa época, a sua natureza e função, bem como os microrganismos eram desconhecidos. A elaboração de produtos fermentados era possível devido às enzimas produzidas pelo crescimento espontâneo dos mesmos. Já em 1940, o desenvolvimento dos bioprocessos tornou possível a produção de enzimas em larga escala, a partir de microrganismos selecionados e com elevado grau de purificação e caracterização. Nesse contexto, a atual produção industrial de enzimas de origem microbiana é o resultado da evolução dos processos biotecnológicos das últimas décadas e emprega uma variedade de diferentes microrganismos para a obtenção de preparações enzimáticas com diferentes purificações e aplicações.

Tais preparações de enzimas microbianas estão sendo amplamente empregadas com inúmeros propósitos na produção dos mais diversos alimentos. Uma aplicação importante é na modificação das matérias-primas alimentícias com o objetivo de melhorar suas qualidades funcionais e nutricionais. As enzimas podem atuar modificando a textura, sabor e cor, valorizando, dessa forma, aspectos funcionais de alguns alimentos e incrementando o valor nutricional de outros pela redução da quantidade ou remoção de constituintes indesejáveis.

Inúmeras pesquisas têm sido realizadas a fim de modificar as características funcionais das proteínas nos alimentos através do emprego de enzimas. Com esse objetivo a transglutaminase (EC 2.3.2.13), enzima de grande importância para a indústria alimentícia, vem sendo estudada há algum tempo. Ela catalisa reações de acil transferência, formando ligações entre proteínas, peptídeos e aminas primárias, através da formação de

ligações covalentes entre resíduos de lisina e glutamina. Devido a essas reações, a transglutaminase atua sobre as proteínas alimentares proporcionando reestruturação do produto; melhora da textura, da elasticidade e da capacidade de gelificação e de emulsificação; aumento da viscosidade, da resistência física e da estabilidade térmica e elevação do valor nutricional mediante a incorporação de aminoácidos limitantes no material original. Além do processamento de alimentos, há possibilidades de utilização dessa enzima em outras áreas, tais como curtimento de peles, imobilização de enzimas e indústria têxtil.

Os estudos sobre transglutaminases microbianas se intensificaram nos últimos 19 anos pela possibilidade de produção em larga escala, através do cultivo de microrganismos em biorreatores e, assim serem amplamente usadas pela indústria. Porém, atualmente, a única transglutaminase disponível comercialmente é produzida pela Ajinomoto S.A. e, em virtude do seu alto custo, a sua utilização ainda é limitada. Dessa forma, o aumento do número de transglutaminases comerciais de origem microbiana, bem como, a redução dos custos de produção das mesmas são diferentes estratégias que podem ser aplicadas a fim de ampliar o uso industrial dessa enzima.

Existem dois métodos principais de cultivo de microrganismos, o processo de cultivo submerso e o de cultivo em estado sólido. O cultivo submerso é mais empregado em bioprocessos industriais, principalmente devido à facilidade de mensuração dos parâmetros de crescimento através de sofisticados controles automatizados por computador. Porém, o cultivo em estado sólido possui um grande potencial para a produção de enzimas, uma vez que, resíduos agroindustriais, abundantes e baratos, podem servir como substrato para o crescimento microbiano, possibilitando a obtenção de produtos com alto valor agregado. Independente do processo de cultivo adotado, a seleção de microrganismos adequados, o desenvolvimento de linhagens eficientes por engenharia genética, a otimização da composição do meio de cultura e a determinação das condições ótimas de cultivo são fatores importantes que afetam a produção de enzimas e que, portanto, devem ser avaliados quando se busca a otimização de processos biotecnológicos.

Estudos de otimização contribuem para o aumento da produtividade dos processos de cultivo de microrganismos, bem como para a diminuição do custo total de produção. A aplicação de metodologias estatísticas pode ser de grande valia na definição dos principais efeitos e interações dos fatores que são importantes em bioprocessos. A combinação de

fatores que geram uma determinada resposta ótima pode ser identificada através de planejamento fatorial e do uso de metodologia de superfície de resposta (MSR). Este método possibilita a determinação das condições ótimas para maximizar a resposta e tem sido aplicado com sucesso para a otimização de processos.

Nesse contexto, o presente trabalho visa contribuir para o desenvolvimento da produção de transglutaminases microbianas. Primeiramente, determinaram-se as condições ótimas de cultivo, como meio de cultura, pH, temperatura, aeração e agitação, para a produção da enzima pelo *Bacillus circulans* BL32 em processo submerso. Num segundo momento, otimizou-se a produção dessa transglutaminase em cultivo no estado sólido, onde foram avaliados diferentes substratos e determinadas as melhores condições de temperatura, aeração e concentração de inóculo, bem como os parâmetros de extração da mesma. Em uma terceira etapa, estudaram-se as características dessa enzima para aplicação no processamento de alimentos e em outras áreas.

2. REVISÃO BIBLIOGRÁFICA

2.1 Transglutaminases

2.1.1 Definição e características

A transglutaminase (TGase) (EC 2.3.2.13) possui o nome sistemático de proteína-glutamina γ-glutamil-transferase e catalisa a reação de acil-transferência, na qual os grupos γ-carboxiamida dos resíduos de glutamina de uma cadeia polipeptídica atuam como acil-doadores (NIELSEN, 1995). Os acil-aceptores podem ser os grupos ε-amino de resíduos de lisina de uma cadeia peptídica, neste caso podendo ocorrer ligações tanto inter como intra-moleculares, uma lisina livre ou outras aminas primárias. Na ausência destas, até mesmo a água pode servir como acil-aceptor, ocorrendo nesse caso a desamidação (BABIKER *et al*., 1996).

Quando o acil-aceptor é a amina terminal de um resíduo de lisina pertencente a um substrato protéico, o resultado da reação é a formação de ligações cruzadas entre cadeias protéicas (reação de polimerização), conseqüentemente alterando diversas propriedades físicas das proteínas. A ligação formada é chamada de ε-(γ-glutamil)lisina. Quando a reação ocorre com lisina livre ou outras aminas primárias o resultado da reação é a incorporação de aminas nas cadeias protéicas, com conseqüente alteração da hidrofobicidade da superfície. Já, devido à reação de desamidação, ocorre um aumento no número de resíduos carboxílicos, o que resulta na alteração da carga da proteína (FOLK & FINLAYSON, 1977).

As reações catalisadas pela transglutaminase estão representadas na Figura 1.

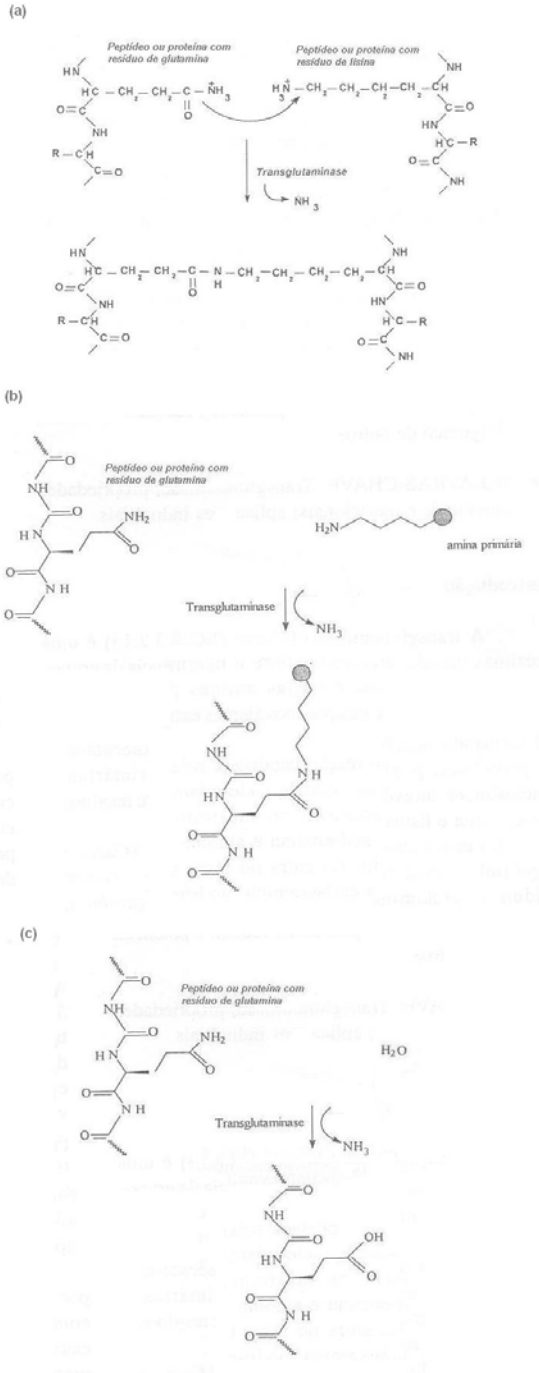

Figura 1: Reações catalisadas pela TGase: (a) ligação cruzada entre glutamina e lisina pertencente a uma cadeia peptídica; (b) reação de acil-transferência com incorporação de lisina ou outra amina livre; (c) reação de desamidação.

As TGases estão largamente distribuídas na natureza, podendo ser encontrada nos tecidos de animais, peixes, plantas e microrganismos (MOTOKI & SEGURO, 1998).

As funções fisiológicas das TGases isoladas dessas várias fontes têm sido investigadas e parecem estar envolvidas em inúmeras reações envolvendo proteínas. A função mais estudada é sua participação no processo de coagulação sanguínea em mamíferos, em que ocorre a formação de ligações cruzadas entre proteínas catalisada pelo Fator XIII, uma forma ativada da TGase do plasma (CHUNG *et al*., 1974). Em tecidos animais também estão envolvidas nos processos de cicatrização e de queratinização da epiderme (AESCHLIMANN & PAULSSON, 1994). A transglutaminase de mamíferos, distribuída naturalmente no fígado e no sangue, é uma enzima Ca^{+2}-dependente (KURTH & ROGERS, 1984).

Já em plantas catalisam reações de formação das estruturas do citoesqueleto e da parede celular (SERAFINI-FRACASSINI *et al*., 1995).

Para fins de aplicação tecnológica, até o final da década de 80, as mais estudadas foram as TGases de fígado de cobaia (MOTOKI & NIO, 1983; IKURA *et al*., 1985) e do fator XIII da coagulação bovina (KURTH, 1983; KURTH & ROGERS, 1984). Mas, as transglutaminases extraídas de mamíferos, mostraram-se inviáveis para aplicação industrial em larga escala, tendo em vista a produção limitada e a dificuldade de purificação (ZHU *et al*., 1995).

2.1.2. Transglutaminases microbianas

ANDO *et al.* (1989) foram os primeiros pesquisadores a investigarem a possibilidade de produção de TGases por microrganismos. Eles identificaram, entre 5000 isolados de solos do Japão, uma cultura de *Streptoverticillium*, que tinha alta capacidade de produção de transglutaminase.

Posteriormente foram isoladas diversas linhagens de *Streptoverticillium,* tais como *S. mobaraense* (LU *et al*., 2003), *S. ladakanum* (HO *et al.,* 2000) e *S. cinnamoneum* (DURAN *et al.,* 1998), que tinham a habilidade de produzir a enzima. Essa também foi detectada em culturas de *Streptomyces*, tais como *Streptomyces platensis* (LIN *et al.,* 2006) e *Streptomyces hygroscopicus* (CUI *et al*., 2007), de *Bacillus,* tais como *B. subtilis* (KOBAYASHI *et al*., 1998b; SUZUKI *et al.,* 2000) e *B. circulans* (SOARES *et al.,* 2003a), e de *Physarum polycephalum* (KLEIN *et al.,* 1992).

Entre as transglutaminases de origem microbiana, até o momento, a obtida a partir do crescimento do microrganismo *S. mobaraense* é a melhor caracterizada. BERNARD *et al.* (1998) realizaram uma avaliação toxicológica dessa enzima, a fim de iniciar a sua comercialização. Os resultados dos estudos de toxicidade oral aguda em ratos e de mutagênese mostraram que a mesma não apresenta potencial mutagênico e toxicológico. Posteriormente, KUTEMEYER *et al.* (2005) investigaram a influência de diferentes sais (KCl, NaCl, $CaCl_2$ e $MgCl_2$) e concentrações (5, 10, 15 e 20 %) desses sais na atividade enzimática e estabilidade térmica dessa preparação comercial de transglutaminase. Concluíram que a adição de KCl e NaCl aumenta a atividade enzimática e a estabilidade térmica, enquanto que o $CaCl_2$ as reduz.

Recentemente, CUI *et al*. (2007; 2008) iniciaram a caracterização da transglutaminase de *Streptomyces hygroscopicus.* Estudos de estabilidade térmica e de mudanças conformacionais mostraram que essa enzima é totalmente inativada quando incubada a 60 °C por 40 min e a sua estrutura terciária adquire uma conformação quase que completamente desenovelada.

A Tabela 1 ilustra as características enzimáticas de algumas tranglutaminases microbianas descritas na literatura.

Tabela 1: Características enzimáticas de algumas TGases microbianas

Microrganismo	pH ótimo de atividade	Temperatura ótima de atividade (°C)	pH de estabilidade	Estabilidade térmica (°C)	Referência
Streptoverticillium sp.	6-7	50	5-9	40-50	ANDO *et al.* (1989)
Streptoverticillium mobaraense	6	52	5-7	20-40	LU *et al.* (2003)
Streptoverticillium ladakanum	5-6	40	5-7	50-55	HO *et al.* (2000)
Streptomyces hygroscopicus	6-7	37-45	5-8	50	CUI *et al.* (2007)
Bacillus subtilis	8,2	60	-	-	SUZUKI *et al.* (2000)

A função da transglutaminase em microrganismos ainda não é totalmente conhecida, mas evidências sugerem que essa enzima catalisa reações de reticulação das proteínas dos esporos (RAGKOUSI & SETLOW, 2004; ZILHÃO *et al.,* 2005), uma vez que esta foi detectada durante a esporulação de *B. subtilis*, entre 6-10 horas após o início da fase estacionária, mas não em células vegetativas durante a fase exponencial. Além disso, foi verificada a presença de uma grande quantidade de ligações isopeptídicas do tipo ε-(γ-glutamil)lisina presentes nas proteínas da capa dos esporos (KOBAYASHI *et al*., 1998b).

A transglutaminase microbiana é uma enzima extracelular dissolvida no meio de cultivo. Portanto, esta pode ser recuperada após centrifugação das células intactas e do material sólido (ZHU *et al*., 1995), não sendo necessária a etapa de ruptura celular para a separação da enzima. Além disso, as atividades dessas TGases são estáveis durante longo tempo de armazenamento e Ca^{+2}-independente (NONAKA *et al.,* 1989; NIELSEN, 1995). Por essa propriedade as transglutaminases microbianas apresentam uma melhor aplicação industrial, já que muitas proteínas de alimentos, como caseínas do leite, globulinas da soja e miosinas da carne, podem facilmente precipitar na presença de cálcio e reduzir sua sensibilidade à TGase (MOTOKI & SEGURO, 1998).

Assim, com a descoberta de TGases microbianas, a maior parte dos estudos de aplicação tecnológica passaram a utilizar enzimas obtidas de cultivos de microrganismos (NONAKA *et al.,* 1989). Dezenove anos se passaram desde que a primeira transglutaminase microbiana oriunda de uma cultura de *Streptoverticillium mobaraense* foi descoberta, mas, segundo ARRIZUBIETA (2007), esta é ainda a única fonte comercial disponível dessa enzima.

Portanto, a utilização da TGase ainda é limitada, tendo em vista o seu alto custo e o fato de que, nem sempre, as características da transglutaminase comercial disponível atualmente são adequadas à aplicação desejada. Com base nisso, vários trabalhos, através do emprego de diferentes estratégias, vêm buscando reduzir os custos de produção e ampliar o número de transglutaminases comerciais de origem microbiana. Seleção de novos microrganismos (SOARES *et al.,* 2003a; ANDO *et al*., 1989), desenvolvimento de linhagens eficientes por engenharia genética (LIN *et al*., 2004), otimização da composição do meio de cultura (TÉLLEZ-LUIS *et al.*, 2004a; ZHU *et al*., 1996), determinação das condições ótimas de cultivo (MEIYING *et al*., 2001; MEIYING *et al*., 2002) e

modificação dos processos de purificação (GERBER *et al*., 1994) são fatores importantes que afetam a produção de enzimas e que, portanto, devem ser avaliados quando se busca a otimização de processos biotecnológicos.

2.1.2.1 Produção de transglutaminases microbianas

Vários pesquisadores mostraram que é possível obter transglutaminase através de manipulação genética usando diferentes microrganismos hospedeiros, tais como *Streptomyces lividans*, *Escherichia coli* e *Saccharomyces cerevisiae* (IKURA *et al*., 1988; BISHOP *et al*., 1990; TAKEHANA *et al.,* 1994; KAWAI *et al*., 1997; WASHIZU *et al*., 1994; YASUEDA *et al*., 1995; KOBAYASHI *et al*., 1998a; LIN *et al*., 2004; LIN *et al.*, 2006).

A expressão do gene de TGase de fígado de cobaia em *E. coli* (IKURA *et al*., 1988), o Fator XIIIa humano expresso em levedura *Saccharomyces cerevisiae* (BISHOP *et al*., 1990), a TGase de *Streptoverticillium mobaraense* em *E. coli* (TAKEHANA *et al.,* 1994; KAWAI *et al*., 1997) e em *Streptomyces lividans* (WASHIZU *et al*., 1994), a transglutaminase do músculo de peixe em *E. coli* (YASUEDA *et al*., 1995), a TGase de *Bacillus subtilis* em *E. coli* (KOBAYASHI *et al*., 1998a), a transglutaminase de *Streptoverticillium ladakanum* (LIN *et al*., 2004) e de *Streptomyces platensis* (LIN *et al*., 2006) em *Streptomyces lividans* são trabalhos que foram realizados na tentativa de obter-se a enzima em grande quantidade e baixo custo.

Porém, nenhuma dessas TGases foram comercializadas devido à fatores como legislação alimentar e aceitabilidade dos consumidores (ARRIZUBIETA, 2007; MOTOKI & SEGURO, 1998). Portanto, os aperfeiçoamentos dos processos de cultivo de isolados selvagens capazes de produzir a transglutaminase são perspectivas promissoras (ZHU *et al*., 1995).

Os estudos dos processos de cultivo para a produção da TGase microbiana relatados na literatura se concentram no microrganismo *Streptoverticillium* spp. e são realizados em sistema de cultivo submerso.

Como fontes de carbono para a produção de transglutaminase podem ser usados glicose, sacarose, amido, glicerol e dextrina. Como fontes inorgânicas e orgânicas de

nitrogênio podem ser utilizados o NH_4NO_3, $(NH_4)_2SO_4$, uréia, $NaNO_3$, NH_4Cl, peptona, extrato de carne, caseína, aminoácidos e extrato de levedura. Os sais minerais necessários são fosfatos, magnésio, potássio, ferro, cobre e zinco (ZHU *et al*., 1995).

O primeiro processo de cultivo para a produção de transglutaminase microbiana foi proposto por ANDO *et al.* (1989). Os autores utilizaram um sistema de cultivo submerso em batelada, em reatores de 20 L com agitação de 250 rpm e aeração de 10 L/min para o crescimento de *Streptoverticillium mobaraense* na temperatura de 30 ºC. O meio de cultura era composto de amido solúvel, peptona, extrato de levedura, KH_2PO_4, K_2HPO_4 e $MgSO_4$. Nessas condições a máxima produção enzimática foi em 70-80 horas.

Partindo do meio basal sugerido por ANDO *et al.* (1989), um novo meio de cultura para a produção de TGase de *S. mobaraense* CBS20778 em cultivos contínuos e em batelada foi proposto por ZHU *et al*. (1996). Os autores utilizaram a modelagem estequiométrica a fim de desenvolver um meio suplementado com inúmeros aminoácidos. Usando essa estratégia eles aumentaram a produção enzimática em quatro vezes e reduziram em 25 horas o tempo total de cultivo.

ZHU *et al.* (1998a) desenvolveram um modelo de fluxos metabólicos para estudar a produção de TGase pelo microrganismo *S. mobaraense* CBS20778. Tal estudo é baseado na concentração dinâmica dos 20 aminoácidos, no consumo de açúcar e na produção de CO_2 e biomassa ao longo de cultivos em batelada em sistemas submersos. Com base nos resultados os autores sugerem que o acúmulo de biomassa é um requisito essencial para a produção de transglutaminase por *S. mobaraense* e esta só ocorre certo tempo após ter cessado a multiplicação celular.

ZHU *et al.* (1998b) propuseram um sistema de cultivo submerso em batetada alimentada para a produção de TGase de *S. mobaraense* CBS20778. O sulfato de amônio, como fonte inorgânica de nitrogênio, foi utilizado durante a fase de alimentação ao invés de peptona, tendo em vista que esta pode ser polimerizada à medida que a enzima está sendo produzida. Dessa forma, a máxima produção de enzima obtida foi de 1,80 U/mL, o que representa um aumento de 80 % na atividade enzimática, em relação ao cultivo em batelada.

Com freqüência, as condições ótimas de temperatura e pH para multiplicação celular de *Streptoverticillium mobaraense* e produção de transglutaminase podem ser diferentes (ZHENG *et al*., 2001; ZHENG *et al*., 2002a). ZHENG *et al*. (2001) estudaram a

produção de TGase pelo *Streptoverticillium mobaraense* WSH-Z2 em várias temperaturas, variando de 25 a 35 °C. Na temperatura de 32 °C a fase lag foi menor e a taxa específica máxima de crescimento maior nos estágios iniciais do cultivo. Já na temperatura de 28 °C a taxa específica de produção de transglutaminase foi maior nos estágios intermediário e final do cultivo. Com base nesses resultados os autores propõem uma estratégia de diferentes temperaturas de cultivo, sendo que nas primeiras 18 horas a temperatura deve ser mantida em 32 °C e após reduzida para 28 °C. ZHENG *et al*. (2002a) também estudaram a produção de TGase de *Streptoverticillium mobaraense* WSH-Z2 em vários pHs, variando de 5,0 a 8,5. Com base nos resultados obtidos os pesquisadores propõem uma estratégia de diferentes pHs durante o cultivo, de forma que nas primeiras 13 horas este deve ser mantido em 7,0 e após alterado para 6,5.

ZHENG *et al*. (2002b) desenvolveram um modelo matemático que relaciona o efeito da temperatura, em sistema de cultivo submerso, com o crescimento celular de *Streptoverticillium mobaraense* WSH-Z2 e a produção de transglutaminase pelo microrganismo. De acordo com o modelo, os máximos valores de biomassa e atividade enzimática poderiam ser obtidos na temperatura de 30 °C.

TÉLLEZ-LUIS *et al.* (2004b) estudaram a produção de TGase de S*treptoverticillium ladakanum* NRRL-3191 num meio de cultura contendo hidrolisado de palha de sorgo e xilose comercial com concentração inicial de 10, 20 ou 30 g/L. A máxima atividade (0,155 U/mL) foi obtida usando hidrolisado e 30 g/L de xilose após 96 horas de cultivo. Usando apenas xilose comercial como fonte de carbono, a máxima atividade obtida foi de 0,282 U/mL, também após 96 horas de cultivo, mas com a concentração inicial de xilose de 20 g/L.

Um estudo sobre o efeito da concentração de oxigênio dissolvido no meio de cultura, em sistema de cultivo submerso em batelada, e a sua relação com o crescimento celular de *Streptoverticillium mobaraense* WSH-Z2 e a produção de transglutaminase foi reportado por YAN *et al.* (2005). Os autores realizaram dois conjuntos de experimentos, num deles a aeração foi fixada em 1vvm e a agitação variou de 250 a 450 rpm, no outro a aeração variou de 0,75 a 2 vvm e a agitação foi fixada em 350 rpm. Com base nos resultados obtidos foi proposta uma estratégia de diferentes agitações durante o cultivo, onde nas primeiras 24 horas esta deve ser mantida em 450 rpm e após alterada para 350 rpm.

2.1.3 Aplicações da transglutaminase

Devido às diferentes reações que catalisam, as TGases são utilizadas para modificar as proteínas, visando diversos objetivos (ZHU *et al*., 1995). A maior aplicação comercial da atividade catalítica das transglutaminases é na indústria de alimentos (YOKOYAMA *et al*., 2004).

2.1.3.1 Aplicações na indústria alimentícia

No domínio alimentar, o interesse concentra-se na alteração das propriedades funcionais e nutricionais. Pode ser utilizada em todo tipo de alimento protéico, tais como produtos cárneos (reestruturados, emulsionados e injetados), produtos lácteos (iogurtes, queijos e sorvetes), produtos farináceos (pães e massas alimentícias), produtos a base de soja (tofu) e gelatinas (NIELSEN, 1995; MOTOKI & SEGURO, 1998; KURAISHI *et al*., 2001).

A reação de acil-transferência, que a TGase pode catalisar, entre os grupos γ-carboxiamida dos resíduos glutamínicos ligados em proteína ou peptídeos e aminas primárias (Figura 1b) pode ser explorada na indústria de alimentos para melhorar o valor nutricional das proteínas alimentares (IKURA *et al.*, 1985). Essa reação pode ser aplicada para introduzir aminoácidos essenciais às proteínas de baixa qualidade nutricional. IKURA *et al.* (1981) avaliaram a incorporação dos aminoácidos etil éster de L-metionina em α_{s1} e β-caseína e nas globulinas 11S e 7S da soja, e L-lisina no glúten do trigo. Após 120 minutos de tratamento, o conteúdo de metionina em α_{s1}-caseína era duas vezes maior do que no material original, e para β-caseína e proteínas 11S e 7S da soja, os valores eram 1,5; 2,4 e 3,5 vezes maiores, respectivamente. Já o conteúdo de lisina no glúten tratado foi 5,1 vezes maior do que no material inicial.

No entanto, na indústria de alimentos a aplicação comercial da atividade catalítica da TGase se concentra, principalmente, na reação de polimerização (ligação cruzada) de proteínas (Figura 1a) (JONG & KOPPELMAN, 2002). Essas ligações cruzadas podem ser tanto intermoleculares como intramoleculares, causando mudanças significativas nas

propriedades físicas dos alimentos protéicos. Como as ligações entre os resíduos de glutamina e lisina são do tipo covalente, estas são mais estáveis que as interações iônicas e hidrofóbicas, e não podem ser quebradas por aquecimento normal e nem por força física. Por esta razão, mesmo uma pequena quantidade de ligações ε-(γ-glutamil)lisina possuem um grande efeito nos alimentos protéicos, aumentando o valor comercial dos mesmos (NIELSEN, 1995; YOKOYAMA *et al*., 2004).

A TGase atua sobre as proteínas alimentares proporcionando melhora da textura e da elasticidade, capacidade de retenção de água, capacidade de emulsificação, capacidade de formar géis protéicos, capacidade de reestruturação; aumento da viscosidade, da resistência física e da estabilidade térmica e a elevação do valor nutricional mediante a incorporação de aminoácidos limitantes no material original (ZHU *et al*., 1995; KURAISHI *et al*., 1997).

2.1.3.1.1 Produtos cárneos

Em produtos cárneos, a grande reatividade entre a transglutaminase e as proteínas actina e miosina leva à formação de ligações covalentes intermoleculares e intramoleculares entre a glutamina e os resíduos de lisina. Esta ligação melhora a textura dos produtos cárneos e torna a estrutura mais resistente ao aquecimento e ao congelamento (MUGURUMA *et al*., 2003).

Uma das principais aplicações da TGase na indústria de carnes é na elaboração de produtos cárneos reestruturados. A partir da aplicação dessa enzima é possível elaborar uma variedade de produtos reestruturados, utilizando carnes de várias espécies e também misturas de carnes com proteínas vegetais em suas formulações (RAMÍREZ-SUÁREZ & XIONG, 2003). Devido à polimerização das proteínas promovida pela transglutaminase, estes produtos têm sua textura melhorada quando a enzima é aplicada. Mesmo na ausência de fosfatos ou de sal, é possível obter produtos com ótima textura e mais homogêneos (KURAISHI *et al*., 1997; MUGURUMA *et al*., 2003). Além disso, a enzima aumenta a capacidade de retenção de água de tais produtos, possibilitando um aumento de suculência e uma redução na perda de água durante a vida-de-prateleira (AHHMED *et al*., 2007).

A adição da TGase na elaboração de salames possibilita uma redução no tempo de maturação, pois a firmeza adequada é obtida mais rapidamente, e há uma melhora na fatiabilidade do produto, sem interferir no desenvolvimento da cultura iniciadora e na variação do pH (GURGEL, 2007).

2.1.3.1.2 Produtos lácteos

Uma outra área de grande importância, na qual a transglutaminase pode contribuir de forma significativa é na elaboração de produtos lácteos (ÖZRENK, 2006). Entre as proteínas do leite, as caseínas, que possuem uma estrutura conformacional aberta, reagem facilmente com a TGase, enquanto as proteínas globulares do soro reagem somente sob condições que favorecem o seu desenovelamento (MATSUMURA *et al*., 1996; LORENZEN *et al*., 1998; SHARMA *et al*., 2002).

Na elaboração de iogurtes, o tratamento do leite com transglutaminase aumenta a firmeza e viscosidade e reduz a sinerese do produto (LAUBER *et al*., 2000). Já no processamento de queijos curados, a adição de TGase no leite possibilita um aumento no rendimento, uma textura mais macia e reduz a separação de soro. Os queijos processados tratados com a enzima apresentam uma maior estabilidade térmica (YOKOYAMA *et al*., 2004).

2.1.3.1.3 Produtos farináceos

Novas perspectivas para a aplicação da transglutaminase incluem a indústria de pães e massas alimentícias. O glúten, proteína da farinha de trigo, pode formar ligações cruzadas quando tratado com TGase, resultando na formação de polímeros de alta massa molecular (LARRÉ *et al.,* 2000). A ação da enzima reforça a estrutura da rede protéica modificando as propriedades viscoelásticas das massas. Consequentemente, massas alimentícias contendo TGase tem uma maior resistência à quebra, ou firmeza. Na elaboração de pães, a enzima aumenta a vida-de-prateleira e o volume do produto (GERRARD *et al*., 1998; GERRARD *et al*., 2000).

2.1.3.1.4 Produtos derivados da soja

A aplicação de TGase tem sido também preconizada na elaboração de produtos à base de proteínas de soja. Estas são adicionadas a muitos alimentos processados, tais como salsicha, hambúrguer e mortadela, a fim de melhorar a textura dos produtos. A formação de ligações cruzadas entre as globulinas da soja modifica as propriedades de gelatinização e de textura dos produtos (NONAKA *et al.,* 1994; KURAISHI *et al*., 2001). No caso do Tofu, produto de soja curado muito consumido na Ásia, obtido por coagulação das proteínas da soja, o tratamento com TGase aumenta a capacidade de retenção de água e o produto obtido apresenta uma textura mais homogênea e firme. Além disso, a adição da enzima permite um melhor controle da reação de coagulação (MOTOKI & SEGURO, 1998).

2.1.3.2 Outras aplicações

Além do processamento de alimentos, trabalhos mostram que esta enzima pode ser aplicada em várias outras áreas, tais como farmacêutica (BERNARD *et al.,* 1998), têxtil (CORTEZ *et al*., 2004), curtimento de couros (COLLIGHAN *et al*., 2002), imobilização de enzimas (JOSTEN *et al*., 1999; KAMIYA *et al*., 2001), formação de imunoconjugados para testes de ELISA (JOSTEN *et al.,* 1998) e de biossensores (JÜLICHER *et al*., 1998), e engenharia de tecidos (ORBAN *et al*., 2004).

2.2 Emprego de metodologias de planejamento experimental e superfície de resposta em processos biotecnológicos

Planejamento experimental é um conjunto de técnicas frequentemente utilizado em estudos de processos para investigações qualitativas ou quantitativas, explorando os efeitos e relações de variáveis de entrada (parâmetros) sobre variáveis de saída (respostas) (BOX *et al*., 1978; KALIL *et al.,* 2000).

Através do planejamento experimental pode-se otimizar um determinado processo utilizando-se um menor número de experimentos, quando comparado às metodologias convencionais, permitindo a investigação do processo em uma faixa ampla de variação, com redução de tempo e custos. Uma outra vantagem do planejamento experimental é a possibilidade de otimizar várias respostas ao mesmo tempo. A maior falta de informação no estudo univariável relaciona-se ao efeito sinérgico ou antagônico entre as variáveis, que só podem ser obtidas pela determinação dos efeitos de interação entre as variáveis através de um planejamento experimental (RODRIGUES & IEMMA, 2005).

Um dos métodos de avaliação do planejamento experimental é a análise e otimização através de superfície de resposta. A metodologia do planejamento experimental associada à análise de superfície de resposta é uma ferramenta fundamentada na teoria estatística fornecendo informações muito mais seguras do processo, minimizando o empirismo das técnicas de tentativa e erro (KALIL *et al.,* 2000). Obtêm-se, assim, relações empíricas entre uma ou mais respostas de interesse, que são medidas analíticas, e um determinado número de fatores, que são controlados e influenciam a resposta do processo. Desta forma, este estudo permite que se verifique, quantifique e otimize esta influência, sendo possível a obtenção das melhores condições de processo (BARROS NETO *et al*., 2003).

Para se aplicar a metodologia de superfície de resposta (MSR) é necessário primeiramente programar ensaios através de um planejamento experimental. Esse método consiste na seleção de um número fixo de níveis para cada um dos fatores ou variáveis de entrada e, então, executar experimentos com todas as combinações possíveis. Como primeira etapa, é usual um planejamento Plackett-Burman (PB) com dois níveis (nível -1 e nível +1) para cada variável (fator) estudada. De posse dos resultados obtidos é possível calcular os efeitos principais, ou seja, especificar os efeitos mais significativos (RODRIGUES & IEMMA, 2005).

2.2.1 Otimização de processos biotecnológicos

Até o final da década de 90, estudos de processos fermentativos eram frequentemente realizados através do emprego de metodologias clássicas, com variação de um fator de cada vez, enquanto todos os outros permanecem fixos. No entanto, técnicas de

planejamento experimental representam uma melhor alternativa para determinar as condições ótimas de cultivo de microrganismos (MYERS & MONTGOMERY, 1995).

Atualmente, a MSR é um dos procedimentos mais empregados nos estudos de otimização de processos biotecnológicos. Além disso, constitui-se em uma ferramenta estatística valiosa para a investigação de processos complexos (BARROS NETO *et al*., 2003). Esta metodologia é eficaz na análise dos efeitos de interação entre vários parâmetros, geralmente resultando em altos rendimentos através de um reduzido número de experimentos (GILMOUR, 2006).

Idealmente, o planejamento de experimentos é um processo seqüencial. Inicialmente, fatores (variáveis) são estudados para determinar quais nutrientes e condições físicas são os mais promissores para otimizar o processo de cultivo. Então, um grande número de fatores (entre 5 a 12) é avaliado através de um planejamento PB. As variáveis não signifivativas nesse experimento serão mantidas fixas na etapa seguinte, dessa forma é possível reduzir o número de fatores que devem ser estudados. As variáveis significativas serão otimizadas através de um planejamento fatorial completo e da MSR. Finalmente, após a obtenção de um modelo matemático e otimização, são determinados os valores ótimos preditos para as variáveis estudadas (MYERS & MONTGOMERY, 1995).

Alguns estudos apresentam o emprego dos métodos citados acima para a otimização de processos biotecnológicos, a fim de maximizar a produção e a atividade de enzimas e de outros metabólitos.

Por exemplo, o PB associado à MSR foi utilizado por VAIDYA *et al.* (2003) para determinar a melhor composição do meio de cultura, com o objetivo de otimizar a produção de quitinase pelo *Alcaligenes xylosoxydans*. Inicialmente, seis nutrientes (Tween 20, extrato de levedura, quitina, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$) foram avaliados através do planejamento PB com 12 experimentos. Os componentes mais importantes para a produção de quitinase, identificados como significativos, a 95 % de confiança, foram Tween 20, extrato de levedura e quitina. Na etapa seguinte a MSR foi empregada para otimizar a produção de quitinase, foram 17 experimentos nos quais as concentrações dos nutrientes não significativos ($(NH_4)_2SO_4$, KH_2PO_4 e $MgSO_4.7H_2O$) foram mantidas constantes. As concentrações ótimas obtidas para o meio de cultura, para a máxima produção de quitinase, foram 1,0 g/L de $(NH_4)_2SO_4$, 1,36 g/L de KH_2PO_4, 0,3 g/L de $MgSO_4.7H_2O$, 0,12 g/L de Tween 20, 0,3 g/L de extrato de levedura e 15 g/L de quitina. O

método estatístico de otimização permitiu o aumento da produção de quitinase de 12 para 29 U/mL.

SHARMA & SATYANARAYANA (2006) também empregaram o PB e a MSR para estudar a produção de pectinase alcalina e termoestável de *Bacillus pumilus*. Os autores reportaram que a relação C:N, a concentração de K_2HPO_4 e o pH do meio são as variáveis que influenciam significativamente na produção, de acordo com o planejamento PB. Consequentemente, os níveis dos três fatores foram otimizados, através da MSR. Para cada variável, cinco níveis foram avaliados e uma matriz com 20 experimentos foi gerada, sendo seis replicatas do ponto central. As concentrações dos nutrientes não significativos (Na_2HPO_4, $MgSO_4$, extrato de levedura, NaCl, e solução de micronutrientes) foram fixadas. Nas condições otimizadas (relação C:N de 11, pH de 8,5 e 0,5 % de K_2HPO_4) foi reportado um aumento de 41 vezes na produção de pectinase pelo *Bacillus pumilus.*

CHAUHAN & GUPTA (2004) utilizaram o planejamento PB juntamente com a MSR a fim de maximizar a produção de protease alcalina pelo *Bacillus* sp. RGR-14. Inicialmente, inúmeras fontes de carbono e nitrogênio foram avaliadas através de experimentos com uma-variável-de-cada-vez. Em seguida, com o auxílio do PB, os autores estudaram os efeitos das concentrações de caseaminoácidos, amido, íons fosfato, cátions metálicos, concentração do inóculo, temperatura de cultivo, agitação e tempo de incubação, em dois níveis (+1 e -1). Na terceira etapa, a otimização dos cinco fatores previamente determinados como significativos (concentração de caseaminoácidos, amido, íons fosfato, concentração do inóculo e tempo cultivo), foi realizada através da MSR, com 32 experimentos, sendo seis replicatas do ponto central. A otimização permitiu um aumento de 12,85 vezes na produção de protease pelo *Bacillus* sp. RGR-14.

2.2.1.1 Otimização da produção de transglutaminase microbiana

Como descrito anteriormente, verifica-se que a produção de TGases microbianas depende de inúmeras variáveis de processo, como temperatura, pH, concentração da fonte de carbono e nitrogênio, inóculo, entre outras. A tentativa de otimização de uma variável (ou fator) mantendo as demais constantes pode acarretar interpretações equivocadas por não considerar o efeito combinado dos diferentes fatores envolvidos. Dentro desse

contexto a metodologia de planejamento experimental e a análise de superfície de resposta são consideradas ferramentas eficazes e indispensáveis.

Alguns trabalhos reportam o uso dos métodos de planejamento citados acima para a produção de TGase.

JUNQUA *et al.* (1997) otimizaram a composição de um meio de cultura para a produção de TGase de *Streptoverticillium cinnamoneum* CBS 683.68 e elucidaram a relação entre a multiplicação celular e a atividade enzimática. Inicialmente, utilizando o método clássico de uma-variável-de-cada-vez, inúmeras fontes de carbono e nitrogênio foram avaliadas. A seguir, os autores estudaram a significância dos nutrientes previamente selecionados, caseína, glicerol, peptona e extrato de levedura. Na terceira etapa, a otimização dos dois fatores previamente determinados como significativos (caseína e glicerol), foi realizada através do planejamento fatorial completo e da MSR, com 13 experimentos, sendo cinco replicatas do ponto central. Em condições ótimas a atividade enzimática foi aumentada três vezes, atingindo 0,33 U/mL.

TÉLLEZ-LUIS *et al.* (2004a) desenvolveram um meio de cultura para a produção de TGase pelo microrganismo *Streptoverticillium ladakanum* NRRL-3191 utilizando um planejamento estatístico e a MSR. Primeiramente, através do método clássico de uma-variável-de-cada-vez, foram determinadas as condições ótimas de agitação e tempo de cultivo. Na etapa seguinte, com agitação constante de 250 rpm e tempo de cultivo de 72 h, foram otimizadas as concentrações do glicerol, xilose e caseína, a fim de maximizar a produção da enzima. Os autores sugerem que o glicerol atua como um indutor para a produção de TGase pelo *Streptoverticillium ladakanum* NRRL-3191, uma vez que a concentração deste nutriente apresentou um efeito positivo na atividade enzimática. O meio de cultura otimizado deveria conter 50,5 g/L de glicerol, 20 g/L de caseína e ausência de xilose.

2.3 O cultivo no estado sólido

Atualmente, o cultivo submerso (CSm) com *Streptoverticillium mobaraense* é empregado para a produção da única transglutaminase disponível comercialmente (ARRIZUBIETA, 2007). Desta forma, o desenvolvimento de novos sistemas de cultivo

para produção desta enzima torna-se uma necessidade. Uma alternativa a ser considerada é o emprego de metodologias de cultivo em estado sólido (CES) utilizando subprodutos agroindustriais. De acordo com PANDEY *et al.* (2000) e LAGEMAAT & PILE (2001) o sistema de CES é uma tecnologia promissora para o desenvolvimento de inúmeros bioprocessos e seus produtos, além de ser uma alternativa mais barata que o CSm.

2.3.1 Definição e características

A origem do CES remonta à produção de pães no antigo Egito. Esse sistema de cultivo é conhecido desde a antiguidade nos países Asiáticos (RIMBAULT, 1998).

O CES caracteriza-se pelo cultivo de microrganismos sobre uma matriz úmida sólida, substrato insolúvel, na ausência de uma fase aquosa macroscópica (água livre) (PANDEY *et al.,* 2000). Esta matriz pode ser obtida a partir de substratos naturais, usados como fonte de energia, ou meios inertes com solução nutriente (PANDEY, 2003). Entende-se por ausência de água livre a não separação da água da matriz sólida, que deve conter umidade suficiente, na forma adsorvida, permitindo o crescimento do microrganismo. O teor de umidade máximo é função do tipo de substrato, ou seja, função das propriedades que o mesmo possui em adsorver água (MITCHELL *et al*., 2003).

O CES oferece inúmeras vantagens sobre o CSm, principalmente econômicas e de engenharia, de forma que nos últimos anos tem aumentado a sua utilização para a produção de inúmeros metabólitos de interesse comercial com resíduos agroindustriais como substrato (PANDEY, 2003). Este tipo de processo de cultivo de microrganismos se torna economicamente interessante para países com abundância de biomassa e resíduos agroindustriais, que podem ser usados como matérias-primas baratas (PANDEY *et al.,* 1999).

Entre as principais vantagens econômicas e de engenharia do CES podemos citar a baixa demanda de água, o uso de equipamentos menos complexos, biorreatores mais compactos devido ao pouco volume de água, pouco espaço necessário para o processo de cultivo, meios de cultura mais simples, baixa demanda de energia e baixo custo operacional. Além disso, outras vantagens incluem a menor probabilidade de contaminação

e a obtenção de preparações enzimáticas mais concentradas (VINIEGRA-GONZÁLEZ *et al*., 2003).

No entanto, estes sistemas podem apresentar alguns problemas como o surgimento de gradientes de temperatura, umidade, oxigênio e nutrientes. Além disso, estes processos apresentam algumas desvantagens tais como: a utilização apenas de microrganismos que se desenvolvam em baixos níveis de umidade e atividade de água (a_w), dificuldades no controle e monitoramento dos parâmetros de cultivo, necessidade de elevadas concentrações de inóculo, pré-tratamento do substrato e dificuldades no escalonamento do processo (PANDEY *et al*., 2000; MITCHELL *et al*., 2000).

2.3.2 Otimização das condições de cultivo em estado sólido

Diversos aspectos, tais como a seleção de microrganismos e substratos e a determinação das condições ótimas de cultivo, devem ser considerados para o desenvolvimento de qualquer processo de CES, a fim de viabilizá-lo, uma vez que estes influenciam na concentração do produto, rendimento e produtividade volumétrica (HECK *et al*., 2005; ADINARAYANA *et al*., 2003a; POORNA *et al*., 2007).

Em geral, o tipo de microrganismo que pode ser empregado em CES é determinado pela sua capacidade de suportar baixas a_w (PANDEY, 2003). Devido aos baixos níveis de água no sistema, os fungos filamentosos têm recebido a maioria das atenções nas pesquisas sobre produção de metabólitos através de CES, uma vez que apresentam capacidade de crescimento nessas condições. Como exemplos, podem ser citados, o uso de culturas de *Rhizopus*, *Trichoderma, Penicillium* ou *Aspergillus* para a produção de enzimas; *Penicillium chrysogenum* para a produção de penicilina e *Gibberella fujikuroi* para a obtenção de ácido giberélico (BORZANI *et al.*, 2001; PANDEY *et al*. 2000; RIMBAULT, 1998). Embora a baixa a_w do CES indique pela não utilização de bactérias, vários trabalhos mostram que tais microrganismos podem ser empregados de forma eficiente nesse tipo de sistema de cultivo para a produção de diferentes substâncias (POORNA & PREMA, 2007; HECK *et al*., 2005; ADINARAYANA *et al*., 2003a; TECHAPUN *et al.,* 2003; HECK *et al*., 2002; NAMPOOTHIRI & PANDEY, 1996).

A seleção adequada do substrato é de fundamental importância para o sucesso de qualquer tipo de processo de cultivo de microrganismos. Diversos são os substratos sólidos que podem ser utilizados no CES. Nesse sistema de cultivo os substratos sólidos são constituídos basicamente de polímeros orgânicos, que se caracterizam pela insolubilidade em água e pela capacidade de possibilitar o crescimento microbiano, mesmo sem a adição de nutrientes complementares. Os resíduos agroindustriais tais como, resíduos de soja e batata, borra e polpa de café, bagaço de mandioca, farelo de cereais, palhas, bagaço de cana, cascas de frutas processadas, são os substratos mais utilizados em processos de CES com diferentes microrganismos (PANDEY *et al*., 2000).

A a_w do substrato também tem influência determinante na atividade microbiana. A capacidade de absorção de água é uma importante característica do substrato, uma vez que a a_w é fundamental para o crescimento microbiano e produção de bioprodutos (DOELLE *et al.,* 1992).

Além do substrato, os processos metabólicos dos microrganismos são influenciados pelas condições de cultivo, tais como temperatura, teor de umidade, aeração, concentração do inóculo e período de incubação. Estas condições variam amplamente de espécie para espécie de microrganismo. Dessa forma, torna-se importante conhecer as condições ambientais ótimas do microrganismo a fim de maximizar a produção do metabólito de interesse em CES (ADINARAYANA *et al*., 2003a).

A temperatura de cultivo e seu controle em CES são fundamentais. Durante o CES uma grande quantidade de calor é gerada, a qual é diretamente proporcional à atividade do microrganismo. Os materiais sólidos usados em CES possuem baixa condutividade térmica, o que faz com que a remoção do calor seja muito lenta (MITCHELL & LONSANE, 1992). Dessa forma o calor produzido durante o processo é acumulado no meio devido à baixa dissipação de calor no substrato sólido. Isto reduz a atividade microbiana e, portanto, diminui o rendimento do produto. Além disso, algumas vezes o acúmulo de calor pode desnaturar o produto formado e acumulado no leito (PANDEY, 2003).

De acordo com OHNO *et al*. (1992) altos teores de umidade resultam numa diminuição da porosidade do substrato, o que impede a penetração do oxigênio. Isso pode favorecer a contaminação por bactérias. Por outro lado, uma baixa umidade pode levar a

uma menor disponibilidade dos nutrientes, resultando num menor crescimento microbiano (MURTHY *et al*., 1999).

A aeração constante do cultivo com ar umidificado é importante para fornecer oxigênio para as células microbianas e para remover o calor gerado no meio devido à atividade do microrganismo. Porém, níveis altos de aeração podem gradualmente secar o meio de cultura do CES resultando numa diminuição da atividade microbiana (DOELLE *et al.,* 1992).

Uma baixa concentração de inóculo pode resultar em formação insuficiente de biomassa causando reduzida formação de produto, enquanto uma alta concentração de inóculo pode produzir muita biomassa resultando em pouca formação de produto (MUDGETTI, 1986).

Normalmente, a otimização das condições de CES era realizada pelo método tradicional de uma-variável-de-cada-vez. Este método não é apenas demorado, mas também leva a um incompleto entendimento do comportamento das variáveis, uma vez que não fornece informações sobre os efeitos de interação entre os fatores (RAO *et al.*, 2000).

Num trabalho pioneiro, SOARES *et al*. (2003b), através da metodologia de uma-variável-de-cada-vez, compararam a produção de TGase por *Bacillus circulans* BL32 em cultivo no estado sólido e submerso, verificando a superioridade do processo sólido. Até então a literatura relatava a produção dessa enzima somente em cultivos submersos.

Atualmente, inúmeros trabalhos mostram o emprego de planejamento experimental para a otimização da produção de bioprodutos em CES.

HECK *et al.* (2005), por exemplo, propuseram um planejamento experimental e MSR para otimizar a produção de xilanase e mananase pelo microrganismo *Bacillus circulans* BL53 em CES, utilizando como substrato o resíduo industrial fibroso de soja. Os autores estudaram os efeitos das variáveis: período de incubação, aeração e temperatura de cultivo. Para cada fator, cinco níveis foram avaliados, empregando um planejamento do tipo fatorial completo com 17 experimentos, sendo três replicatas do ponto central. Nas condições otimizadas (5 dias de cultivo, aeração de 0,5 L/min e temperatura de cultivo de 25 °C) a atividade máxima obtida de xilanase foi de 0,953 U/mg, já a atividade máxima obtida de mananase foi de 0,540 U/mg nas condições de cultivo determinadas como ideais (7 dias de cultivo, aeração de 0,8 L/min e temperatura de cultivo de 30 °C).

ADINARAYANA *et al*. (2003b) avaliaram, através da metodologia de uma-variável-de-cada-vez, os efeitos de vários nutrientes suplementares (fontes de carbono e de nitrogênio) no substrato trigo moído para a produção de neomicina pelo microrganismo *Streptomyces marinensis* NUV-5. Em seguida, as concentrações dos nutrientes previamente escolhidos, dextrina, pó de semente de framboesa e solução de sais minerais, foram otimizadas pela MSR. Nessa etapa uma matriz com 20 experimentos foi gerada, sendo seis replicatas do ponto central, e cada variável foi avaliada em cinco níveis. As concentrações ótimas obtidas foram: dextrina, 14,1 g/kg substrato; pó de semente de framboesa, 64,91 g/kg substrato e solução de sais minerais, 172,6 mL/kg substrato. Nestas condições, foi obtida uma máxima produção de antibiótico de 17.150 mg/kg de trigo moído.

2.3.3 Otimização das condições de extração do produto em CES

Os sólidos, ao final de um CES, contêm, além do produto desejado, células microbianas, partículas do substrato sólido e todos os outros metabólitos produzidos concomitantemente (GHILDYAL *et al*., 1991). Dessa forma, a extração de bioprodutos em meios sólidos é uma operação crítica e a eficiente recuperação é essencial para a viabilidade do processo (CASTILHO *et al*., 2000).

Como na produção industrial de enzimas microbianas a partir de CES, a recuperação do produto constitui cerca de 80 % dos custos totais de processo, torna-se necessário desenvolver técnicas eficientes de extração para tornar o CES aplicável para a produção de enzimas com um elevado grau de pureza e que possibilite sua exploração comercial (KUMAR & LONSANE, 1987; RAMADAS *et al.*, 1995; CASTILHO *et al*., 1999).

As técnicas de extração do produto desejado dos sólidos cultivados têm como objetivo principal a obtenção de um extrato bruto o mais concentrado possível, reduzindo, portanto, os custos dos processos de *downstream* (GHILDYAL *et al*., 1991).

Vários fatores podem influenciar a extração de bioprodutos em CES. Os mais importantes são tipo de solvente, pH do solvente, relação soluto/solvente, tempo de contato soluto/solvente, temperatura de extração, número de re-extrações, condição estática ou

agitada e velocidade do sistema agitado (IKASARI & MITCHELL, 1996; SINGH *et al.,* 1999; TUNGA *et al*., 1999; CASTILHO *et al.*, 2000; AIKAT *et al.,* 2000).

Segundo SINGH *et al.* (1999), o solvente ideal extrai a enzima seletivamente e completamente, à temperatura ambiente, com o mínimo tempo de contato e preferencialmente no pH do substrato sólido de cultivo.

Muitas estratégias têm sido eficientemente empregadas para aumentar a extração de enzimas. No entanto, a otimização das condições durante o processo de extração parece ser uma das mais promissoras (IKASARI & MITCHELL, 1996; TUNGA *et al*., 1999; CASTILHO *et al.*, 2000; REDDY *et al.,* 2000).

REDDY *et al.* (2000) para a otimização da recuperação da pululanase produzida por *Clostridium thermosulfurogenes* SV2 em CES realizaram um planejamento fatorial completo com os seguintes fatores: relação soluto/solvente, temperatura de extração, pH do solvente, velocidade de agitação e tempo de contato. Entre as cinco variáveis estudadas, a relação soluto/solvente, o pH do solvente e a velocidade de agitação foram identificadas como significativas através da MSR. Os níveis identificados como ótimos para recuperação da pululanase termoestável de *Clostridium thermosulfurogenes* SV2 foram: soluto/solvente 1:9, pH 6 e 200 rpm.

CASTILHO *et al*. (2000) investigaram a melhor combinação de temperatura de extração, tempo de extração e tipo de solvente, a fim de otimizar as operações de recuperação de pectinases produzidas em CES por *Aspergillus niger*. O tampão acetato, com pH 4,4, a temperatura de extração de 35 °C e o tempo de contato soluto/solvente de 30 minutos permitiram uma melhor recuperação. A partir dos resultados obtidos, os autores concluíram que a otimização do processo de extração de enzimas produzidas em CES é uma forma simples de obter extratos enzimáticos mais concentrados.

Segundo FERNÁNDEZ-LAHORE *et al.* (1998), água como solvente, relação soluto/solvente de 1:10, temperatura de extração de 25 °C, agitação de 220 rpm e tempo de contato soluto/solvente de 45-60 minutos foram as condições ideais para a extração de protease produzida por *Mucor bacilliformis* em CES.

AIKAT *et al.* (2000) estudaram o efeito de repetidas extrações sobre a recuperação de protease produzida em CES por *Rhizopus oryzae*, usando farelo de trigo como substrato. Segundo os autores, na segunda extração foi obtida menos que 10 % da atividade total recuperada na primeira extração.

3. OBJETIVOS

3.1. Objetivo geral

Este trabalho teve por objetivo contribuir para o desenvolvimento da produção em processos de cultivo submerso e estado sólido e a caracterização de transglutaminases de origem microbiana.

3.2. Objetivos específicos

- Desenvolver um meio de cultura para a produção em cultivo submerso da transglutaminase pelo microrganismo *Bacillus circulans* BL32, visando maximizar a atividade enzimática e minimizar o custo do meio de cultura.

- Estudar em sistema submerso a influência dos parâmetros de cultivo, como temperatura e pH, visando otimizar a produção da transglutaminase.

- Avaliar em processo de cultivo submerso a influência de parâmetros como taxa de aeração e agitação, através do coeficiente de transferência volumétrica de oxigênio, visando minimizar o tempo de bioprocesso e viabilizar a produção dessa enzima em larga escala.

- Estudar a viabilidade e eficiência de produção desta enzima em sistema de cultivo no estado sólido, através do emprego de diferentes substratos.

- Avaliar o efeito de parâmetros de processo, como aeração, temperatura e concentração do inóculo, para a produção da transglutaminase pelo isolado *Bacillus circulans* BL32 em sistema de cultivo no estado sólido.

- Desenvolver metodologias reprodutíveis de otimização do processo de extração da enzima produzida em cultivo no estado sólido.

- Caracterizar a enzima parcialmente purificada, no que se refere ao pH e temperatura de atuação, a estabilidade térmica, ao efeito de sais sobre a atividade enzimática, a toxicidade e a atuação sobre sistemas protéicos alimentares.

- Estudar a cinética de inativação térmica da enzima purificada, bem como o efeito de sais sobre esse processo.

4. MATERIAIS E MÉTODOS

4.1. Instalações e reagentes

O trabalho foi realizado em sua maior parte nos Laboratórios de Biotecnologia I (Laboratório 212) e Biotecnologia II (Laboratório 216), do Instituto de Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul.

Todos os reagentes químicos utilizados nas diversas etapas desse trabalho foram do melhor padrão analítico disponível nos laboratórios.

A água utilizada na preparação de tampões e soluções era de qualidade ultra pura proveniente de um sistema de purificação da marca TKA, modelo LAB – UPW.

4.2. Instrumentação e equipamentos

Todos os equipamentos, meios de cultura e vidrarias foram esterilizados em autoclave vertical modelo AV-75 da marca Phoenix Equipamentos.

O cultivo do microrganismo em frascos Erlenmeyer realizou-se em incubadora do tipo “shaker” com agitação orbital e temperatura controlada da marca Nova Técnica.

As leituras espectrofotométricas foram feitas em aparelhos modelo U-1100 da marca Hitachi e modelo Ultrospec 3100 da marca Amersham Biosciences.

As determinações de pH foram realizadas em potenciômetro modelo NT pH da marca Nova Técnica.

As amostras foram concentradas em um sistema de evaporação por centrifugação modelo Centrivap combinado com bomba de vácuo e resfriamento modelo Cold Trap da marca Labconco Corporation.

As centrifugações foram realizadas em microcentrífuga com velocidade fixa de 14.000 rpm (12.800 *g*) modelo 5410 da marca Eppendorf e para volumes maiores foi usada a centrífuga modelo Himaq CR21E da marca Hitachi, empregando um dos seguintes rotores: R12A3 (6 x 250 mL), R22A2 (6 x 35 mL) e R21A (10 x 12 mL).

Para a purificação da transglutaminase nas etapas de cromatografia líquida utilizou-se um sistema composto por bomba peristáltica modelo P-1 Peristaltic Pump da marca Amersham Pharmacia Biotech e um coletor de frações modelo 2128 Fraction Collector da marca Bio-Rad Laboratories.

4.3. Microrganismo

A linhagem de microrganismo utilizada neste trabalho *Bacillus circulans* BL32 faz parte de uma coleção de microrganismos isolados em ambientes da bacia amazônica pelo Professor Spartaco Astolfi-Filho, do Instituto de Ciências Biológicas da Universidade Federal do Amazonas. O isolado foi selecionado em trabalho anterior (SOARES *et al*., 2003a). A cultura mantida em glicerol foi recuperada em placa de meio Mueller-Hinton e repicada uma vez por semana.

4.4. Produção de transglutaminase em cultivo submerso

4.4.1 Meio de cultivo submerso

A composição do meio de cultivo celular empregado nos bioprocessos submersos foi otimizada no item Resultados I. Esse é composto de glicerol (9 g/L), sacarose (2 g/L), peptona (7 g/L), triptona (1 g/L), Na_2HPO_4 (1 g/L), $MgSO_4.7H_2O$ (1 g/L) e $FeSO_4.7H_2O$ (0,1 g/L). Adicionou-se antiespumante (0,005 %) antes da esterilização por 20 minutos a 121 ºC em autoclave. Os cultivos iniciaram quando da adição de 5 % do volume útil total de um inóculo elaborado a partir do mesmo meio acima citado.

4.4.2. Condições de cultivo dos bioprocessos submersos

Os cultivos submersos foram realizados em biorreatores "Biostat B" (B.Braun Biotech International) de 2 L, possuindo 4 defletores internos e com agitação fornecida por duas turbinas do tipo "Rushton" no eixo central.

Os biorreatores possuem ajuste automático de temperatura, aeração e agitação; o pH e o antiespumante são controlados por bombas peristálticas integradas à unidade central. A otimização do pH e temperatura de cultivo para a produção de transglutaminase pelo *B. circulans* BL32 nos cultivos submersos será apresentada no item Resultados II. Já a determinação das melhores condições de aeração e agitação será descrita no item Resultados III.

4.5. Produção de transglutaminase em cultivo no estado sólido

4.5.1. Cultivo em biorreatores cilíndricos verticais estáticos

Todos os cultivos em estado sólido foram realizados em biorreatores cilíndricos verticais estáticos (BCVE) de fluxo ascendente com capacidade de 500 mL (60 mm de diâmetro x 170 mm de altura), desenhados em nosso laboratório. O corpo do biorreator, conforme Figura 2, é feito de vidro e o fundo é composto de vidro sinterizado (de porosidade G1). Na parte superior encaixa-se uma rolha de borracha com filtro acoplado para saída do ar e dos gases formados durante o cultivo. Foram montados em conjuntos de quatro unidades com distribuidor de ar central, que recebe ar umidificado e estéril com vazão controlada por rotâmetro. Os biorreatores foram colocados em estufa para permitir o controle de temperatura. As condições de temperatura e aeração empregadas para a produção de transglutaminase nos cultivos em estado sólido serão apresentadas no item Resultados IV. A Figura 3 mostra um conjunto de BCVEs. As zonas claras dentro do biorreator são células bacterianas.

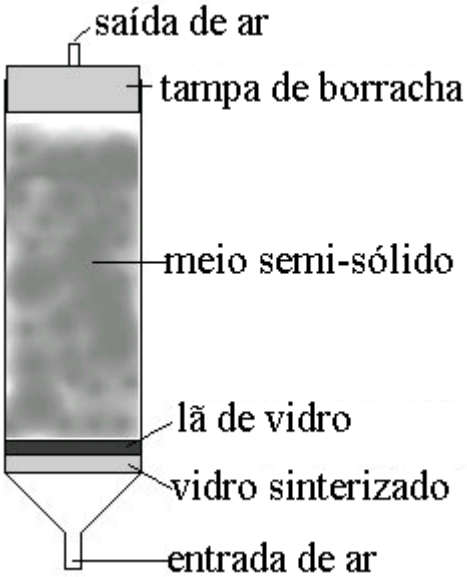

Figura 2. Desenho simplificado de um Biorreator Cilíndrico Vertical Estático.

Figura 3. Biorreatores Cilíndricos Verticais Estáticos.

4.5.2. Meio de cultivo sólido

Para os cultivos no estado sólido empregaram-se diferentes substratos para crescimento celular, conforme será descrito no item Resultados IV.

Preparava-se o meio de cultivo da seguinte forma: em um copo de Becker pesavam-se 20 g de substrato sólido e adicionava-se meio mineral mínimo Bushnell-Hass, num volume correspondente a capacidade máxima de absorção do substrato. Este material era esterilizado em autoclave por 15 minutos a 121 ºC, e, em seguida, adicionavam-se 20 mL de inóculo com diferentes concentrações celulares, conforme será apresentado no item Resultados IV. Por último, a mistura era transferida assepticamente para os biorreatores de cultivo sólido descritos no item anterior.

Meio mineral mínimo Bushnell-Hass: NH_4NO_3 (1 g /L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), $MgSO_4$ (0,2 g/L), $FeCl_2$ (0,03 g/L) e $CaCl_2$ (0,02 g/L).

4.5.3. Extração da enzima

A recuperação da transglutaminase produzida foi feita adicionando-se um determinado volume de água destilada, conforme será apresentado no item Resultados V, ao conteúdo total do biorreator. Esta mistura foi transferida para frascos Erlenmeyer e agitada em placa agitadora orbital. Em seguida o material sólido foi separado da fase líquida por centrifugação a 4 ºC por 15 minutos e 17.000 *g*. O sobrenadante foi recolhido e utilizado como extrato enzimático.

4.6. Purificação da transglutaminase

Quando a máxima produção enzimática era atingida o meio de cultivo era centrifugado a 4 ºC por 20 minutos e 17.000 *g*. A transglutaminase de *B. circulans* BL32 foi purificada segundo procedimento descrito por SOARES *et al*. (2003a).

Primeiramente, procedeu-se a precipitação com 60-90 % (p/v) de sulfato de amônio em temperatura próxima a 0ºC e após diálise contra o tampão Tris-HCl 20 mM pH 8,0, por no mínimo 3 vezes em 2 litros.

Em seguida, a cromatografia de troca iônica foi realizada em coluna empacotada com Q-Sepharose Fast Flow (amônia quaternária, forte trocadora de ânions) e equilibrada com tampão Tris-HCl 20 mM pH 8,0. A eluição da coluna foi feita com gradiente linear crescente de zero a 1,0 M de NaCl. As frações que apresentaram atividade enzimática foram dialisadas contra o mesmo tampão. Essa preparação enzimática, com ausência de proteases, foi utilizada nos experimentos de caracterização da transglutaminase parcialmente purificada, conforme será apresentado nos itens Resultados VI e VII.

A cromatografia de interação hidrofóbica foi realizada em coluna empacotada com Octyl Sepharose 4 Fast Flow e equilibrada com 3,5 M de sulfato de amônio em tampão Tris-HCl 20 mM pH 8,0. As frações recolhidas da etapa anterior receberam adição de sulfato de amônio até atingir 3,5 M e foram aplicadas na coluna. A eluição foi feita com gradiente linear decrescente de 3,5 a 0 M no mesmo tampão. Essa preparação enzimática foi utilizada nos experimentos de caracterização da transglutaminase purificada a homogeneidade, conforme será apresentado no item Resultados VIII.

4.7. Determinações analíticas

4.7.1. Determinação da atividade de transglutaminase

A atividade enzimática foi determinada pela técnica de formação de hidroxamato segundo GROSSOWICZ *et al.* (1950).

Em 400 μL de amostra adicionou-se a mesma quantidade do reagente A. Após homogeneização incubou-se por 10 min a 37 °C e interrompeu-se a reação adicionando 400 μL do reagente B. Em seguida, procedeu-se a leitura da absorbância a 525 nm. A curva padrão foi feita com ácido L-glutâmico-γ-monohidroxâmico. Uma unidade de atividade enzimática (U) foi definida como a quantidade de enzima capaz de formar 1,0 μmol de hidroxamato por minuto nas condições do ensaio.

Reagente A: Tampão Tris-HCl 200 mM contendo 100 mM de hidroxilamina, 50 mM de $CaCl_2$, 10 mM de glutationa reduzida e 30 mM de CBZ-Gln-Gly.

Reagente B: solução 1:1:1 (v/v/v) de HCl 3 N; ácido tricloroacético 12 %, $FeCl_3.6H_2O$ 5 % dissolvido em HCl 0,1 N.

4.7.2. Dosagem de proteína solúvel

A proteína solúvel em todas as amostras foi determinada pelo método proposto por LOWRY *et al*. (1951).

4.7.3. Dosagem de açúcares redutores

Os açúcares redutores totais foram quantificados pelo método do ácido 3,5-dinitrosalicílico conforme MILLER (1959).

4.7.4. Determinação da atividade proteolítica

A atividade proteolítica foi determinada pelo método adaptado de SARATH *et al*. (1989) com azocaseína como substrato.

Em tubos de microcentrífuga contendo 250 μL de uma solução-substrato com 2 % de azocaseína em tampão fosfato de sódio 50 mM e pH 7,0 adicionaram-se 150 μL de extrato enzimático. A mistura foi incubada a 40 ºC por 40 minutos e no final deste período adicionou-se ácido tricloroacético (TCA) a 10 % em cada tubo. Para cada amostra enzimática preparou-se um branco, misturando, nesta ordem, enzima, TCA e substrato azocaseína. Após 15 minutos de repouso centrifugaram-se os tubos a 14000 *g* por 5 minutos. Transferiu-se 1 mL do sobrenadante para um tubo de ensaio contendo 1 mL de hidróxido de sódio 1,0 M. A absorbância desta solução foi determinada a 440 nm em espectrofotômetro.

Uma unidade de atividade enzimática (U) foi definida como a quantidade de enzima necessária para produzir, em cubeta de 1 cm, a variação de uma unidade na absorbância, sob as condições do método.

4.7.5. Dosagem de nitrogênio amino

A quantidade de grupos amino livres foi determinada pela técnica proposta por ADLER-NISSEN (1979) que faz uso do ácido trinitrobenzenosulfônico (TNBS). O procedimento está descrito em detalhes em Resultados VI.

4.7.6. Capacidade de formação de emulsão

A atividade emulsificante e a estabilidade da emulsão formada foram determinadas de acordo com PIERCE & KINSELLA (1978). Os detalhes da técnica e as modificações estão descritas em Resultados VI.

4.8. Análises estatísticas

Nas análises estatísticas dos experimentos empregaram-se os *software* Statistic 5.0 e 7.0.

5. RESULTADOS E DISCUSSÃO

Os resultados deste trabalho serão apresentados na forma de artigos científicos, formato este que é altamente incentivado pelo PPGBCM para organização da tese. Os referidos artigos estão apresentados nos itens Resultados I, II, III, IV, V, VI, VII e VIII.

No primeiro artigo (Resultados I - "Optimization of medium composition for the production of transglutaminase by *Bacillus circulans* BL32 using statistical experimental methods") foi determinada a composição de um meio de cultura para a produção de transglutaminase pelo isolado *Bacillus circulans* BL32 em cultivo submerso. Este artigo já está publicado - Process Biochemistry, 41:1186-1192, 2006.

No segundo artigo (Resultados II - "Environmental effects on transglutaminase production and cell sporulation in submerged cultivation of *Bacillus circulans*") foram determinadas as melhores condições de cultivo, tais como pH e temperatura, para a produção de transglutaminase pelo isolado *Bacillus circulans* BL32 em cultivo submerso. Este artigo está aceito para publicação - Applied Biochemistry and Biotechnology, no prelo.

No terceiro artigo (Resultados III - "Effects of oxygen volumetric mass transfer coefficient on transglutaminase production by *Bacillus circulans* BL32") foram determinadas as melhores condições de aeração e agitação, avaliadas através do coeficiente de transferência volumétrica de oxigênio (k_La), para a produção de transglutaminase pelo isolado *Bacillus circulans* BL32 em cultivo submerso. Este artigo foi submetido para publicação no periódico Biotechnology and Bioprocess Engineering em abril de 2008.

No quarto artigo (Resultados IV - "Solid state bioreactor production of transglutaminase by Amazonian *Bacillus circulans* BL32 strain") foram determinadas as melhores condições de cultivo, tais como temperatura, aeração e concentração do inóculo, para produção de transglutaminase pelo *Bacillus circulans* BL32 em cultivo no estado sólido. Este artigo está aceito para publicação - Journal of Industrial Microbiology and Biotechnology, no prelo.

O quinto artigo (Resultados V - "Optimization of transglutaminase extraction produced by *Bacillus circulans* BL32 on solid-state cultivation") trata do estudo das condições de extração da transglutaminase produzida em cultivo no estado sólido pelo *Bacillus circulans* BL32. Este artigo está aceito para publicação – Journal of Chemical Technology and Biotechnology, 83:1306-1313, 2008.

O sexto artigo (Resultados VI - "Enzymatic properties of transglutaminase from a newly isolated *Bacillus circulans* BL32") trata da caracterização da transglutaminase produzida pelo *Bacillus circulans* BL32, no que se refere à temperatura, pH, estabilidade térmica, efeitos de sais sobre a atividade enzimática e a aplicação em diferentes proteínas alimentares. Este artigo foi submetido para publicação no periódico Journal of the Science of Food and Agriculture em julho de 2008.

O sétimo artigo (Resultados VII – "Toxicological evaluation of transglutaminase from a newly isolated *Bacillus circulans* BL32") relata a caracterização *in vivo* e *in vitro* da transglutaminase de *Bacillus circulans* BL32 em relação a alguns aspectos toxicológicos. Este artigo foi submetido para publicação no periódico Food Control em julho de 2008.

O oitavo artigo (Resultados VIII – "Kinetics of thermal inactivation of transglutaminase from a newly isolated *Bacillus circulans* BL32") trata da modelagem e dos parâmetros termodinâmicos da cinética de inativação térmica da transglutaminase de *Bacillus circulans* BL32. Este artigo foi submetido para publicação no periódico Journal of Biotechnology em julho de 2008.

5.1. RESULTADOS I. "Optimization of medium composition for the production of transglutaminase by *Bacillus circulans* BL32 using statistical experimental methods" - Process Biochemistry, 41:1186-1192, 2006.

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Process Biochemistry 41 (2006) 1186–1192

Process Biochemistry

www.elsevier.com/locate/procbio

Optimization of medium composition for the production of transglutaminase by *Bacillus circulans* BL32 using statistical experimental methods

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Received 5 October 2005; received in revised form 2 December 2005; accepted 20 December 2005

Abstract

Medium composition for transglutaminase (MTGase) production by *Bacillus circulans* BL32, a recently isolated strain from the Amazon basin, was optimized using a stepwise strategy. Effects of carbon, nitrogen and mineral sources were investigated by screening experiments. Plackett–Burman (P–B) statistical design was applied to find the key ingredients for the best medium composition among glycerol, sucrose, peptone, tryptone, Na_2HPO_4, $MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$. Results indicated that sucrose, negatively, and peptone, positively, have significant effects on MTGase production, with confidence levels above 90%. Central composite design (CCD) was used to determine the optimal concentrations of these two components and the experimental results were fitted with a second-order polynomial model at 90% level ($P < 0.10$). Under the proposed optimized conditions, the model predicted the MTGase activity of 0.280 U/mL, very closely matching experimental activities of 0.306 U/mL.

Keywords: *Bacillus circulans* BL32; Medium optimization; Microbial transglutaminase; Plackett–Burman design; Response surface methodology

1. Introduction

Transglutaminase (TGase; protein–glutamine γ-glutamyl-transferase; EC 2.3.2.13) is an enzyme capable of catalysing acyl transfer reactions by introducing covalent cross-links between proteins, as well as peptides and various primary amines [1,2]. Recent developments on the production and uses of this enzyme are on the increase due to its attractive applications in the food industries [2], pharmaceuticals [3], textiles [4], and in the formation of immunoconjugates for application in ELISA tests and biosensors [5]. Until recently, guinea-pig liver was the only source of commercial TGase. The scarce source and the complicated separation and purification process for obtaining tissue TGase have resulted in an extremely high price of these enzymes [6] making its use prohibitive in food processing and other low-priced products. In the last few years efforts are been made to search for microbial transglutaminases, to modify downstream processing and improve fermentation processes in order to boost productivity and reduce production costs [6–10].

In biotechnology-based industrial processes, the formulation of cultivation media is of critical importance because their composition affects product concentration, yield and volumetric productivity. It is also important to reduce the costs of the medium as this may affect the overall process economics.

Cultivation media studies that use conventional single factor optimization, which is done by maintaining all but one of the variables involved unchanged at a time, does not allow for the interaction of these variables with each other. This method is also time consuming and require large numbers of experiments to be carried out in order to determine the optimal level of each factor [11]. The response surface methodology (RSM) is a time saving method, which reveals the interactions that occur among the components of the medium [12]. Therefore, RSM is a good sense application of statistical techniques for designing experiments, building models, evaluating the effects of several factors in order to obtain high productivity in bioprocesses. Although the successful applications of RSM in several

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doi:10.1016/j.procbio.2005.12.019

biotechnological projects [13–17], there are only a few reports on media optimization for the production of MTGase [18,19].

For screening of more than five factors, Plackett–Burman (P–B) statistical design is recommended. This technique cannot determine the exact amount, but can provide indications and tendencies regarding the necessity of each factor needing relatively few experiments [20]. When optimization is required, a process optimization tool, such as RSM, whereby the optimum values of the factors studied are determined, follows P–B. These two methods are used to statistically optimize processes [20–26]. However, there are no reports on the literature of MTGase medium definition using P–B statistical design.

The aim of this work was to optimize the medium to maximize MTGase volumetric production by *Bacillus circulans* BL32, a recently isolated strain from the Amazon environment [27]. A stepwise optimization was performed including: (1) screening carbon, nitrogen and mineral sources; (2) elucidation of the medium component that significantly affect MTGase production using a P–B statistical design; (3) RSM optimization of these significant ingredients by central composite design (CCD).

2. Material and methods

2.1. Microorganism

A *B. circulans* strain, coded BL32, isolated from Amazon environment [27], was used in this study. The stock culture was maintained at 4 °C on Mueller–Hinton agar plate (infusion from meat, 2 g/L; casein hydrolysate, 17.5 g/L; starch, 1.5 g/L; agar, 13 g/L) and was monthly sub cultured.

2.2. Inocula preparation

Erlenmeyer flasks (250 mL) filled with 50 mL of medium composed of peptone, 10 g/L, yeast extract, 5 g/L, and NaCl, 5 g/L, were inoculated with a single colony from a stock culture and incubated at 30 °C in a rotatory shaker at 100 rpm and grown to optical density (OD) of 1.0 at 600 nm (approximately 20 h). This culture procedure was then used as the standard inocula preparation for all experiments.

2.3. Cultivation procedures

An amount of 2.5 mL of the standard inocula were transferred to 250 mL Erlenmeyer flasks containing 50 mL of different production media. The composition of media varied according to the experimental design described in this work. The initial pH of media was adjusted to 6.5 and the media were sterilized by autoclaving at 121 °C for 15 min. Cultivations were conducted at 30 °C in a rotatory shaker at 100 rpm. Samples were collected for analysis at the 8th day of growth since this was the highest enzyme activity point. All experiments were carried out in triplicates.

2.4. Biomass and enzyme assays

Samples of 10 mL the culture broth were centrifuged at 3500 × *g* for 20 min at 4 °C. The cell-free supernatant was used for the estimation of enzyme activity and for pH determinations using a standard lab pHmeter.

MTGase activity was determined by the hydroxamate procedure [28]. A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One enzymatic unit (U) causes the formation of 1 μmol hydroxamic acid per minute at 37 °C. Enzyme was expressed as volumetric activity (U/mL medium).

Biomass was quantified gravimetrically as dry weight of cells. Samples were centrifuged, twice washed with cold distilled water, and dried in pre-weighed plastic tubes at 80 °C to a constant weight in vacuum-ovens.

2.5. Optimization procedure and experimental design

2.5.1. Pre-selection of carbon, nitrogen and mineral sources

Seven different carbon sources at concentration of 20 g/L (soy fiber, soluble starch, sucrose, lactose, fructose, glucose and glycerol), six sources of nitrogen (peptone, meat peptone, yeast extract, meat extract, tryptone and urea) at concentrations of 20 g/L (also 5 g/L for urea), and seventeen minerals (NaCl, $CaCl_2$, NH_4Cl, Na_2SO_4, $ZnSO_4{\cdot}7H_2O$, K_2SO_4, $FeSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $MgSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$, Na_2HPO_4, $(NH_4)_2HPO_4$, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, NH_4NO_3 and $NaNO_3$), 2 g/L for all (also 0.2 g/L for $ZnSO_4{\cdot}7H_2O$ $7H_2O$ and $CuSO_4{\cdot}5H_2O$) were pre-tested. In the investigation of carbon sources, cultivations were carried out with media containing KH_2PO_4, 2 g/L; K_2HPO_4, 2 g/L; $MgSO_4$, 2 g/L, supplemented with 20 g/L of peptone. In the screening of nitrogen sources, cultivation was carried out with media containing the same salts above mentioned supplemented with the optimal carbon sources, while for the investigation of mineral sources, cultivation media contained the optimal carbon and nitrogen sources (see results). In all experiments, MTGase activity, dry cell weight and final pH were determined. All experiments were performed in triplicates for the mean calculation.

Table 1
Plackett–Burman experimental design matrix with MTGase production

Trial no.	Variables[a]/levels[b]											MTGase activity (U/mL)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	D_1	D_2	D_3	D_4	
1	+	−	+	−	−	−	+	+	+	−	+	0.081
2	+	+	−	+	−	−	−	+	+	+	−	0.000
3	−	+	+	−	+	−	−	−	+	+	+	0.221
4	+	−	+	+	−	+	−	−	−	+	+	0.081
5	+	+	−	+	+	−	+	−	−	−	+	0.000
6	+	+	+	−	+	+	−	+	−	−	−	0.022
7	−	+	+	+	−	+	+	−	+	−	−	0.189
8	−	−	+	+	+	−	+	+	−	+	−	0.143
9	−	−	−	+	+	+	−	+	+	−	+	0.022
10	+	−	−	−	+	+	+	−	+	+	−	0.013
11	−	+	−	−	−	+	+	+	−	+	+	0.015
12	−	−	−	−	−	−	−	−	−	−	−	0.123

[a] X_1 sucrose at a high concentration of 15 g/L and a low concentration of 3 g/L; X_2 glycerol at a high concentration of 15 g/L and a low concentration of 3 g/L; X_3 peptone at a high concentration of 15 g/L and a low concentration of 3 g/L; X_4 tryptone at a high concentration of 2 g/L and a low concentration of 0.2 g/L; X_5 Na_2HPO_4 at a high concentration of 2 g/L and a low concentration of 0.2 g/L; X_6 $MgSO_4{\cdot}7H_2O$ at a high concentration of 2 g/L and a low concentration of 0.2 g/L; X_7 $FeSO_4{\cdot}7H_2O$ at a high concentration of 1 g/L and a low concentration of 0.1 g/L; D_1, D_2, D_3 and D_4 are dummy variables.

[b] (+) High concentration of variable; (−) low concentration of variable. The results are the mean of three replications.

Table 2
Independent variables and the concentration levels studied in CCD

Variable	Components	Levels of variables (g/L)				
		−1.41	−1	0	+1	+1.41
X_1	Sucrose	1	2	5	8	9
X_3	Peptone	3	7	16.5	26	30

2.5.2. Identification of important nutrient components

To determine which nutrients had a significant effect on MTGase production, P–B design was used [26], with the designed matrix shown in Table 1. In our work, seven variables and four dummy variables were screened in 12 trials. Each row represents a trial and each column represents an independent or dummy variable. All experiments were performed in triplicates for the mean calculation. The mean of the MTGase production obtained was taken as the response. Variables with confidence levels >90% were considered to influence MTGase production significantly.

2.5.3. Optimization of key ingredients

The optimization of key components concentration to maximize the MTGase activity was determined with a CCD. The variables and the coded and uncoded values of the variables at various levels are given in Table 2. Table 3 shows 12 trials of the two variables, each at five levels. The design was made up of a central composite design ($k = 2$) with four replications of the central points (all factors at level 0) and the four star points, that is, points having for one factor an axial distance to the centre of $\pm\alpha$, whereas the other factor is at level 0. The axial distance α was chosen to be 1.41 to make this design orthogonal. In each case, the MTGase activity was determined. All experiments were performed in triplicates for the mean calculation. The mean value of the MTGase production obtained was taken as the response. Quadratic equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (1)$$

where Y is the response variable, β_0 the constant, β_i the coefficient for the linear effect, β_{ii} the coefficient for the quadratic effect, β_{ij} the coefficient for the interaction effect, and x_i and x_j the coded level of variable X_i and X_j. The above quadratic equation was used to plot surfaces for the variables.

The test factors were coded according to the following regression equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i}\right) \quad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the ith independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value.

Table 3
Experimental design and results of CCD

Trial no.	Variables/levels		MTGase activity (U/mL)	
	Sucrose (x_1)	Peptone (x_3)	Experimental	Predicted
1	−1	−1	0.311	0.280
2	−1	1	0.076	0.083
3	1	−1	0.284	0.249
4	1	1	0.112	0.114
5	−1.41	0	0.173	0.203
6	1.41	0	0.205	0.203
7	0	−1.41	0.235	0.276
8	0	1.41	0.054	0.042
9	0	0	0.175	0.180
10	0	0	0.171	0.180
11	0	0	0.182	0.180
12	0	0	0.194	0.180

The results are the mean of three replications. $x_1 = (X_1 - 5)/3$; $x_3 = (X_3 - 16.5)/9.5$, x_1 and x_3 are coded values; X_1 and X_3 are the actual values; X_1 = sucrose (g/L); X_3 = peptone (g/L).

2.6. Time course of MTGase production

To confirm the above predictions, further experiments were performed comparing the optimized medium in this work and a commonly, non-optimized medium used for the production of MTGase by *Streptoverticillium mobaraense* [7], also previously used by us to study MTGase by *B. circulans* BL32 [27]: starch 20 g/L; peptone 20 g/L; yeast extract 2 g/L; KH_2PO_4 2 g/L; K_2HPO_4 2 g/L; $MgSO_4$ 2 g/L.

Inoculation was done by transferring 20 mL of the standard inoculum to 2 L Erlenmeyer flasks containing 400 mL of production medium. The initial pH of media was adjusted to 6.5. The media were sterilized by autoclaving at 121 °C for 15 min. Cultivations were conducted at 30 °C in a rotatory shaker at 100 rpm for 10 days. Samples were periodically removed and MTGase activity and dry cell weight were determined as described above. All the experiments were carried out in triplicates.

2.7. Data analysis

All statistical experimental designs and results analyses were carried out using Statistica 5.0 software (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities $p(t)$ were determined by Student's t-test; the second order model equation significance was determined by Fisher's F-test. The variance explained by the model is given by the multiple determination coefficient, R^2. For each variable, the quadratic models were represented as contour plots (2D).

3. Results and discussion

3.1. Screening of carbon, nitrogen and mineral sources

3.1.1. Effect of carbon sources on MTGase production

Fig. 1(A) shows the effect of different carbon sources on *B. circulans* BL32 growth and MTGase production. Glycerol, sucrose and soluble starch were the best carbon sources for MTGase production: enzyme activity reached 0.194, 0.183 and 0.162 U/mL, respectively, after 8 days of cultivation. Interestingly, glycerol allowed for the highest biomass formation of 2.19 g/L, while sucrose or soluble starch did not stimulate growth. Lactose, fructose and glucose produced low activities of MTGase. Except for the medium with glucose, pH increased after 8 days of cultivation (data not shown). Although not previously reported for *Bacillus*, Junqua et al. [18], working with several carbon sources, found that MTGase activity of *Streptoverticillium cinnamoneum* was only detectable when the medium contained either glycerol (0.019 U/mL) or glucose (0.015 U/mL), while sucrose produced no activity at all. Téllez-Luis et al. [19], working with a strain of *S. ladakanum*, also found that glycerol had the strongest effect on transglutaminase activity. These findings, in accordance with our results, may suggest a common pathway among these bacteria on the synthesis of MTGase. Glycerol and sucrose were then chosen as the carbon sources for further experiments.

3.1.2. Effect of nitrogen sources on MTGase production

The effect of nitrogen sources on the MTGase production by *B. circulans* BL32 was investigated using glycerol (15 g/L) and sucrose (5 g/L) combined as carbon source. The results of MTGase activity and biomass are shown in Fig. 1(B).

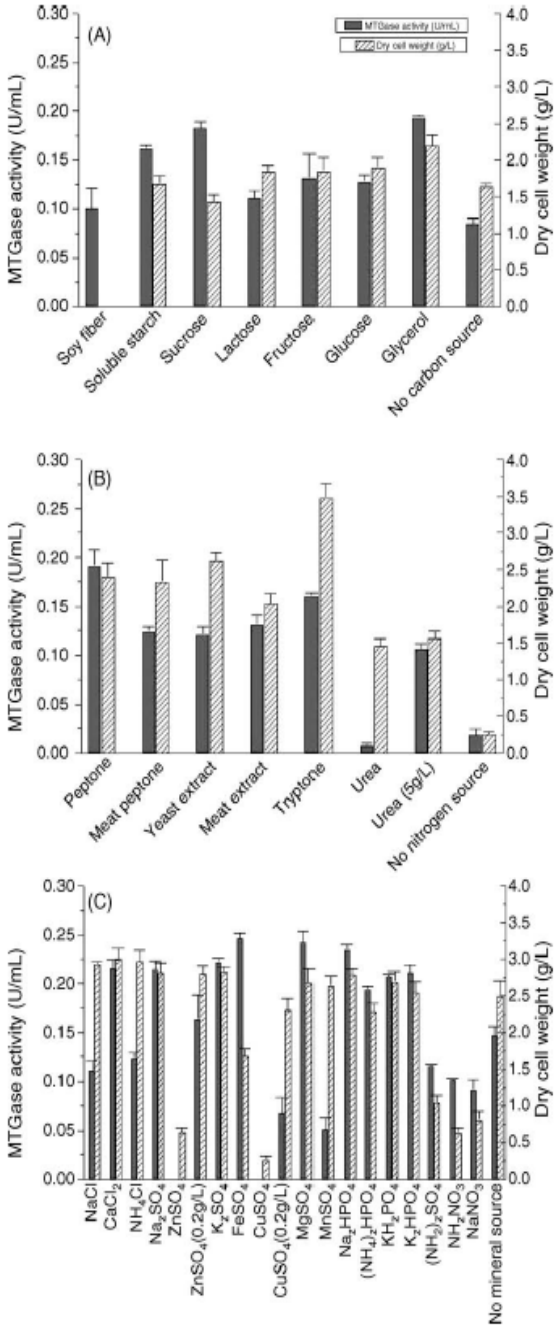

Fig. 1. Effect of different carbon (A), nitrogen (B) and mineral (C) sources on MTGase production and dry cell weight. The results are the mean of triplicates. Initial pH is 6.5. Incubation at 30 °C in a rotatory shaker at 100 rpm for 8 days, 50 mL medium containing: (A) peptone 20 g/L; KH_2PO_4 2 g/L; K_2HPO_4 2 g/L; $MgSO_4$ 2 g/L and the carbon source to be investigated; (B) glycerol 15 g/L; sucrose 5 g/L; KH_2PO_4 2 g/L; K_2HPO_4 2 g/L; $MgSO_4$ 2 g/L and the nitrogen source to be investigated; (C) glycerol 15 g/L; sucrose 5 g/L; peptone 15 g/L; tryptone 5 g/L and the mineral source to be investigated.

The highest MTGase activity (0.191 U/mL) was obtained with peptone, while tryptone produced the highest biomass (3.48 g/L) with MTGase activity of 0.161 U/mL. When meat peptone, yeast extract and meat extract were used, MTGase production was low, while urea had a poor effect on growth and MTGase activity. Zhu et al. [9] tested the effect of nitrogen sources on MTGase activity of *S. mobaraense* and found that inorganic nitrogen sources and free amino acids failed to induce MTGase synthesis, suggesting that certain peptides and some amino acids present in complex nitrogen sources, such as peptone, are necessary in the metabolic pathway of this enzyme, much in the same way found for *S. cinnamoneum* [18]. Peptone and tryptone were thus chosen as the nitrogen sources for further experiments.

3.1.3. Effect of mineral sources on MTGase production

The effect of minerals on the production of MTGase was also investigated using glycerol (15 g/L) and sucrose (5 g/L) as carbon sources and peptone (15 g/L) and tryptone (5 g/L) as nitrogen sources. The results of MTGase activity and cell growth are shown in Fig. 1(C). $FeSO_4{\cdot}7H_2O$, $MgSO_4{\cdot}7H_2O$ and Na_2HPO_4 were the best mineral sources for MTGase production, with activity reaching 0.246, 0.242 and 0.235 U/mL, respectively, after 8 days of cultivation. MTGase production with the addition of NaCl, NH_4Cl, $ZnSO_4{\cdot}7H_2O$, $7H_2O$, $CuSO_4{\cdot}5H_2O$, $MnSO_4{\cdot}H_2O$, $(NH_4)_2SO_4$, NH_4NO_3 or $NaNO_3$ was found to be low when compared with not supplemented medium. Therefore, the following minerals were chosen for further studies: Na_2HPO_4, $MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$. In comparison, the optimized medium proposed for the production of MTGase by *S. cinnamoneum* contained $MgSO_4{\cdot}7H_2O$ (0.5 g/L), KH_2PO_4 (2 g/L) and Na_2HPO_4 (5 g/L) [18].

3.2. Plackett–Burman experimental design

Plackett–Burman was designed to evaluate the impact of sucrose, glycerol, peptone, tryptone, Na_2HPO_4, $MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$, in the MTGase production by *B. circulans* BL32. Table 1 represents the Plackett–Burman experimental design for 12 trials with two levels of concentrations for each variable and corresponding MTGase activity. The variables X_1 to X_7 represent media constituents and D_1 to D_4 represents the dummy or unassigned variables. MTGase activity varied markedly in a range of 0–0.221 U/mL with different levels of the components in the media. This variation reflected the importance of optimization to attain higher production. The lowest values of MTGase activity were obtained when the maximal level of sucrose and the minimal level of peptone were used. Table 4 shows the statistical analysis of the studied variables on MTGase production. The effect of variables X_1 (sucrose), X_2 (glycerol), X_3 (peptone), X_4 (tryptone), X_5 (Na_2HPO_4), X_6 ($MgSO_4{\cdot}7H_2O$) and X_7 ($FeSO_4{\cdot}7H_2O$) were found to be −0.086, −0.003, 0.094, −0.007, −0.011, −0.038, −0.005, respectively. As can be seen from Table 4, the factor sucrose (X_1) and the peptone (X_3) were found to be significant at the level of 90% for MTGase production. The main effects of these variables were negative (X_1) and positive (X_3), respectively. The other components in the media showed no significant influence MTGase production.

Table 4
Effect estimates for MTGase production from the result of Plackett–Burman design

Factors	Medium components	Effect	S.E.	*t*-value	*P*-value	Confidence level (%)
X_1	Sucrose	−0.086	0.0317	−2.7130	0.0534	94.66
X_2	Glycerol	−0.003	0.0317	−0.0841	0.9370	6.30
X_3	Peptone	0.094	0.0317	2.9654	0.0413	95.87
X_4	Tryptone	−0.007	0.0317	−0.2103	0.8437	15.63
X_5	Na_2HPO_4	−0.011	0.0317	−0.3575	0.7388	26.12
X_6	$MgSO_4{\cdot}7H_2O$	−0.038	0.0317	−1.1883	0.3005	69.95
X_7	$FeSO_4{\cdot}7H_2O$	−0.005	0.0317	−0.1472	0.8901	10.99

3.3. Central composite designs (CCD)

Based on the results of P–B design (Table 1), glycerol, tryptone, Na_2HPO_4, $MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$ were found to be non-significant. Therefore, for the next step of optimization, the concentrations of glycerol, tryptone, Na_2HPO_4 and $MgSO_4{\cdot}7H_2O$ were set to approximately their mean level; meanwhile $FeSO_4{\cdot}7H_2O$ was set to its lower level. The variables showing confidence level above 90% in the P–B design (sucrose, x_1, and peptone, x_3) were selected and their concentrations were further optimized using CCD. Table 3 represents the experimental design with the results obtained and predicted by model for MTGase production.

MTGase activity varied from 0.054 to 0.311 U/mL according to the different levels of the components in the medium. The lowest MTGase activity was observed for the highest peptone concentration (30 g/L), while the highest activities of 0.311 and 0.284 U/mL were obtained with low peptone concentrations (7 g/L). These results suggest that the concentration of peptone strongly affects the MTGase production by *B. circulans* BL32 showing an inverse correlation between peptone concentration and enzyme activity. In fact, when the C:N ratio is considered, it is interesting to note that MTGase activity increases exponentially from 1 to 5 (C:N initial ratio), with no significant variations for ratios above that, suggesting that nitrogen limitation is important for enzyme production, probably by stimulating the sporulation process of cells (results not shown). The highest activity (0.311 U/mL) experimentally obtained according to the CCD was similar to activities obtained for *S. cinnamoneum* (0.331 U/mL) [18].

Table 5
Coefficient estimates by the regression model for optimization of MTGase production

Independent variables (parameter)	Coefficient (β)	Standard error (β)	*t*-value	*P*-value
Intercept (β_0)	0.1804	0.0050	35.79	<0.0001
x_1	0.0068	0.0036	1.90	0.1535
x_1x_1	0.0116	0.0040	2.91	0.0622[b]
x_3	−0.0830	0.0036	−23.25	0.0002[a]
x_3x_3	−0.0107	0.0040	−2.69	0.0747[b]
x_1x_3	0.0157	0.0050	3.12	0.0523[b]

[a] Statistically significant at 99% of confidence level.
[b] Statistically significant at 90% of confidence level.

The significance of each regression coefficient was determined by *t*-values and *P*-values, which are listed in Table 5. The *P*-values suggest that the negative coefficient for linear effect of peptone, with a value < 0.0002, is the most significant. This confirms that MTGase production decreases with elevated concentration of peptone. The coefficients for quadratic effects of sucrose and peptone had high significance on correlation of coefficients with the low *P*-values < 0.10. The quadratic terms for both sucrose and peptone indicated that they can act as limiting factors and even small increases in their concentrations will significantly increase or decrease MTGase production, respectively [29]. However, their interaction showed a significant effect, which indicates that both factors are required for MTGase production as is shown in Fig. 2. The contour plot indicates that an optimum production is obtained with either a minimum peptone and sucrose concentrations, or high sucrose and low peptone concentrations. Whenever possible, models are simplified by the elimination of statistically insignificant terms. We proposed then, that the quadratic model should be reduced to:

$$Y = 0.1804 - 0.0830x_3 + 0.0116x_1^2 - 0.0107x_3^2 + 0.0157x_1x_3 \quad (3)$$

where Y is the predicted response, and x_1 and x_3 are the coded values of sucrose and peptone, respectively.

For MTGase production, ANOVA shows that the model is very reliable, with R^2 value of 0.9215, indicating good agreement between experimental and predicted values of MTGase production [14]. The model explains 92.15% of the

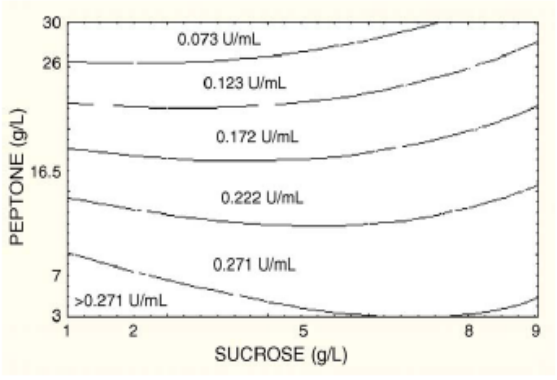

Fig. 2. Contour plot showing the effect of peptone and sucrose on MTGase production by *Bacillus circulans* BL32.

variability in the response. The computed F-value (14.09) was greater than the tabulated F-value, $F_{5,6,0.1} = 3.11$, reflecting the statistical significance of the model equation. This shows that the model as expressed in Eq. (3) provides a suitable model to describe the response of the experiment pertaining to MTGase production.

The final optimized medium composition is that of trial no. 1, Table 3, of the CCD and contains, in g/L: glycerol, 9.0; sucrose, 2.0; peptone, 7.0; tryptone, 1.0; Na_2HPO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 1.0; and $FeSO_4{\cdot}7H_2O$, 0.1.

In order to verify the predicted results, an experiment was performed with the optimized levels of nutrients producing 0.306 U/mL of MTGase (mean result of five repetitions), suggesting that experimental and predicted (0.280 U/mL) values of MTGase production were in good agreement, validating the model.

3.4. Growth kinetics and MTGase activity in batch cultures

The time course of biomass and MTGase production by *B. circulans* BL32 was investigated by the cultivation in two different media: (1) optimized in this work; (2) a classical, non-optimized, medium used for the production of MTGase by *S. mobaraense* [7], which was also previously used for the production of transglutaminase by *B. circulans* BL32 in our laboratory [27]. Fig. 3 shows the results.

B. circulans BL32 grew rapidly reaching stationary phase after 24 h of cultivation in the optimized medium but only after 72 h in the classical medium. Although biomass was higher in the classical medium (1.45 g/L) compared with our optimized medium (0.55 g/L), MTGase production after 192 h of cultivation reached 0.32 U/mL in the second, compared to only 0.20 U/mL in the first, an increase of approximately 60%, further supporting the usefulness of the P–B and CCD as optimization techniques to improve the production of MTGase.

The analysis of results in Fig. 3 further supports the suggestion that the MTGase production of *B. circulans* BL32 is a manifestation of nitrogen and carbon limitation at the onset of death phase. This phenomenon could be explained due to the fact that this enzyme is expressed during sporulation of *B. circulans* [30]. In contrast, Junqua et al. [18], reporting the results for MTGase optimization, found that enzyme activity of *S. cinnamoneum* reached its maximum at the end of exponential phase, which might indicate different mechanisms of sporulation of these two bacteria.

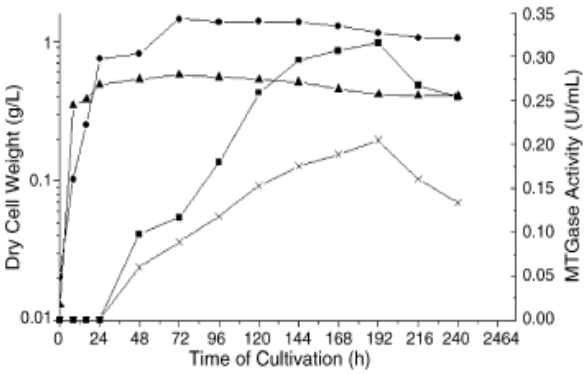

Fig. 3. The time course of production of MTGase by *B. circulans* BL32 cultivated in the optimized medium composition (■) MTGase activity, (π) dry cell weight and in the literature medium, (×) MTGase activity, (●) dry cell weight.

4. Conclusions

In the present study, sucrose and peptone were identified as most influencing components for enhancing MTGase production by using P–B statistical design, and then their optimal concentrations were obtained by using response surface methodology. It was shown that the model was adequate to predict the optimization of MTGase production of *B. circulans* BL32. From the present study, it is evident that the use of P–B statistical design and CCD can be used to determine the significant variables and optimum condition for MTGase production, respectively. The optimization of the medium resulted not only in a 60% higher MTGase activity than media previously cited in the literature but also in a reduction of constituents costs, besides solving the problem of foam production, which is abundantly produced when starch is present in the medium [27]. This is particularly important for scaling-up the cultivation process. The information obtained is considered fundamental and useful for the development of *B. circulans* BL32 cultivation process for efficient production of MTGase on a large scale.

Further studies on the C:N ratio are granted in order to enhance both biomass and MTGase production.

Acknowledgments

The authors wish to thank CNPq, FAPERGS and CAPES for their financial support.

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5.2. RESULTADOS II. “Environmental effects on transglutaminase production and cell sporulation in submerged cultivation of *Bacillus circulans*” - Applied Biochemistry and Biotechnology, *in press*.

Environmental Effects on Transglutaminase Production and Cell Sporulation in Submerged Cultivation of *Bacillus circulans*

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Abstract

In this research, it was investigated the effects of pH, temperature, and oxygen on growth kinetics of a newly isolated strain of *Bacillus circulans* from Amazon and their correlations with transglutaminase (TGase) production and cell sporulation. Statistical experimental methods were used to optimize these parameters, while induction of sporulation was achieved by oxygen culture control. Full factorial composite experimental design and response surface methodology were experimentally tested. The model showed that temperature has a positive and significant effect on TGase production, ($P < 0.05$), while pH and temperature, associated with anoxic conditions, have a marked effect on cell sporulation, which is consistently linked with TGase production. The contour plot of results showed that the best culture conditions for TGase production of *B. circulans* were 30 °C, initial pH 8.5 and the highest production was obtained in late-stationary culture phase, with maximal specific enzyme activity of 655 $U.g^{-1}$ of cells (0.37 U/mL). It was also demonstrated a correlation between enzyme production and cell sporulation, as mediated by oxygen culture conditions and, although demonstrated only for *B. subtilis*, it corroborates the molecular mechanisms involved in this process. It can be suggested that *B. circulans* BL32 is a strong biological system for the industrial production of TGases.

Keywords: *Bacillus circulans* BL32; cell sporulation; culture conditions optimization; microbial transglutaminase; submerged bacterial cultivation.

Introduction

Transglutaminase ([TGase]; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) is an economically important enzyme capable of catalyzing acyl transfer reactions by introducing covalent cross-links between proteins, as well as peptides and various primary amines [1,2]. Recent developments on the production and uses of this enzyme are on the increase due to its attractive applications in the food industries [2], pharmaceuticals [3], textiles [4], and in the formation of immunoconjugates for ELISA tests and biosensors [5]. For long time, non-microbial sources of TGase, especially guinea-pig liver, were the only commercial preparations available of this enzyme. The scarce source and the complicated separation and purification process for obtaining tissue TGase have resulted in an extremely high price for this enzyme [6] making its use prohibitive in food processing and other low-priced products. In the last few years, however, biotechnological research aiming at the production of microbial TGases [7], along with downstream processing developments [8] and improved fermentation processes [6, 9, 10] are on the increase to boost productivity and to reduce separation costs. Notwithstanding, only *Streptoverticillium* species have so far been used for microbial TGase production.

The efforts to improve TGase production has also to take into account the physiological aspects of spore formation. Although not yet elucidated for *B. circulans*, recent reports on the relationship between cell sporulation and TGase production have been well established for *Bacillus subtilis*. Nutrient limitations will trigger *B. subtilis* sporulation, a process in which TGase has a role in the cross-linking of proteins such as GerQ to generate high-molecular-mass proteins involved in the assembly of the spore coating [11, 12].

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of several factors in order to obtain high productivity in several industrial processes. This method has been successfully applied in many areas of biotechnology and bioprocesses, including some studies on optimization of medium to TGase production by *Streptoverticillium* species [13, 14] and *Bacillus circulans* [15]. In biotechnology-based industrial processes, the culture conditions are of critical importance because they affect product concentration, yield and volumetric productivity. In developing an optimal process for commercial production of microbial metabolites, the major aspects usually considered for improvement are effects of

environmental conditions, such as pH and temperature, and the selection of suitable nutrient media. For instance, TGase production by *Streptoverticillium mobaraense* is greatly influenced by physical factors, such as pH [10] and temperature [6], nevertheless the factors were analyzed independently, without an appropriate methodology allowing the overall study of the effects, as well as of their interactions.

In previous works [15, 16], it were investigated the nutritional factors affecting the TGase production by *Bacillus circulans* BL32 in submerged cultivation, and TGase chemical characterization. In this work, it was investigated the effects of temperature, pH and oxygenation of medium, at different incubation times, on cell sporulation and TGase induction.

Materials and methods

Microorganism

A strain of *B. circulans*, coded BL32 was used in this study. It has been recently isolated from the Amazon environment and its isolation and characterization were described elsewhere [15, 16]. This strain is deposited in culture collections of The Federal University of Rio Grande do Sul State (Brazil). Previously to culture, cells were recovered from frozen stocks in 50 % glycerol (v/v) and were kept at 4 °C on Mueller-Hinton agar (Merck) plates.

Inocula preparation

Erlenmeyer flasks (250 mL) containing 50 mL medium (M1), optimized in our previous work [15], composed of ($g.L^{-1}$): glycerol 9.0, sucrose 2.0, peptone 7.0, tryptone 1.0, Na_2HPO_4 1.0, $MgSO_4.7H_2O$ 1.0 and $FeSO_4.7H_2O$ 0.1, were inoculated with a single colony from a stock culture and incubated at 30 °C in a rotatory shaker at 100 rpm and grown to optical density (OD) of 1.0 at 600 nm (approximately 20 h). This culture procedure was then used as the standard inoculum preparation for all experiments.

Cultivation procedures in shaker

The effects of pH and temperature on TGase production were investigated on shaker cultures. The initial pH of medium M1 was adjusted according to the experimental design described in this work and were sterilized by autoclaving at 121 °C for 15 min. 20

mL of the standard inoculum were transferred to 2 L Erlenmeyer flasks containing 400 mL of medium. Cultivations were conducted in a rotatory shaker at 100 rpm under different temperatures for each set of experiment. Samples were periodically removed. TGase activity, biomass, and frequency of sporulation were determined as described below.

Bioreactor cultivations

The induction of cell sporulation was studied in bioreactor cultures. These cultures were run on a 2 L bioreactor (BBraun model B, Germany), fully equipped with probes for pH, temperature, and dissolved oxygen tension, aeration and agitation systems. The vessel was filled with 1.9 L of medium M1 and inoculated with 100 mL of a 1.0 OD cell suspension of pre-inoculum as described above. Conditions of culture were 30 ^{o}C, pH 8.5, controlled with addition of 1 M NaOH solution, and varying conditions of oxygen tension: (a) 400 rpm, 0 vvm air (anoxic conditions throughout cultivation); (b) 400 rpm, 2 vvm air (aerobic conditions throughout cultivation); (c) 400 rpm, initial air supply of 2 vvm up to the end of exponential growth phase, followed by no air supply during the stationary phase.

Experimental design

The response surface methodology by using a set of experimental design was performed to determine the optimal levels of the variables, temperature and initial pH, on TGase production. The upper and lower limits were set to be in the range described in the literature and also based on our previous experience. A 2^2 full factorial central composite design for two independent variables, each one at five levels with four star points and three replicates at the central point, was employed to fit a second-order polynomial model in which 11 experiments were required for this procedure. In the statistical model, *Y* denotes either TGase specific activity (U.g^{-1} of dry cells) or dry cell weight (g.L^{-1}). Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. The enzyme activity and biomass values were determined after 5 to 9 incubation days but the regression equation was made with the values obtained after 8 days (maximal value of the TGase specific activity).

This design is represented by a second-order polynomial regression model:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Where: Y = response variable, β_0 = constant, β_i = coefficient for the linear effect, β_{ii} = coefficient for the quadratic effect, β_{ij} = coefficient for the interaction effect, and x_i and x_j = the coded level of variable X_i and X_j. The above quadratic equation was used to plot surfaces for the variables.

The test factors were coded according to the following equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \quad (2)$$

Where: x_i is the *coded* value and X_i is the *actual* value of the *i*th independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value.

All statistical experimental designs and results analyses were carried out using Statistica 5.0 software (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities $p(t)$ were determined by Student's t-test; the second order model equation significance was determined by Fisher's F-test. The variance explained by the model is given by the multiple determination coefficient, R^2. For each variable, the quadratic models were represented as contour plots (2D).

Assay methods

The colorimetric hydroxamate procedure described by Grossowicz et al. [17] was used for transglutaminase activity determination. Aliquots of 2 mL of the culture were centrifuged at 12,000 *g* for 5 min at 4 °C. The cell-free supernatant was used for the estimation of enzyme activity over the substrate carbobenzoxy-L-glutaminyl-glycine [17]. A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One enzymatic unit (U) causes the formation of 1 μmol hydroxamic acid per minute. Enzyme was expressed as specific activity ($U.g^{-1}$ of dry cells).

The biomass concentration was determined by measuring turbidity at 600 nm and correlating with a dry cell weight calibration curve. To prepare the curve, 10 mL of culture was centrifuged at 3,500 *g* for 20 min at 4 °C. Cell pellet was washed twice with cold distilled water and cells were dried in pre-weighed plastic tubes at 80 °C to a constant weight in vacuum-ovens.

The frequency of sporulation (% $spores.cells^{-1}$) was measured using the technique described by Mansour and Millière [18]. Aliquots of the culture were spread on agar

nutrient (Merck) before and after heat treatment at 80 °C for 10 min in order to evaluate total cell (before heating) and spore counts (after heating). Plating was made after a serial of decimal dilutions in tryptone salt broth (tryptone: 1 $g.L^{-1}$; sodium chloride: 8.5 $g.L^{-1}$). Plates were incubated at 30 °C for 24 h and then enumerated.

All the experiments in this research were carried out in triplicates.

Results and discussion

The experimental design matrix and results obtained for enzyme activity and dry cell weight are shown in Table 1.

Table 1 Process variables used in the CCD, showing the treatment combinations and the mean experimental responses

Run No.	Coded setting levels		Actual levels		TGase specific activity ($U.g^{-1}$ of dry cells)					Dry cell weight ($g.L^{-1}$)				
	x_1	x_2	X_1	X_2	Day 5	Day 6	Day 7	Day 8	Day 9	Day 5	Day 6	Day 7	Day 8	Day 9
1	-1	-1	5.1	23	174	200	220	237	297	1.20	1.03	0.94	0.97	0.73
2	-1	1	5.1	37	440	531	481	518	552	0.46	0.44	0.43	0.39	0.31
3	1	-1	7.9	23	184	218	343	397	430	1.11	0.93	0.63	0.59	0.50
4	1	1	7.9	37	480	571	618	566	528	0.51	0.46	0.45	0.50	0.46
5	-1.41	0	4.5	30	504	480	606	576	598	0.59	0.58	0.48	0.50	0.48
6	1.41	0	8.5	30	475	540	568	655	636	0.65	0.58	0.56	0.56	0.50
7	0	-1.41	6.5	20	106	120	137	171	218	1.20	1.19	1.10	1.08	0.83
8	0	1.41	6.5	40	411	475	490	369	356	0.50	0.46	0.42	0.45	0.48
9	0	0	6.5	30	333	404	415	434	460	0.88	0.75	0.72	0.70	0.62
10	0	0	6.5	30	400	543	530	528	544	0.76	0.55	0.56	0.57	0.54
11	0	0	6.5	30	341	472	469	497	493	0.85	0.60	0.61	0.59	0.56

The results are the mean of three replications.

$x_1 = (X_1 - 6.5)/1.4$; $x_2 = (X_2 - 30)/7$;

x_1 and x_2 are coded values;

X_1 and X_2 are the actual values;

X_1 = initial pH; X_2 = temperature (^{0}C).

The bacterial growth for the interval of days 5 to 9, which corresponds to the higher enzymatic activities, was smaller for temperatures around 37-40 °C (experiments 2, 4 and 8) than that observed in lower temperatures (20 and 23 °C) (experiments 1, 3 and 7). However, the ratio enzyme/dry cell weight ($U.g^{-1}$) (Table 1) and volumetric activity ($U.mL^{-1}$) were, in general, higher in temperatures around 37-40 °C. The enzyme activity and biomass values were determined after 5 to 9 days of incubation. The highest value for TGase specific activity was obtained after 8 days, consequently the regression equations were made to this incubation time. Treatment 6 showed the highest level of TGase specific activity (655 $U.g^{-1}$ of dry cells; 0.37 $U.mL^{-1}$). This production was obtained at a moderate incubation temperature (30 °C) and at high medium initial pH (8.5). Comparatively, for *S. mobaraense*, so far the only bacterium used for the commercial preparation of TGase, the highest activities reported for optimized cultivations were approximately 117 $U.g^{-1}$ cells (2.94 $U.mL^{-1}$) [6]. The highest activity experimentally obtained according to the CCD was 35 % higher than the activity obtained under conditions previously used in our laboratory (central point of this study); 486 $U.g^{-1}$ (0.30 $U.mL^{-1}$) of dry cells (mean of three replications at the central point) at 8 days, initial pH 6.5 and 30 °C.

Statistical analysis of results showed that, in the studied range, temperature exerts a strong effect on TGase production and biomass formation by *B. circulans* BL32. The significance of each regression coefficient was determined by *t*-values and *P*-values, which are listed in Table 2.

Table 2 Coefficient estimates by the regression model for optimization of tgase production and biomass formation

Independent variables (parameter)	TGase specific activity ($U.g^{-1}$)			Dry cell weight ($g.L^{-1}$)		
	Coefficient (β)	*t*-value	*P*-value	Coefficient (β)	*t*-value	*P*-value
Intercept	486.33	17.58	0.0032	0.6200	15.34	0.0042
x_1	39.96	2.36	0.1422	-0.0231	-0.93	0.4484
$x_1.x_1$	61.27	3.04	0.0933	-0.0537	-1.82	0.2096
x_2	91.25	5.39	0.0327[a]	-0.1951	-7.88	0.0157[a]
$x_2.x_2$	-111.48	-5.53	0.0312[a]	0.0637	2.16	0.1629
$x_1.x_2$	-28.00	-1.17	0.3628	0.1225	3.50	0.0728

[a] Statistically significant at 95% of confidence level.

The *P*-values suggest that the negative and positive coefficients for the linear effect of temperature are significant for biomass (P = 0.0157) and TGase production (P = 0.0327), respectively. This confirms that dry cell weight of *B. circulans* BL 32 decreases while enzyme production increases with the elevation of temperature. The high significance for the quadratic effect of temperature in TGase formation (P = 0.0312) indicates that even small variations of temperature will significantly affect the enzyme production by *B. circulans*. The experimental results of the CCD design were fitted with second-order polynomial equations by applying multiple regression analysis on the experimental data. Whenever possible, the models were simplified by the elimination of statistically insignificant terms. However, the x_1^2 term ($P\ x_1.\ x_1$ = 0.0933) for TGase specific activity and the $x_1.x_2$ term ($P\ x_1.x_2$ = 0.0728) for dry cell weight were maintained in the models because of their magnitudes. We then proposed that the quadratic models should be reduced as described in equations 3 and 4.

The production of TGase may be best predicted by the following model:

$$Y_{TGase} = 486.33 + 61.27x_1^2 + 91.25x_2 - 111.48x_2^2 \quad (3)$$

Where: Y_{TGase} is the predicted response for TGase specific activity ($U.g^{-1}$ cell), x_1 in the initial medium pH, and x_2 is the incubation temperature, as coded settings.

The formation of biomass may be best predicted by the following model:

$$Y_{DryCell} = 0.62 - 0.1951x_2 + 0.1225x_1x_2 \quad (4)$$

Where: $Y_{DryCell}$ is the predicted response for dry cell weight ($g.L^{-1}$), x_1 is the initial medium pH, and x_2 is the incubation temperature, as coded settings.

For TGase production and biomass formation, ANOVA shows that the models are very reliable, with R^2 values of 0.9553 and 0.9256, respectively, indicating good agreement between experimental and predicted values. The models explain 95.53 and 92.56 % of the variability in the responses. The computed *F*-value of 21.36 for enzyme production and of 12.45 for biomass formation were greater than the tabulated *F*-value, $F_{5,5,0.05}$ = 5.05, reflecting the statistical significance of the model equations. The lack of fit *F*-values were

found to be 0.74 and 1.65 for TGase specific activity and dry cell weight, respectively, they were smaller than the tabulated *F*-value, $F_{3,2,0.05}$ = 19.16, which implies that the lack of fit is non-significant. This shows that the models, as expressed in equations 3 and 4, provide suitable mathematical models to describe the responses of the experiments concerning TGase production and dry cell weight.

Fig. 1a and 1b, shows the shapes of contour plots of incubation temperature against initial pH for TGase production and biomass formation by *B. circulans* BL32, respectively.

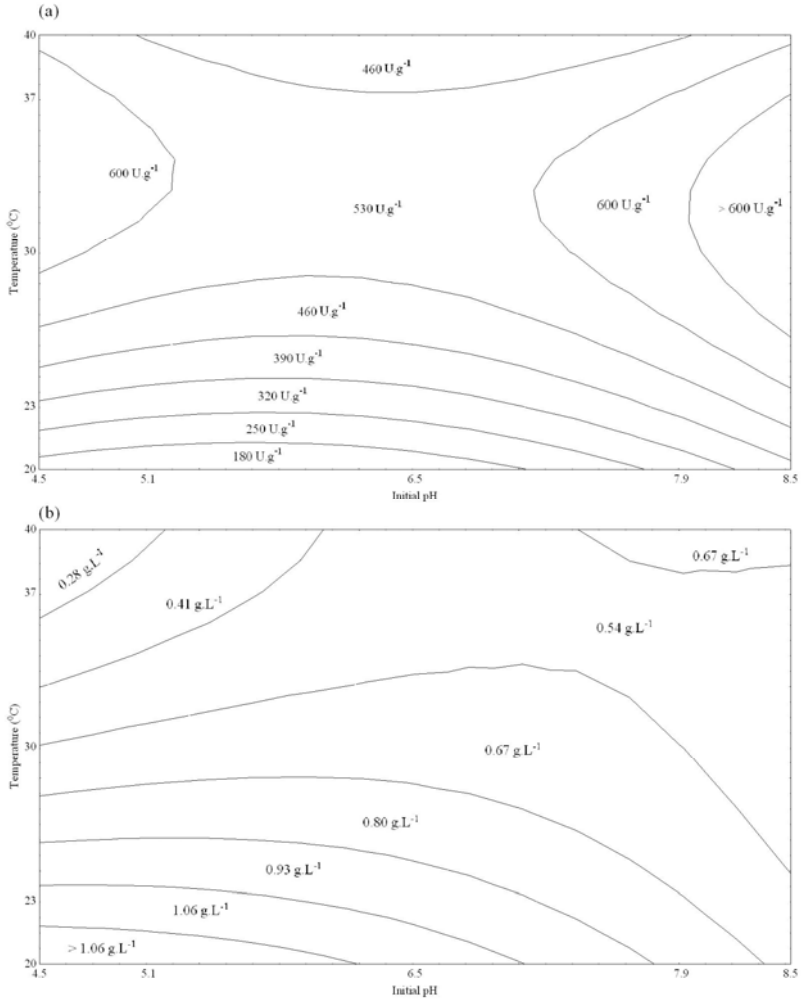

Fig. 1 Contour plot for the effect of temperature vs. initial pH on (a) TGase specific activity and (b) dry cell weight

The plots demonstrate that the production of enzyme could be increased by elevating the medium initial pH, even under moderate temperatures (Fig. 1a). There is a great flexibility for enzyme productivity, either varying pH or temperature, which is

interesting for industrial applications. However, maximal enzyme production was achieved at initial pH in between 8.3 and 8.5 and incubation temperatures in between 28 and 36 °C. Biomass formation, however, was inversely affected (Fig. 1b). Maximal biomass was achieved at lower values of initial pH, between 4.5 and 5.1, and at lower temperatures, between 20 and 21 °C. The optimal response factor levels for TGase production differed from those for the biomass concentration, as is generally the case for secondary metabolites. The effects of pH on TGase production and biomass formation by *B. circulans* BL32 found in our work contrast strongly with results obtained for *S. mobaraense* by Zheng et al. [10]. These authors, evaluating the effects of pH varying in the range of 5.0 to 8.5, observed that, at lower than 6.0 and higher than 7.5, almost no cell growth occurred, and that the best pH for TGase production and biomass formation were between 6.5 and 7.0. Concerning temperature and its effect on biomass and enzyme production, the results for *B. circulans* obtained in our work contrast with those by Zhu et al. [19], who reported that biomass accumulation is essential for enzyme production by *Streptoverticillium mobaraense*, so far the only microorganism used in commercial-scale production of TGase. Zheng et al. [6] studying the cell growth and TGase production by *S. mobaraense* in a one-factor-at-a-time optimization experiment at temperatures ranging from 25 to 35 °C, found that the ideal temperature for both cell growth and enzyme production by that bacterium was 30 °C. These observations might be indicating different mechanisms of these bacteria on the synthesis of TGase.

We speculated whether TGase production by *B. circulans* BL32 would possibly be involved in the sporulation process of this bacterium. In a early study, Kobayashi et al. [20] suggested that this enzyme is expressed during sporulation and plays a role in the assembly of the spore coat proteins of *Bacillus* spp. Recently, Ragkousi and Setlow [11], and Zilhão et al. [12], demonstrated the molecular mechanisms involved in the assembly of spore coat proteins in *B. subtilis* and have shown that TGase plays an essential role in the cross-linking of one of the coat proteins, GerQ. These authors also demonstrated that TGase is produced in the mother cells during the process of sporulation [11, 12]. Our results were showing that temperature and pH were strongly affecting TGase content of *B. circulans* BL32. Probably, the increment of both variables could be acting as inducers of TGase synthesis by mother cells before and during spore formation of *B. circulans*. To test this

possibility, we measured the independent effects of pH and temperature on the frequency of sporulation of our strain and results are shown on Fig. 2.

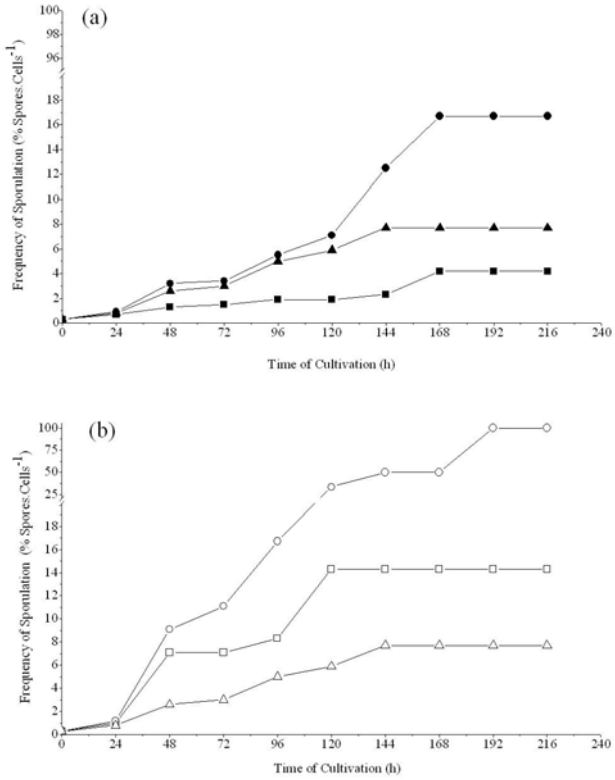

Fig. 2 (a) Effect of temperature in the sporulation of *B. circulans* BL32 at pH 6.5: (■) 20 °C, (▲) 30 °C, (●) 40 °C. (b) Effect of pH in the sporulation of *B. circulans* BL32 at temperature of 30 °C: (□) 4.5, (△) 6.5, (○) 8.5.

As it can be seen, sporulation is linearly stimulated by the increase in temperature, reaching its maximal at 40 ^{o}C. The effect of pH on cell sporulation was somewhat more complex, with a non-linear response, with the highest value tested (pH 8.5) inducing 100 % of spores formation at 192 hours of cultivation. This non-linear response can be explained by the fact that extremes of pH will be detrimental to cell metabolism thus inducing sporulation. To further confirm the role of spore formation on TGase activity, we cross-checked the effects of varying oxygen concentration of cultures under the optimized values of pH and temperature (Fig. 3). As can be seen, anoxic conditions (Fig. 3 a) will

have little effect on cell formation and TGase induction, while fully aerated cultures (Fig. 3 b) will produce higher amounts of biomass but fails to induce TGase. However, when combining aeration during cell formation (exponential growth) with anoxic conditions during stationary phase, this will result in high spore induction and TGase production.

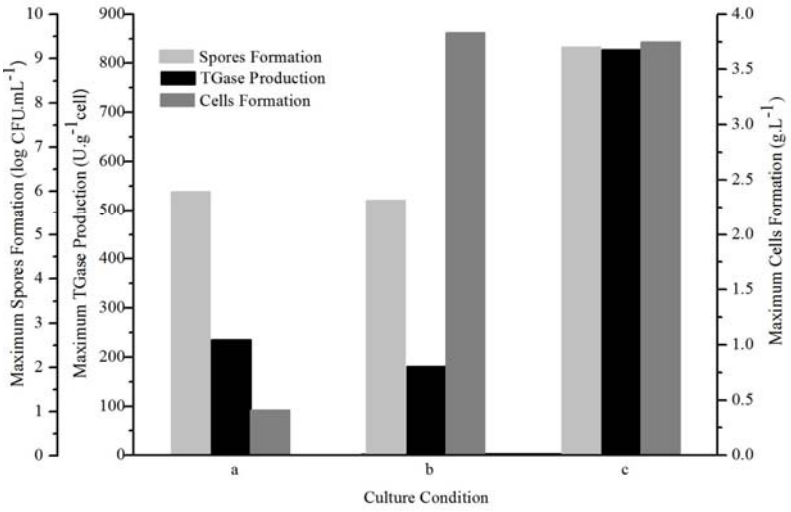

Fig. 3 Effect of oxygen on **s**pore formation, TGase and biomass production in batch cultivations of *B. circulans* BL32 growing at the optimal conditions of pH (8.5) and temperature (30 ^{o}C); (a): 400 rpm, 0 vvm; (b): 400 rpm, 2 vvm throughout cultivation; (c) 400 rpm, 2 vvm up to the end of exponential phase followed by 400 rpm, 0 vvm to induce sporulation. Results represent the mean of two experiments.

Further experiments were performed in order to validate the model for the predicted optimal culture conditions for TGase production by our strain (pH 8.5 and temperature 30 °C) and results are shown in Fig. 4.

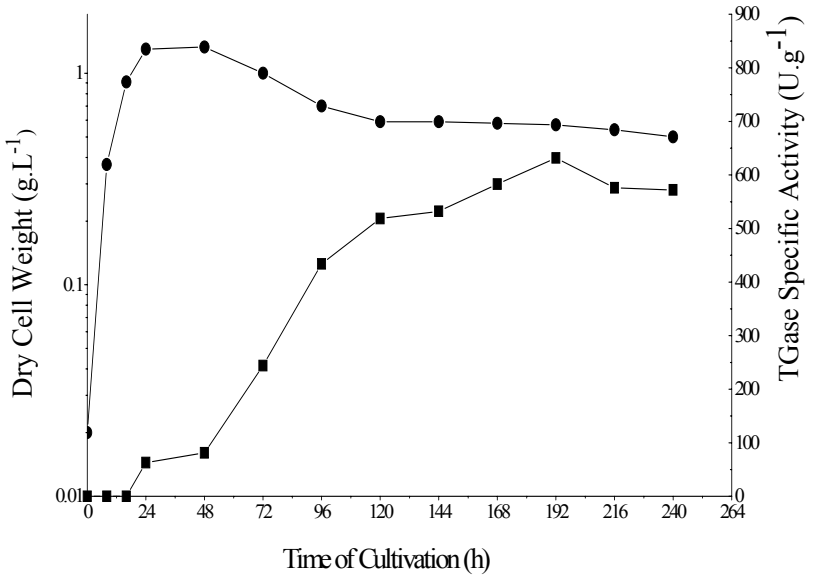

Fig. 4 The growth kinetics of TGase production (model validation) on shaker culture. TGase activity (■) and cell growth (●) profile under the optimal conditions suggested by the model (30 ^{o}C, pH 8.5). Results are the mean of three experiments.

After 8 days of cultivation, the TGase specific activity and biomass were 633 U.g^{-1} cells (0.37 U.mL^{-1}) and 0.58 g.L^{-1}, respectively. In this case, the coded settings of the tested variables were $x_1 = +1.41$, $x_2 = 0$, with the models predicting TGase activity of 608 U.g^{-1} (0.38 U.mL^{-1}) and biomass of 0.62 g.L^{-1}. Therefore, the models developed were considered to be accurate and reliable for predicting the production of both enzyme and biomass. As expected from our observations on cell sporulation, TGase production is not related to cell growth, being produced from the onset of stationary phase and reaching its maximal after 192 hours of cultivation. This time coincides of the maximal frequency of sporulation for all tested conditions (Fig. 2), both for variations in pH and temperature, further demonstrating the correlation between TGase activity and cell sporulation.

Conclusions

The optimal design of process parameters is a relevant aspect to be considered in the development of bioprocesses. The culture conditions for TGase production by *B. circulans* BL32 were optimized using statistical methods. In this study, the experimental results clearly showed that the TGase production by this bacterium is dependent on pH and

temperature. Our results showed that the process is flexible concerning good productivities of this enzyme but at 30 °C, initial pH 8.5 and 8 days of incubation, the TGase specific activity reached 655 $U.g^{-1}$ (0.37 $U.mL^{-1}$) of cells, which is 35 % higher than the activity previously obtained in our laboratory and comparable to activities reported for *S. mobaraense*, presently the bacterial model for the production of this enzyme. Our results also demonstrate a correlation between enzyme production and cell sporulation, as mediated by culture conditions and, although demonstrated only for *B. subtilis*, it corroborates the molecular mechanisms involved in this process. Based on our results, it can be suggested that *B. circulans* BL32 is a strong biological system for the industrial production of TGases.

Acknowledgements

The authors wish to thank CNPq for their financial support.

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5.3 RESULTADOS III. “Effects of oxygen volumetric mass transfer coefficient on transglutaminase production by *Bacillus circulans* BL32” - Biotechnology and Bioprocess Engineering, submetido em abril de 2008.

Effects of oxygen volumetric mass transfer coefficient on transglutaminase production by *Bacillus circulans* BL32

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Abstract

The effects of oxygenation in cultures of *Bacillus circulans* BL32 on transglutaminase (TGase) production and cell sporulation were studied by varying the agitation speed and the volume of aeration. Kinetics of cultivations have been studied in batch systems using a 2 L bioreactor and the efficiency of agitation and aeration was evaluated through the oxygen volumetric mass transfer coefficient (k_La). It was adopted a two-stage aeration rate control strategy: first stage to induce biomass formation, followed by a second stage in which cell sporulation was stimulated. A correlation of TGase production, spores formation, and oxygen concentration was established. Under the best conditions (500 rpm; 2 vvm air flow, followed by no air supply during stationary phase; k_La of 33.7 h^{-1}), TGase production reached a volumetric production of 589 U/L after 50 h of cultivation and the enzyme yield was 906 U/g cells. These values are 61 % higher than that obtained in shaker cultures and TGase productivity increased 82 % when k_La varied from 4.4 h^{-1} to 33.7 h^{-1}. The maximal cell concentration increased four times in relation to shaker cultures and the cultivation time for the highest TGase activity was reduced from 192 h to just 50 h. These results show the importance of bioprocess design for the production of microbial TGase, especially concerning the oxygen supply of cultures and the induction of cell sporulation.

Keywords: Transglutaminase; Spores formation; *Bacillus circulans* BL32; Aeration; Agitation; Volumetric oxygen mass transfer coefficient (k_La).

INTRODUCTION

Transglutaminases (TGase, EC 2.3.2.13, γ-glutamyl-peptide, amine-γ-glutamyl transferase) belong to the group of acyltransferases, which catalyze acyl-transfer reactions between a γ-carboxyamine group of a peptide- or protein-bound glutamyl residue (acyl donors) and a primary amino group (acyl acceptors) of a variety of substrates, including the ε-amino group of lysine or lysyl residues in proteins, resulting in amine incorporation or polymerization [1]. In the absence of amine substrates, TGase catalyses the deamidation of glutamyl residues during which water molecules are used as acyl acceptors [2].

These enzymes are widely distributed in various animal or vegetable tissues and body fluids and are involved in several biological processes, such as blood clotting, wound healing, epidermal keratinization, and stiffening of the erythrocyte membrane [3]. TGase has been isolated from animal tissues, plants, and microorganisms [4]. Microbial TGases are the most industrially important due to their properties, such as, calcium independence, relatively low molecular weight, and because they are excreted making for simple separation and purification processes [1,5].

Microbial TGases have many potential applications in food processing [6]. A variety of food proteins can have their functional properties altered by TGase-mediated cross-linking [4]. They can promote protein texturisation and gelation, modify solubility and nutritional value, and improve water-holding capacity, elasticity, and appearance [6]. Moreover, several works have shown that TGases can be used in many other attractive non-food applications, such as pharmaceuticals [7], textiles [8], immobilization of enzymes [9], formation of immunoconjugates for ELISA tests [10], biosensors [11], and tissue engineering [12]. Notwithstanding its importance, the microbial TGase obtained from *Streptoverticillium mobaraense* is so far the only commercial source of TGase [13].

In biotechnology-based industrial processes, the culture conditions have a critical importance because they affect product concentration, yield and volumetric productivity. The economics of TGase production may be enhanced by the optimization of the culture conditions in large-scale processes and the cultivation is affected by medium composition, pH, temperature, aeration, and agitation [14-21]. Currently, TGase production and its improvement has been conducted only in submerged cultures of *Streptoverticillium* sp. However, the optimization of cultivation processes from a wide variety of microbial

TGases would allow for more extensive and effective use of this enzyme in food and non-food industrial applications [22].

In a previous work, we determined the optimal culture temperature and pH for the production of TGase by *Bacillus circulans* BL32 and observed that the enzyme is produced during sporulation [23]. Since some researches point to the possible interactions between culture oxygenation and spores formation, in this work we investigate the possible role of aeration and agitation conditions, as measured by the volumetric oxygen mass transfer coefficient (k_La), on the induction of cell sporulation and TGase production by *B. circulans* BL32 in bioreactor cultures.

MATERIALS AND METHODS

Materials

Unless otherwise mentioned, all chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany). N-carboxybenzoyl-L-glutaminyl-glycine (N-CBZ-Gln-Gly) was purchased from Sigma-Aldrich Co. Ltd. (MO, USA).

Microorganism and maintenance

A strain of *B. circulans*, coded BL32 was used in this study. It has been recently isolated from the Amazon environment and its isolation and characterization were described elsewhere [23,24]. Previously to culture, cells were recovered from frozen stocks in glycerol and were kept at 4 °C on Mueller-Hinton agar plates.

Inocula preparation

Inocula were prepared in 250 mL Erlenmeyer flasks filled with 50 mL of LB medium and inoculated with a loopfull from a stock culture. Cells were cultivated at 100 rpm, 30 °C for 18 h. After this procedure, 10 mL of this culture was transferred to Erlenmeyer flasks (500 mL) containing 100 mL medium (M1), optimized in our previous work [23], and composed of (g/L): glycerol 9.0, sucrose 2.0, peptone 7.0, tryptone 1.0, Na_2HPO_4 1.0, $MgSO_4.7H_2O$ 1.0 and $FeSO_4.7H_2O$ 0.1, and incubated at 30 °C in a rotatory shaker, 100 rpm, until it reached OD 1.0 (600 nm), and subsequently inoculated into the

bioreactor. This procedure was used as the standard inoculum procedure for all experiments.

Bioreactor cultivation conditions

Batch cultivations were carried in a 2 L stirred tank bioreactor (Biostat B model, B. Braun Biotech International, Germany) containing 2 L of medium. The bioreactor was equipped with temperature, agitation, aeration and pH controllers and two Rushton turbines with six flat-blades. The vessel was filled with 1.9 L of medium M1, sterilized by autoclaving at 121 °C for 20 min, and inoculated with 100 mL of a 1.0 OD cell suspension of pre-inoculum as described above. Conditions of culture were 30 ^{o}C, initial pH 8.5. Dissolved oxygen concentration of cultures was measured using a polarographic electrode and was expressed as percentage of O_2 saturation. The oxygen transfer rate was studied as the volumetric oxygen mass transfer coefficient (k_La). The estimation of k_La was carried out by the dynamic gassing-out method [25]. We investigate the effect of a two-stage strategy: the agitation and aeration rates were set at different values, characterizing different oxygen transfer conditions: 4.4 h^{-1} (200 rpm, 1 vvm), 14.2 h^{-1} (400 rpm, 1 vvm), 17.2 h^{-1} (400 rpm, 2 vvm), 33.7 h^{-1} (500 rpm, 2 vvm), 35.2 h^{-1} (500 rpm, 3 vvm), and 39.3 h^{-1} (500 rpm, 4 vvm); followed by no air supply during the stationary phase, in order to stimulate cell spores formation.

Samples were periodically removed along time of cultivation in order to determine TGase activity, glycerol and sucrose concentrations, biomass, and number of spores.

Analytical methods

Aliquots of the culture were centrifuged at 12,000 *g* for 5 min at 4 °C. The cell-free supernatant of the culture medium was used for the estimation of enzyme activity and for glycerol and sucrose concentration determinations.

The colorimetric hydroxamate procedure described by Grossowicz *et al.* [26] was used for TGase activity determination. The cell-free supernatant was used for the estimation of enzyme activity over the substrate carbobenzoxy-L-glutaminyl-glycine. A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One enzymatic unit (U) causes the formation of 1 μmol hydroxamic acid/min.

Glycerol and sucrose concentrations were determined by HPLC with a refractive index (RI) detector (Perkin Elmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300 x 7.8 mm), at 80 ºC, using ultrapure water as eluting fluid, flow of 0.6 mL/min and sample volume of 20 µL.

The biomass concentration was determined as dry cell weight. Aliquots of the culture were centrifuged at 3,500 *g* for 20 min at 4 °C. Cell pellet was washed twice with cold distilled water and cells were dried in pre-weighed plastic tubes at 80 °C to a constant weight in vacuum-ovens.

The number of spore was measured using the technique described by Mansour and Millière [27]. Aliquots of the culture were spread on agar nutrient after heat treatment at 80 °C for 10 min. Plating was made after a serial of decimal dilutions in tryptone salt broth (tryptone: 1 g/L; sodium chloride: 8.5 g/L). Plates were incubated at 30 °C for 24 h and then enumerated.

All the experiments in this research were carried out in triplicates.

RESULTS AND DISCUSSION

According to various authors, the sporulation process from *Bacillus* spp. may be induced by the absence of oxygen [28-30]. However, cultures of this bacterium under anoxic conditions will halt biomass formation and affect negatively TGase production, as it has been demonstrated by one experiment run by us at 400 rpm and 0 vvm air (0 % dissolved oxygen concentration, results not shown). On the other hand, fully oxygenated cultures of *B. circulans* BL32, while enhancing biomass formation, will enter stationary phase repressing cell sporulation, again causing low production of TGase. Therefore, we investigate the effect of various aeration and agitation conditions, which were evaluated through the volumetric oxygen mass transfer coefficient (k_La), and induction of cell sporulation, by no air supply during the stationary phase, on TGase production by *B. circulans* BL32 in bioreactor cultures. A two-stage aeration control strategy with biomass production under the growth conditions in the first stage, followed by a second anoxic stage was adopted.

The kinetics of cell growth, TGase production, glycerol and sucrose consumption by *B. circulans* BL32 at k_La of 4.4, 14.2, 17.2, and 33.7 h^{-1}, followed by the interruption of air supply during the stationary phase, are depicted in Fig. 1 to 4, respectively.

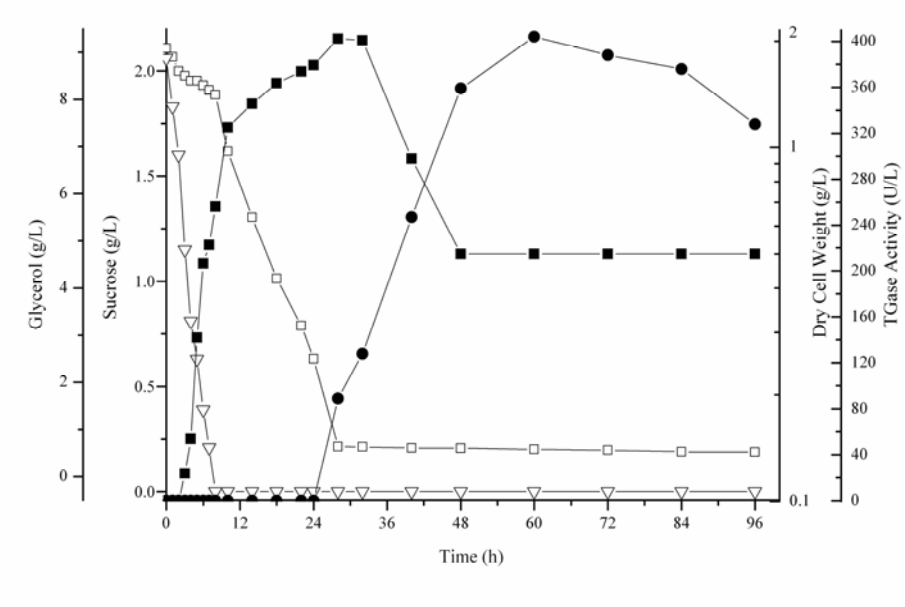

Fig. 1. Cultivation of *B. circulans* BL32 at 200 rpm, initial air supply of 1 vvm up to the end of exponential growth phase (k_La of 4.4 h^{-1}), followed by no air supply during the stationary phase. Glycerol (□); sucrose (∇); cell mass (■); TGase activity (●).

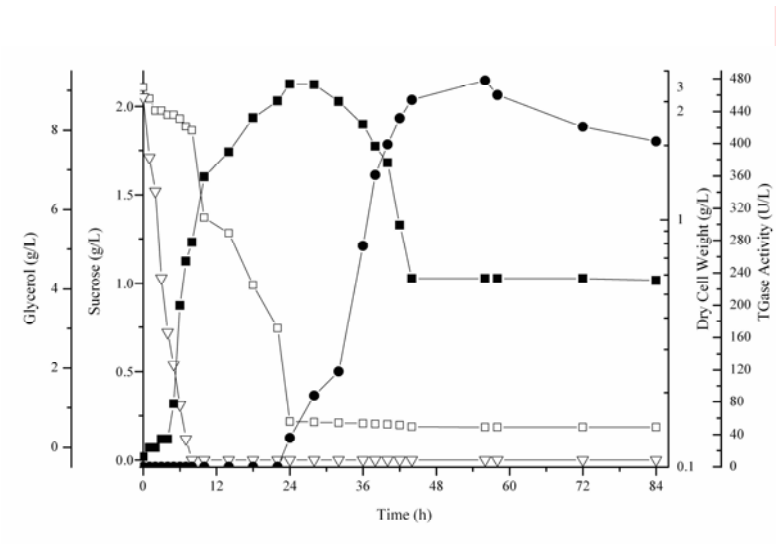

Fig. 2. Cultivation of *B. circulans* BL32 at 400 rpm, initial air supply of 1 vvm up to the end of exponential growth phase (k_La of 14.2 h^{-1}), followed by no air supply during the stationary phase. Glycerol (□); sucrose (∇); cell mass (■); TGase activity (●).

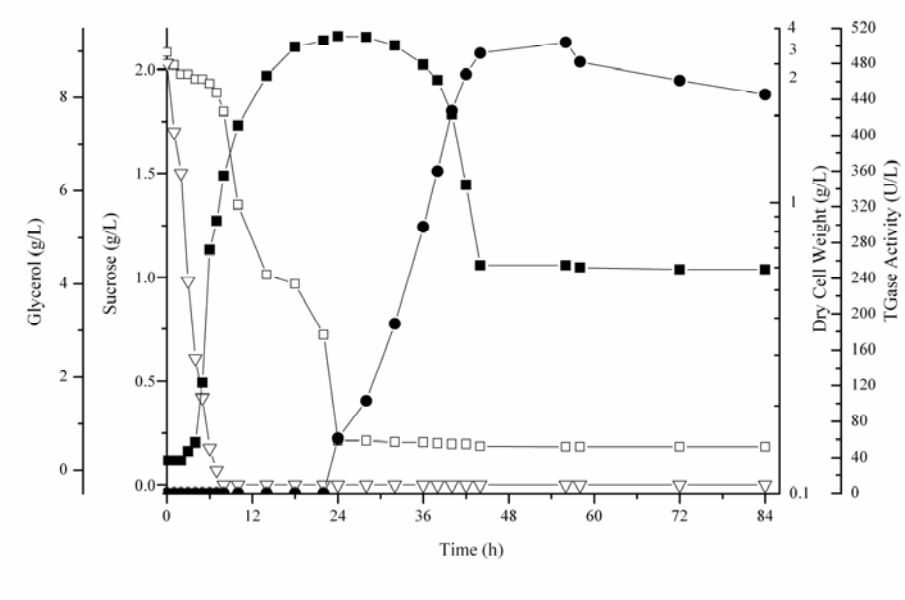

Fig. 3. Cultivation of *B. circulans* BL32 at 400 rpm, initial air supply of 2 vvm up to the end of exponential growth phase (k_La of 17.2 h^{-1}), followed by no air supply during the stationary phase. Glycerol (□); sucrose (∇); cell mass (■); TGase activity (●).

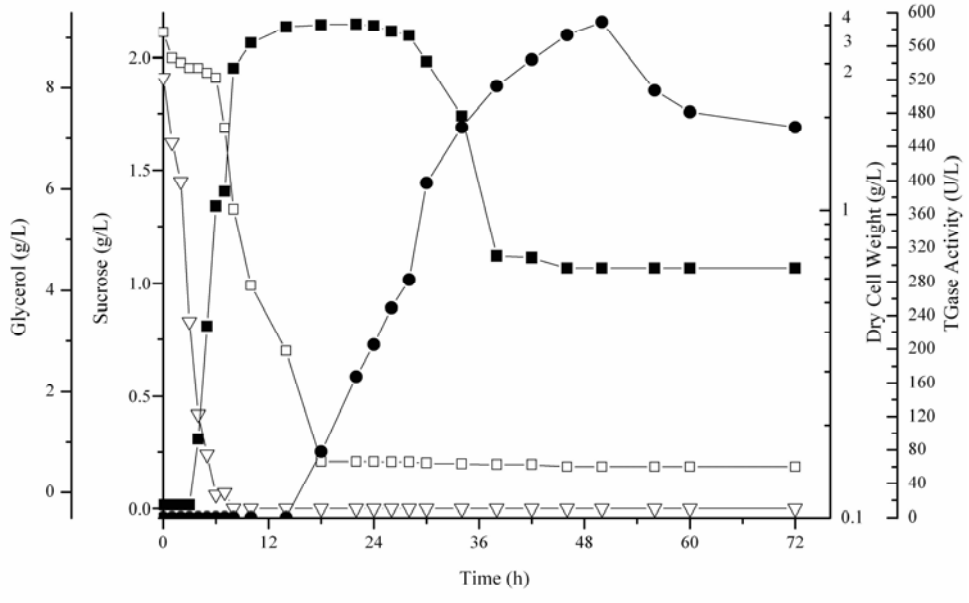

Fig. 4. Cultivation of *B. circulans* BL32 at 500 rpm, initial air supply of 2 vvm up to the end of exponential growth phase (k_La of 33.7 h^{-1}), followed by no air supply during the stationary phase. Glycerol (□); sucrose (∇); cell mass (■); TGase activity (●).

Biomass concentration and TGase production by *B. circulans* BL32 were strongly affected by volumetric oxygen mass transfer coefficient in the range studied. At 200 rpm and 1 vvm (k_La of 4.4 h^{-1}), the cell concentration reached 2.0 g/L and the maximum TGase production was at 404 U/L (Fig. 1). Under these conditions, the enzyme production was 10 % higher than the obtained in previous results in shake flask. More important though, the incubation time required for maximum TGase activity was reduced from 192 to only 60 h of cultivation. When the conditions were 500 rpm and 2 vvm (k_La of 33.7 h^{-1}), TGase production and dry cell weight reached 589 U/L and 4.0 g/L (Fig. 4). Compared to shake flask experiments, this culture condition resulted in an improved TGase volumetric production of approximately 61 %, the cell concentration increased four times, and the cultivation time for the maximal TGase activity was reduced from 192 to only 50 h. This TGase activity is around two times higher than the obtained by Junqua *et al.* [15], who showed that *Streptoverticillium cinnamoneum* had a peak of TGase production in approximately 175 h of cultivation at 140 rpm, in a culture medium also containing glycerol as carbon source. Yan *et al.* [21] reported TGase production by *Streptoverticillium mobaraense* in bioreactor at different agitation speeds (250, 350, and 450 rpm) and fixed aeration rate of 1 vvm. They observed highest dry cell weight and TGase activity at 30 h for 450 rpm and at 40 h for 350 rpm, respectively. Thus, these authors proposed a two-stage agitation speed control strategy in which the agitation speed was controlled at 450 rpm in the first 24 h of cultivation to facilitate the cell growth, and then switch it to 350 rpm after 24 h to enhance the TGase production. In these works however, k_La of cultures were not measured and consequently comparisons are difficult to be established.

The growth kinetics determinants and conversion rate parameters for the TGase production by *B. circulans* BL32 obtained for different oxygen volumetric mass transfer coefficients are presented in Table 1.

Table 1. Influence of k_La on cultivation parameters of growing and TGase production by *B. circulans* BL32.

Parameter	k_La (h^{-1})			
	4.4	14.2	17.2	33.7
μ_{max} (h^{-1})	0.48	0.59	0.76	0.87
Q_{st} (g/L.h)	0.50	0.53	0.61	0.80
Q_p (U/L.h)	7.48	10.32	11.18	13.62
Q_x (g/L.h)	0.10	0.14	0.19	0.35
$Y_{P/st}$ (U/g substrate)	38.22	45.01	48.00	56.04
$Y_{X/st}$ (g/g substrate)	0.24	0.36	0.47	0.51
$Y_{P/X}$ (U/g cell)	1,010	1,017	1,050	1,091

μ_{max}, maximal growth rate; st, total substrate (glycerol, sucrose); Q_{st}, total substrate consumed; Q_p, TGase productivity; Q_x, biomass productivity; $Y_{P/st}$, yield coefficient for TGase over total substrate; $Y_{X/st}$, yield coefficient for biomass over total substrate; $Y_{P/X}$, yield coefficient for TGase over biomass; Yield coefficients were calculated using values of ΔX, ΔSt, and ΔP (difference between maximal and initial values).

The results indicate that as k_La increases, both the dry cell weight and TGase activity increased. The μ_{max} value was found to be highest at k_La of 33.7 h^{-1} (0.87 h^{-1}), demonstrating that this k_La value was favorable for cell growth compared to the other k_La values. At this k_La the consumed substrate (Q_{st}) and biomass productivity (Q_X) were the highest. An eight-fold increase in k_La led to a two-fold increase in TGase productivity (Q_p). These findings show that the inadequate supply of oxygen to biomass until the stationary phase is a critical factor to the productivity of TGase by *B. circulans* BL32 in submerged cultivations. The highest yield coefficients for TGase on substrate ($Y_{P/st}$), for cells on substrate ($Y_{X/st}$), and for product on biomass ($Y_{P/X}$) were attained with k_La of 33.7 h^{-1}. The highest $Y_{P/st}$ (56.04 U/g substrate) obtained with this k_La at 50 h is 8 % higher than that observed by Téllez-Luis *et al.* [5] in a cultivation of *Streptoverticillium ladakanum* at 48 h under 150 rpm, in a culture medium also containing glycerol as carbon source. Fig. 5 shows the relationship between cell growth and TGase production. This is a confirmation that TGase production by *B. circulans* BL32 is a non-associated product, dependent on cell sporulation.

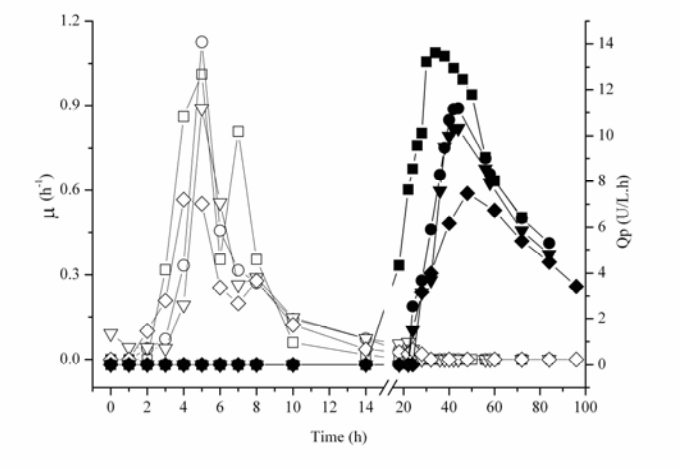

Fig. 5. Specific growth rates (opened symbols) and TGase productivity (closed symbols) along culture time and different k_La: (◇, ◆) 4.4 h^{-1}; (▽, ▼) 14.2 h^{-1}; (○, ●) 17.2 h^{-1}; (□, ■) 33.7 h^{-1}.

Fig. 6 shows the effects of different k_La on the spore counts and TGase production by *B. circulans* BL32.

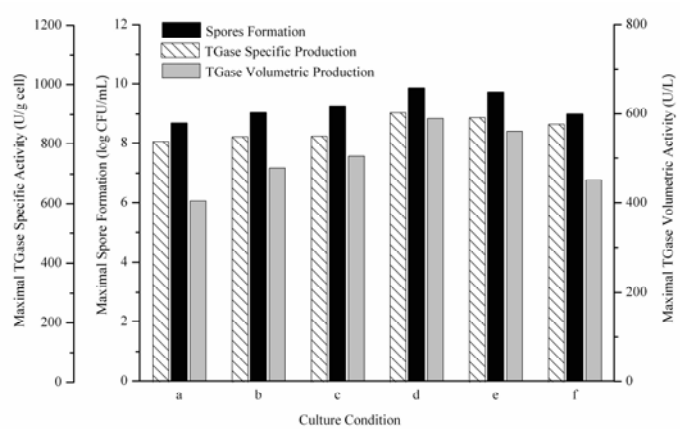

Fig. 6. Effects of different oxygen supply strategies on spores formation and TGase production by *B. circulans* BL32 in batch cultivations. k_La: (a) 4.4 h^{-1} (200 rpm, 1 vvm); (b) 14.2 h^{-1} (400 rpm, 1 vvm); (c) 17.2 h^{-1} (400 rpm, 2 vvm); (d) 33.7 h^{-1} (500 rpm, 2 vvm); (e) 35.2 h^{-1} (500 rpm, 3 vvm); (f) 39.3 h^{-1} (500 rpm, 4 vvm). In all conditions, air supply was suppressed during the stationary phase. The maximal TGase specific activity (U/g cell) was considered at the time where enzyme activity was the highest.

The sporulation process initiated in the early stationary phase, for all culture conditions evaluated, and at the end of cultivations 100 % of spores were obtained. According to Nicholson and Setlow [31], under ideal conditions, the culture will initiate sporulation at a cell density of about 10^8 cells/mL, and typical sporulation efficiencies will be in the range of 30 - 100 %. Sarrafzadeh and Navarro [30], studying the effect of oxygen on the sporulation of *Bacillus thuringiensis* H14, reported that the highest rate of sporulation was observed in the absence of oxygen and the mature spores were the only population present under this condition at the end of culture. The highest spore formation and TGase production were obtained for k_La of 33.7 h^{-1}, which coincides with the highest cell concentration obtained with this k_La (Fig. 4). These results are in agreement with the investigations performed by Zhu *et al.* [17], who studying the fed-batch fermentation process to microbial TGase production by *Streptoverticillium mobaraense*, reported that biomass accumulation is essential for enzyme production. Another important observation is the fact that the maximal number of spores (k_La at 33.7 h^{-1}) was 15-fold higher than that for k_La 4.4 h^{-1}. The main observable difference between these two conditions may be their effect on cell growth rates: k_La 4.4 h^{-1} had μ_{max} = 0.476 h^{-1},while for k_La 33.7 h^{-1} μ_{max} was 0.872 h^{-1}. According to Goldberg *et al.* [32] fast cell growth in batch cultures supported better sporulation. It is likely that cells growing fast contain enough energy and other factors necessary for sporulation, while slowly growing cells do not. Some researches have shown that this enzyme plays a role in the assembly of the spore coat proteins of the genus *Bacillus* sp. [33-35]. According to Kang *et al*. [36], one reasonable way to increase spore production is to achieve high cell density cultivation and subsequently allow sporulation to occur.

CONCLUSIONS

The availability of TGases with appropriate characteristics for specific industrial applications is still limited, and the improvement of TGase production continue to be important research topics. The optimal design of process parameters is a relevant aspect to be considered in the development of bioprocesses. In this work we improved TGase production by *B. circulans* BL32 in bioreactor cultures by determining the best aeration and agitation conditions and expressed this as the oxygen volumetric mass transfer

coefficient (k_La), which is a scaling-up parameter. The results demonstrated that the optimal process conditions are different for biomass and spore production. Therefore, a two-stage strategy, with biomass production under aerobic growth conditions in the first stage, followed by a second anoxic stage for sporulation and TGase induction was proposed in the present study. Comparing to previous shake flask experiments, these culture conditions resulted in a 61 % increase in TGase volumetric production, four times higher cell concentration, and the cultivation time for the maximal TGase activity was reduced from 192 to only 50 h. These results are important because researches on TGase production at bioreactor scale are scarce. Further studies are granted to scale-up bioreactor work for TGase production by *B. circulans* and the development of high cell density cultivations with special care on oxygen supply strategies.

Acknowledgements

The authors wish to thank CNPq and CAPES for their financial support.

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5.4 RESULTADOS IV. “Solid state bioreactor production of transglutaminase by Amazonian *Bacillus circulans* BL32 strain” - Journal of Industrial Microbiology and Biotechnology, *in press*.

Solid state bioreactor production of transglutaminase by Amazonian *Bacillus circulans* BL32 strain

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Abstract

In this work, we investigated the production of transglutaminase (TGase) by an Amazonian isolated strain of *Bacillus circulans* by solid-state cultivation (SSC). Several agro-industrial residues, such as untreated corn grits, milled brewers rice, industrial fibrous soy residue, soy hull, and malt bagasse, were used as substrates for microbial growth and enzyme production. Growth on industrial fibrous soy residue, which is rich in protein and hemicellulose, produced the highest TGase activity (0.74 U g^{-1} of dried substrate after 48 h of incubation). A 2^3 central composite design was applied to determine the optimal conditions of aeration, cultivation temperature and inoculum cell concentration to TGase production. The best culture conditions were determined as being 0.6 L air min^{-1}, 33 °C and 10 $\log_{10}$ CFU g^{-1} of dried substrate, respectively. Under the proposed optimized conditions, the model predicted an enzyme production of 1.16 U g^{-1} of dried substrate, closely matching the experimental activity of 1.25 U g^{-1}. Results presented in this work point to the use of this newly isolated *Bacillus circulans* strain as a potential alternative of microbial source for TGase production by SSC, using inexpensive culture media.

Keywords: Transglutaminase; *Bacillus circulans* BL32; Solid-state cultivation; Industrial fibrous soy residue; optimization.

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Introduction

Transglutaminases (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) are a family of enzymes that catalyses acyl transfer reactions using peptide-bond glutamine residues as acyl donors and several primary amines as acceptors. These reactions introduce covalent cross-links between proteins, as well as peptides and various primary amines [18]. TGase has been found in animal and plant tissues and microorganisms [31]. The microbial enzyme has widespread and growing applications in the food processing industry. Recent research has shown that TGase can be employed in many others attractive applications such as, pharmaceuticals [3], textiles [6], tissue engineering [19], and in the formation of immunoconjugates to be applied in ELISA and biosensors [11]. To date, only one microbial TGase, that from the actinomycete *Streptoverticilium mobarense*, has met commercial applications [31]. However, the potential commercial uses of this enzyme have not been achieved because a lack of mass production and also because some of its properties, such as specificity of pH and temperature, are not consistent with a variety of substrates and processes [3].

Commercial TGase production depends largely on culture conditions [13, 14], medium composition [29, 32] and downstream processing [7, 28]. Therefore, the development of low-cost processes for the production of transglutaminases would allow for more extensive industrial applications. Presently, submerged cultivation (SmC) is the only technology used for the commercial production of TGase. However, solid-state cultivation (SSC) systems offer many advantages over commonly used SmC, and it has gained renewed interest in recent years, often being employed for the production of many metabolites due to several economical and engineering advantages [20] including higher productivity, simplified operation, low investments, low energy requirements, less water output, better product recovery, and lack of foam build-up [10, 22, 30]. The latter is a problem when TGase is obtained in SmC [27]. Furthermore, agro industrial residues are generally considered to be the best substrates for the enzyme production by SSC. Thus, these processes are of special economic interest for countries with abundant biomass and agro industrial residues, as they can be used as cheap raw materials [5].

In SSC-based industrial bioprocesses, the determination of optimal culture conditions is of critical importance because it affects product concentration, yield and volumetric productivity. The studies of culture conditions by SSC that use conventional

single factor optimization does not allow for the interaction of these variables with each other. This method is also time consuming and requires large numbers of experiments to be carried out in order to determine the optimal level of each factor [4]. The response surface methodology (RSM) is a time saving method, which reveals the interactions that occur among the different culture conditions, building models and evaluating the effects of several factors in order to obtain high productivity [16]. So far there have been no reports in the literature on the optimization of culture conditions using statistical design concerning the production of TGase by SSC.

In a previous study, we compared the TGase production of *Bacillus circulans* BL32 on SmC and SSC, in which productivity by SSC was higher than in SmC [27]. However, in that early work, conditions for both forms of cultures were not optimized. It is important to optimize the process parameters in order to obtain high TGase production by this microorganism by SSC. Therefore, the purpose of this study was to evaluate the effects of different solid substrates and culture conditions such as aeration, cultivation temperature, and inoculum cell concentration on this enzyme production in order to determine its viability as a production system.

Material and methods

Microorganism and inoculum preparation

A strain of *Bacillus circulans*, coded BL32, which was isolated from the aquatic environment of the Amazon rain forest, was used in this study. Its isolation and characterization were described elsewhere [28, 29]. Previously to culture, cells were recovered from frozen stocks in glycerol and were kept at 4 °C on Mueller-Hinton agar plates (Merck, Germany). The inoculae for all experiments by SSC were prepared in 1000 mL Erlenmeyer flasks containing 160 mL of Luria–Bertani (LB) medium. Flasks were inoculated from a single colony from agar plates and incubated for 24 h, 37 ºC and 150 rpm.

Bioreactors cultivations

Bioreactor experiments were carried out in 500 mL cylindrical bioreactors (60 mm diameter; 170 mm height) designed and constructed in our laboratory [9]. Bioreactors were loaded with the solid substrates soaked with basic salt solution with the following

composition (g L^{-1}): 0.2 $MgSO_4$; 1.0 KH_2PO_4; 1.0 K_2HPO_4; 1.0 NH_4NO_3; 0.02 $CaCl_2$; 0.05 $FeCl_2$. Bioreactors were inoculated with 20 mL of inoculum.

Screening of solid substrates

Preliminary studies were carried out in order to evaluate the effect of the different solid substrates, such as corn grits (CG), milled brewers rice (MBR), industrial fibrous soy residue (IFSR, which is a by-product of isolated soybean protein production, rich in sugars and proteins), soy hull (SH) and malt bagasse (MB) on the TGase production by *B. circulans* BL32. These substrates were obtained locally. Soy hull was milled and sieved to obtain particles smaller than 1 mm in size and the malt bagasse was oven dried at 60 ºC for 12 h, in order to prevent decomposition during storage. The substrates were used without any other pre-treatments. Twenty grams of the dried substrates were soaked with basic liquid medium until they were saturated and then sterilized at 121 ºC for 15 min. The inoculum cell concentration was adjusted to 10^6 CFU g^{-1} of dried substrate (DS). The initial pH of cultures was that of the respective substrate without adjustment, while the moisture of cultures was that attained by the maximum water absorption capacity of each substrate. Cultures were run at 30 ºC for up to 168 h. During cultivation, water-saturated sterile air was supplied at a constant flow of 0.25 L air min^{-1}. The substrate in which the highest enzyme production was achieved in this step was selected and used in the subsequent experiments.

Optimization of process parameters

The influence of the process parameters on TGase production by *B. circulans* BL32 by SSC was tested performing a statistical experimental design. A 2^3 full factorial central composite design for three independent variables, each one at five levels with six star points and four replicates at the central point, was employed to fit a second-order polynomial model in which 18 experiments were required for this procedure. The test variables chosen in this study were: aeration, cultivation temperature and inoculum cell concentration. Five levels of each variable were chosen, the upper and lower limits of them were set to be in the range described in the literature and also based on our previous experience. In the statistical model, *Y* denotes units of TGase activity (U g^{-1} of dried substrate).

Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. The enzyme activity values were determined from inoculation time (0 h) to 96 h of incubation, but the regression equation was constructed with the values obtained after 48 h, when maximal values of the TGase activity were achieved.

Table 1 Process variables used in the CCD, showing the treatment combinations and the mean of experimental responses

Treatment	Coded setting levels			Actual levels			TGase activity (U g^{-1} of dried substrate)			
	x_1	x_2	x_3	X_1	X_2	X_3	24 h	48 h	72 h	96 h
1	-1	-1	-1	0.2	30	6	0.72 ± 0.05	0.77 ± 0.06	0.55 ± 0.04	0.55 ± 0.04
2	-1	-1	+1	0.2	30	9	0.76 ± 0.05	1.06 ± 0.09	0.91 ± 0.09	0.83 ± 0.07
3	-1	+1	-1	0.2	44	6	0.22 ± 0.01	0.29 ± 0.02	0.29 ± 0.01	0.27 ± 0.01
4	-1	+1	+1	0.2	44	9	0.35 ± 0.02	0.42 ± 0.03	0.31 ± 0.02	0.30 ± 0.02
5	+1	-1	-1	0.8	30	6	0.82 ± 0.07	0.89 ± 0.08	0.49 ± 0.04	0.44 ± 0.01
6	+1	-1	+1	0.8	30	9	0.81 ± 0.05	0.95 ± 0.06	0.83 ± 0.03	0.52 ± 0.02
7	+1	+1	-1	0.8	44	6	0.42 ± 0.01	0.51 ± 0.03	0.46 ± 0.02	0.43 ± 0.02
8	+1	+1	+1	0.8	44	9	0.47 ± 0.03	0.77 ± 0.07	0.64 ± 0.06	0.59 ± 0.04
9	-1.68	0	0	0	37	7.5	0.09 ± 0.01	0.12 ± 0.01	0.29 ± 0.01	0.27 ± 0.01
10	+1.68	0	0	1	37	7.5	0.77 ± 0.05	0.82 ± 0.04	0.61 ± 0.05	0.57 ± 0.04
11	0	-1.68	0	0.5	25	7.5	0.21 ± 0.02	0.57 ± 0.03	0.53 ± 0.04	0.51 ± 0.04
12	0	+1.68	0	0.5	49	7.5	0.03 ± 0.00	0.18 ± 0.01	0.18 ± 0.01	0.15 ± 0.01
13	0	0	-1.68	0.5	37	5	0.78 ± 0.05	0.84 ± 0.07	0.50 ± 0.04	0.39 ± 0.04
14	0	0	+1.68	0.5	37	10	0.77 ± 0.08	0.96 ± 0.08	0.64 ± 0.06	0.59 ± 0.06
15	0	0	0	0.5	37	7.5	0.86 ± 0.07	0.91 ± 0.07	0.75 ± 0.05	0.65 ± 0.03
16	0	0	0	0.5	37	7.5	0.81 ± 0.06	0.88 ± 0.06	0.73 ± 0.03	0.51 ± 0.01
17	0	0	0	0.5	37	7.5	0.78 ± 0.07	0.82 ± 0.07	0.72 ± 0.05	0.54 ± 0.04
18	0	0	0	0.5	37	7.5	0.79 ± 0.05	0.81 ± 0.06	0.77 ± 0.04	0.60 ± 0.03

The results are the mean of three replications.

x_1, x_2 and x_3 are coded values. X_1, X_2 and X_3 are the actual values. X_1 = aeration (L min^{-1}); X_2 = temperature (ºC); X_3 = inoculum cell concentration ($\log_{10}$ CFU g^{-1} of DS).

$$x_1 = \frac{X_1 - 0.5}{0.3};\ x_2 = \frac{X_2 - 37}{7};\ x_3 = \frac{X_3 - 7.5}{1.5}$$

The experimental design is represented by a second-order polynomial regression model, Eq. (1), to generate contour plots:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Where: Y = response variable, β_0 = constant, β_i = coefficient for the linear effect, β_{ii} = coefficient for the quadratic effect, β_{ij} = coefficient for the interaction effect, and x_i and x_j = the coded level of variable X_i and X_j. The above quadratic equation was used to plot surfaces for the variables.

The test factors were coded according to the following equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \quad (2)$$

Where: x_i is the *coded* value and X_i is the *actual* value of the *i*th independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value [4].

All statistical experimental designs and results analyses were carried out using Statistica 7.0 software (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities $p(t)$ were determined by Student's *t*-test. The variance explained by the model is given by the multiple determination coefficient, R^2. For each variable, the quadratic models were represented as contour plots (2D).

Enzyme extraction

Enzyme recovery from cultivated medium was performed by the addition of 140 mL of distilled water to the total content of the bioreactors and extracted under agitation at 150 rpm during 30 min. The enzymatic extract was then centrifuged at 17,000 *g* for 15 min. The extract was filtered through a Whatman No. 1 filter paper to obtain a clear extract and assayed for TGase activity.

Assay techniques

The chemical composition of the substrates was determined as described by AOAC [2]. The initial moisture of cultures was determined by drying samples of saturated substrates at 105 ºC for 24 h. The pH value of the sample of cultures was assayed following dilution of 5 g in 50 ml of distilled water, and by using a pHmeter DM 20

(Digimed, Brazil). The water activity was determined using an Aqua Lab CX-2 Water Activity System (Decagon Devices, USA). Reducing sugars were measured according to Miller [15]. Proteolytic activity was measured by the azocasein method, according to Sarath et al. [26], in which one enzymatic unit (U) is defined as the amount of enzyme needed to produce the change in one unit of absorbance under the conditions of the method (40 ºC, 30 min). TGase activity was determined by the hydroxamate procedure [8], in which a calibration curve is prepared with L-glutamic acid γ-monohydroxamate and one enzymatic unit (U) causes the formation of 1 μmol hydroxamic acid per minute at 37 °C. Enzyme was expressed as specific activity (U g^{-1} of dried substrate).

Results and discussion

Selection of substrate

Substrates provide the required energy and nutrients for the microorganism to grow and produce the desired metabolites [21]. The selection of a suitable solid substrate for the production of enzyme is a primary-key factor, which involves the choice of agro industrial residues for microbial growth and product formation. In the present study we tested five different substrates, which are abundant worldwide: corn grits (CG), milled brewers rice (MBR), industrial fibrous soy residue (IFSR), soy hull (SH), and malt bagasse (MB). Their complete chemical composition, as dry matter, is shown in Table 2 and results for enzyme production by *B. circulans* BL32 on them are shown in Figure 1.

Table 2 Chemical composition of solid substrates tested for TGase production by *B. circulans* BL32

Components (%)	Substrates				
	CG	MBR	IFSR	SH	MB
Moisture	11.5	10.3	9.1	9.9	6.6
Ash	0.3	0.2	3.3	4.0	3.9
Proteins	7.0	6.6	23.3	10.8	19.1
Lipids	0.6	0.6	0.2	1.1	6.2
Total Carbohydrates	80.6	82.3	64.1	74.2	64.2
Fibers	0.2	0.2	14.1	34.8	14.3
Reducing sugars	n.d.	n.d.	n.d.	n.d.	2.2
No-reducing sugars	n.d.	n.d.	2.7	2.3	2.5

The results are the mean of three replications.

n.d.: not detected

CG: corn grits; MBR: milled brewers rice; IFSR: industrial fibrous soy residue; SH: soy hull; MB: malt bagasse.

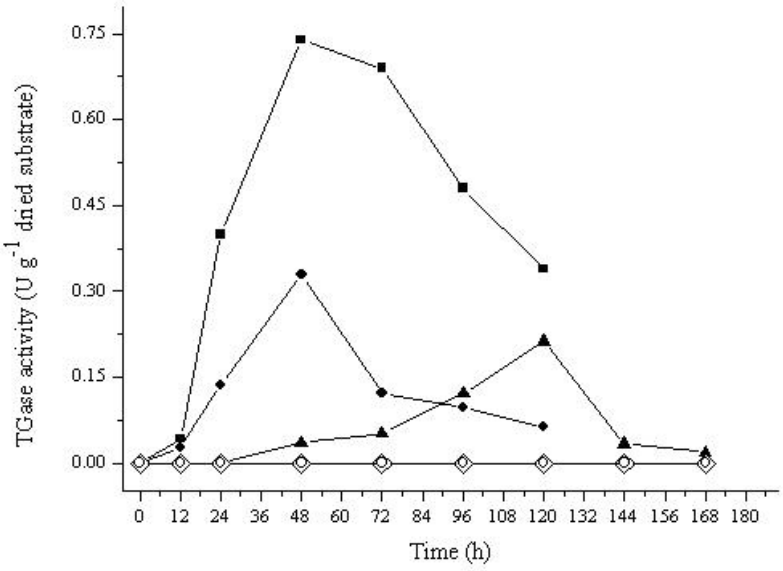

Fig. 1. SSC TGase production by *B. circulans* BL32 on different substrates. IFSR: industrial fibrous soy residue (■); MB: malt bagasse (●); SH: soy hull (▲); CG: corn grits (○); MBR: milled brewers rice (◊)

The highest TGase production (0.74 U g^{-1} of dried substrate) was achieved with IFSR after an incubation time of 48 h. Maximum TGase activities for MB and SH were 0.33 and 0.21 U g^{-1} of dried substrate after 48 h and 120 h, respectively, while for CG and MBR there were no detectable activities. The results suggest that the carbohydrate:protein ratio is an important factor for TGase production. The carbohydrate:protein ratio of enzyme-producing substrates are: IFSR (2.7), MB (3.4) and SH (6.9), whereas for the non-producing ones the ratios are CG (11.5) and MBR (12.5). In a previous work, we observed that C:N initial ratio of the culture medium influence in the TGase production by *B. circulans* BL32 [29]. The activity increases exponentially from 1 to 5 C:N ratio, suggesting that nitrogen limitation is important for enzyme production, probably by stimulating the sporulation process of cells.

Table 3 shows the initial conditions of cultivation for each of the tested substrates. The volume of added water was the matched capacity of absorption without free-water left. Although the differences in absorbed water, all substrates presented similar water activities (statistically significant at 95 %), and there were no important variations of pH at the beginning and during cultivations for all substrates, strongly suggesting that the higher enzymatic activity obtained with IFSR is due to nutritional composition of this substrate. Therefore, in subsequent experiments, IFSR was used as the substrate for the production of transglutaminase.

Table 3 Initial conditions of cultivation for each substrate tested for TGase production by *B. circulans* BL32

Assays	Substrates				
	CG	MBR	IFSR	SH	MB
pH	5.6	6.5	6.4	5.3	5.6
Moisture (% w/w)	78.2	74.8	89.4	74.9	74.3
Water activity	0.996	0.986	0.983	0.983	0.986
Reducing sugars (mg glucose g^{-1} substrate)	2.5	1.3	4.2	7.4	12.6

The results are the mean of three assays. CG: corn grits; MBR: milled brewers rice; IFSR: industrial fibrous soy residue; SH: soy hull; MB: malt bagasse.

Optimization of solid-state cultivation conditions

Cultivation factors that strongly influence microbial growth and enzyme activities by SSC are reported to be the temperature, aeration, incubation time and inoculum cell concentration [1, 9, 17, 21, 23]. Therefore, in this work, Central Composite Design (CCD) was used to investigate the best conditions of these three variables for TGase production by *B. circulans* BL32 and determined as growth kinetics.

The experimental design matrix and results obtained for enzyme activities are shown in Table 1. TGase activities values were determined in a spam time of 96 h of incubation, being the highest values of TGase specific activity observed in 48 h. Therefore, the regression equations were constructed considering this incubation time.

TGase activity varied from 0.12 to 1.06 U g^{-1} according to different levels of process parameters. The lowest values of TGase activity were obtained without aeration and at the highest incubation temperature (49 ºC). The upper temperature level tested probably impaired cell metabolism, while cultivation without aeration might be indicating either the need of air for cell metabolism and enzyme production, or the increased difficulty of heat dissipation, therefore negatively affecting the results. Heat dissipation in SSC bioreactors have been reported as major scale-up problems and must be adequately addressed [20, 22]. Treatment 2 (Table 1) showed the highest TGase specific activity (1.06 U g^{-1} of dried substrate), which was obtained at a moderate incubation temperature (30 °C). In SmC, the best temperatures for maximum transglutaminase activities by *B. circulans* BL32 were within the range of 28 and 36 °C (our results, not published yet). Meiying et al. [13] studying the TGase production by *S. mobaraense* in SmC, using a one-factor-at-a-time experimental procedure, testing temperatures in between 25 to 35 °C, have found highest enzyme production at 30 °C. For this same substrate, IFSR, Heck et al. [9] have shown that temperature significantly affects xylanase activities of *B. circulans*, the enzyme that is involved in the hydrolysis of xylans to xylo-oligosaccharides.

The significance of each regression coefficient was determined by *t*-values and *P*-values, listed in Table 4.

Table 4 Effect and coefficient estimates by the regression model for optimization of TGase production on SSC by *B. circulans* BL32

Independent variables (parameter)	Effect	Coefficient (β)	Standard error (β)	*t*-Value	*P*-value
Intercept (β_0)	0.8481	0.8481	0.0239	35.42	< 0.0001
x_1*	0.2573	0.1287	0.0260	9.92	0.0022
$x_1.x_1$*	-0.2106	-0.1053	0.0270	-7.81	0.0044
x_2*	-0.3421	-0.1710	0.0260	-13.18	0.0009
$x_2.x_2$*	-0.2777	-0.1389	0.0270	-10.30	0.0020
x_3**	0.1379	0.0690	0.0260	5.31	0.0130
$x_3.x_3$**	0.0935	0.0467	0.0270	3.47	0.0404
$x_1.x_2$**	0.1400	0.0700	0.0339	4.13	0.0258
$x_1.x_3$	-0.0250	-0.0125	0.0339	-0.74	0.5144
$x_2.x_3$	0.0100	0.0050	0.0339	0.29	0.7873

x_1, x_2 and x_3 are the coded values of variables aeration (L min^{-1}), temperature (ºC) and inoculum cell concentration ($\log_{10}$ CFU g^{-1} of DS), respectively.

* Statistically significant at 99 % of confidence level.

** Statistically significant at 95 % of confidence level.

Although the *P*-values for the negative coefficient for the linear effect of temperature ($P\ x_2 = 0.0009$), and the positive coefficient for the linear effect of aeration ($P\ x_1 = 0.0022$), were highly significant, the model also shows the significant interaction between them ($P\ x_1.x_2 = 0.0258$). Therefore, treating them separately may not reflect their real influence on the TGase production (e.g., optimum aeration changes along with incubation temperature). This interaction is essential and it would be of difficult solution using the *one-variable-at-a-time* approach and possibly explains the differences of results found in this work with those obtained by Soares et al. [27] in relation to the effect of aeration on TGase production by *B. circulans* BL32 by SSC.

The inoculum cell concentration was also an important factor for the production of TGase. The positive coefficient for the linear effect of inoculum cell concentration, with a value $P\ x_3 = 0.0130$, is highly significant. This result indicates that TGase production by *B.*

circulans increases with elevated initial inoculum cell concentration. The physiological role of microbial transglutaminase has recently been linked to the sporulation process in which it has a role in the cross-linking of proteins such as GerQ to generate high-molecular-mass proteins involved in the assembly of the spore coating [24, 34]. According to Kobayashi et al. [12] this enzyme is expressed during sporulation and plays a role in the assembly of the spore coat proteins of the genus *Bacillus* sp. Zhu et al. [33] reported that biomass accumulation is essential for enzyme production, due to the fact that TGase is produced before the sporulation phase. One possible explanation for the positive influence of inoculum size on TGase activity might be that more biomass is formed, triggering a faster sporulation process. TGase production contrasts with findings for other bio-products for which higher concentrations of inoculae are reported to be inhibitory when done by SSC. According to Adinarayana et al. [1] high inoculum cell concentrations are inhibitory for the production of cephalosporin C by *Acremonium chrysogenum* by SSC. The same was observed by Murthy et al. [17] for cyclosporine-A produced by *Tolypocladium inflatum*, and by Ramachandran et al. [25] for phytase produced by *Rhizopus* spp.. On the other hand, a 10 % (v/w) inoculum of 3.6 x 10^6 cells mL^{-1} was found to be optimum for xylanase production by *Bacillus pumilus* when using SSC with wheat bran as substrate [23].

The second-order effect of aeration, temperature, and inoculum cell concentration were highly significant ($P\ x_1 x_1 = 0.0044$, $P\ x_2 x_2 = 0.0020$ and $P\ x_3 x_3 = 0.0404$, respectively). The high significance of second-order variables in the model indicates that they can act as limiting factors. Therefore, even small variations in their values, will alter TGase production to a considerable extent.

Whenever possible, the model was simplified by the elimination of statistically insignificant terms. Then, the quadratic model should be reduced to:

$$Y = 0.8481 + 0.1287\ x_1 - 0.1053\ x_1 x_1 - 0.1710\ x_2 - 0.1389\ x_2 x_2 + 0.0690\ x_3 + 0.0467\ x_3 x_3 + 0.0700\ x_1 x_2 \quad (3)$$

Where: Y is the predicted response to the specific TGase activity (U g^{-1} of dried substrate), x_1 the aeration (L min^{-1}), x_2 the temperature (ºC) and x_3 is the inoculum cell concentration ($\log_{10}$ CFU g^{-1} of dried substrate) as coded settings.

Analysis of variance (ANOVA) was employed to determine the significance of the second-order polynomial model. The model was highly significant ($P < 0.01$), with an R^2 =

0.86, explaining 86 % of the total variation. This shows that equation 3 provides a suitable model to describe the response of the experiment relative to TGase production by SSC.

The contour shapes (Figure 2) were plotted on the basis of the model equation in order to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum TGase production by *B. circulans* BL32. In the Figure 2a is depicted the shapes contour of aeration versus temperature of incubation, showing that there is a significant mutual interaction between these two variables. The TGase production tends to increase with both moderate aeration and cultivation temperature. When incubation temperatures were set at the extremes of the studied interval, low TGase activities were obtained. Low temperature may lead to slow bacterium metabolism, whilst high temperatures might induce enzyme inactivation. Figure 2b shows contour plot of aeration against inoculum cell concentration. The production of TGase is increased for high inoculum cell concentration under moderate aeration. Figure 2c shows contour of temperature against inoculum cell concentration with results suggesting an optimum TGase production at temperatures between 27 and 35 ºC and again at high inoculum cell concentration. The non-elliptical nature of contour plots (Figures 2b and 2c) depicts that there is no mutual interaction between inoculum cell concentration and each of the other two variables.

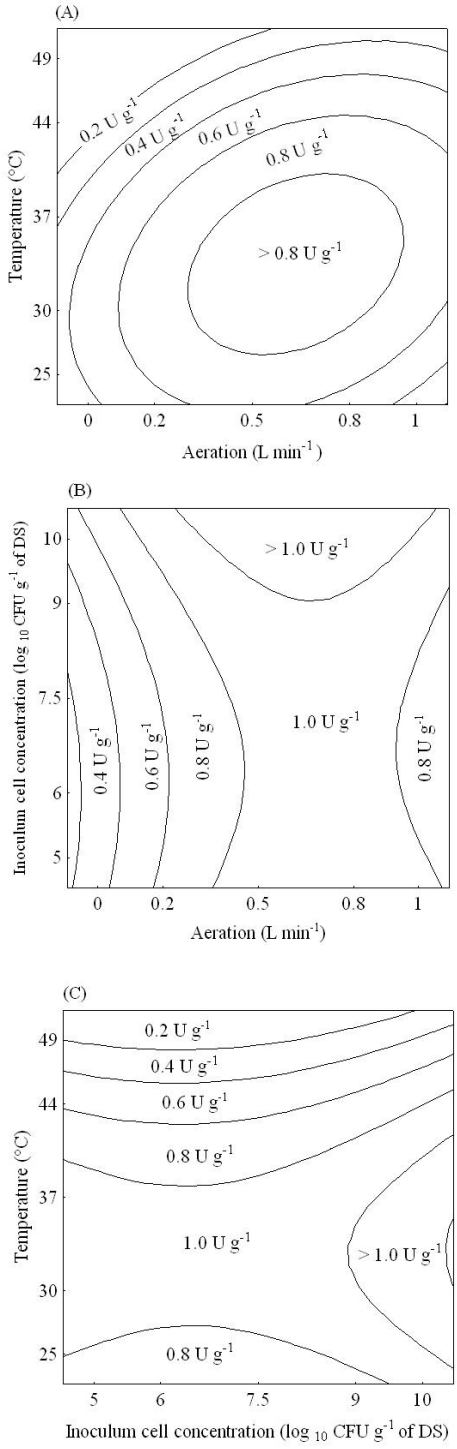

Fig. 2. Contour plot for the effect of (a) aeration x temperature, (b) aeration x inoculum cell concentration and (c) inoculum cell concentration x temperature on transglutaminase production by *B. circulans* BL32 under SSC. The variable that is not plotted is fixed at zero level in all of the three graphs.

Thus, the contour plots revealed that the optimal range of process values for TGase production by *B. circulans* on IFSR is: aeration, 0.55 – 0.65 L air min^{-1}; temperature, 30 - 37 ºC; and inoculum cell concentration, 9.5 – 10 $\log_{10}$ CFU g^{-1} DS. The polynomial model gives the optimal levels for the three process variables to be: aeration of 0.6 L air min^{-1}, temperature of 33 ºC, and inoculum cell concentration of 10 $\log_{10}$ CFU g^{-1} DS, with a predicted highest TGase production of 1.16 U g^{-1} of activity. The experimental validation of the model, at the optimized process parameters, produced TGase activity of 1.25 U g^{-1} of dried substrate (mean of three experiments). The specific activity of transglutaminase achieved under optimized culture conditions was 1.7 fold higher than the non-optimized conditions (0.74 U g^{-1} of dried substrate, Figure 1).

Production of TGase under the optimized conditions

Figure 3 shows the kinetics of *B. circulans* BL32 on IFSR. It shows that the enzyme production increased progressively with incubation time to a maximum of 1.28 U g^{-1} dried substrate at 48 h of cultivation. TGase activity declined during further incubation, which could have been due to either increased proteolytic activity during cultivation or the complete cell sporulation towards the end of the run. Variations in reducing sugars concentration and pH followed expected patterns of hydrolysis and liberation of basic aminoacids by proteolytic enzymes. However, the most important observation is the fact that, in submerged cultivations of *B. circulans* BL32 on starch [27] and on glycerol [29], it takes six to eight days for a maximum enzyme activity. In contrast, for the solid-state substrate bioprocess, this time is reduced to only two days, which is advantageous in terms of cost for enzyme production on a large scale. This effect might be explained by faster sporulation by SSC versus SmC. Moreover, in the SmC, the maximum TGase activity was 27.6 U g^{-1} reducing sugars (for starch [27]), whereas in SSC we obtained 47.4 U g^{-1} reducing sugars, which represents an increase of 42 % in productivity.

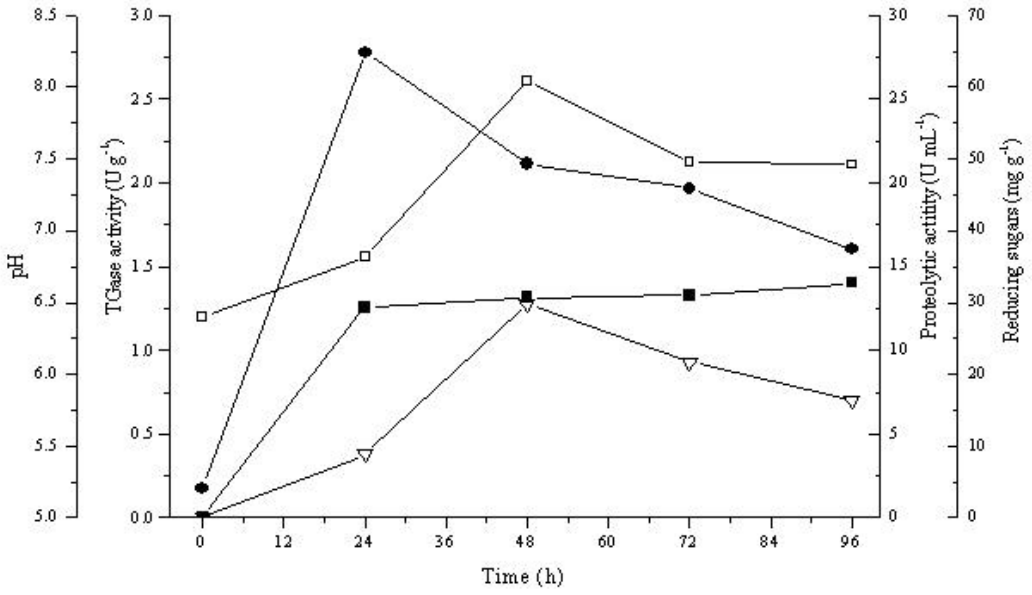

Fig. 3. Kinetics of TGase production by *B. circulans* BL32 under the optimal conditions suggested by the model. Aeration = 0.6 L min^{-1}; Temperature = 33 ºC; and Inoculum cell concentration = 10 $\log_{10}$ CFU g^{-1} of dried substrate. pH (□); TGase activity (▽); proteolytic activity (■); reducing sugars (●)

Conclusions

The search for alternative sources and the improvement of enzyme production and productivity are major goals of bioprocess engineering. These are essential features for cost reductions and cleaner production that ultimately leads to wider industrial uses of a specific enzyme. Microbial TGase is a case in particular, since it is entirely marketed by one single company from one single microorganism. *Bacillus circulans* BL32, recently isolated from the Amazonian environment, is a promising source for this highly valued enzyme. Furthermore, if SSC with agro-industrial residues used as substrates can be employed for enzyme production, then inexpensive and efficient bioprocess alternatives are possible. In the present work, several cultivation parameters were studied through the use of CCD, in order to improve TGase production by *Bacillus circulans* BL32 by SSC. The highest productivity of TGase (1.28 U g^{-1} of dried substrate) was achieved in only 48 h of cultivation, under optimized process parameters. This activity is 70 % higher than with non-optimized SSC conditions and 42 % higher than activities obtained in long time SmC.

Presented results show the potential of SSC using an abundant and inexpensive agro-residue (IFSR) as substrate, and also points to the use of *B. circulans* BL32 as an alternative biological source for the efficient production of TGase on a large scale, commercially viable process.

Acknowledgments

The authors wish to thank the Brazilian Bureau for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq) for the financial support of this research.

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Journal of Chemical Technology and Biotechnology *J Chem Technol Biotechnol* (2008)

Optimization of transglutaminase extraction produced by *Bacillus circulans* BL32 on solid-state cultivation

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Abstract

BACKGROUND: This paper reports investigations of the extraction of transglutaminase (TGase) produced by *Bacillus circulans* BL32 on solid-state cultivation in order to obtain a crude extract with the highest possible specific activity. The optimization of downstream processing parameters for the effective recovery of the enzyme was carried out using response surface methodology based on the central composite rotatable design (CCRD) to reduce losses in the cultivated solids and to obtain a crude extract as concentrated as possible. Several solvents and temperatures were tested, followed by a 2^3 factorial design performed to optimize conditions extraction time, mechanical agitation, and solid:liquid ratio.

RESULTS: The mathematical model showed that solid:liquid ratio has a significant negative effect on transglutaminase recovery. The optimal conditions for extraction were: water as solvent at 7 °C; 5 min extraction time; agitation speed 250 rpm; and 1:6 solid:liquid ratio. Under these conditions the model predicts a maximum response of 0.291 U mg^{-1} of protein of transglutaminase activity recovery, very closely matching experimental activity of 0.285 U mg^{-1} of protein. TGase recovery achieved under the optimized extraction conditions, according to the CCRD, was 2.5-fold higher than that obtained under non-optimized conditions previously employed.

CONCLUSION: Results show that TGase can be produced in cheap solid state cultivations and the optimization of its downstream processing parameters can improve enzyme recovery in crude extracts and may have important impacts on enzyme costs.

Keywords: transglutaminase; *Bacillus circulans* BL32; solid-state cultivation; industrial fibrous soy residue; enzyme recovery; extraction optimization

INTRODUCTION

Transglutaminases (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) are a family of enzymes that catalyze acyl transfer reactions using peptide-bonded glutamine residues as acyl donors and several primary amines as acceptors. These reactions introduce covalent cross-links between proteins, as well as peptides and various primary amines.[1] TGase has been isolated from animal and plant tissues and from microorganisms.[2] Microbial transglutaminases have found widespread and growing applications in the food processing industry. Recent research has shown that TGase can be used in many other applications, such as pharmaceuticals,[3] textiles,[4] tissue engineering,[5] and in the formation of immunoconjugates for ELISA tests and biosensors.[6] At present, only the microbial TGase from actinomycete *Streptoverticilium mobarense* has found commercial applications.[2] Optimal commercial uses of this enzyme are not being achieved owing to its low yield and because some of its characteristics, such as its pH and temperature optima, are not consistent with a variety of substrates and processes.[3] However, in the last few years efforts are been made to search for new sources of microbial transglutaminases,[7] along with the improvement of cultivation techniques and downstream processing[8–13] in order to boost productivity and reduce production costs. Most of the research has been carried out using microbial submerged cultivations (SmC) to produce TGase, which is also the industrial standard for its commercial preparation.

Recently, the use of solid-state cultivation (SSC) to produce TGase was reported.[14] The SSC system

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(*Received 15 January 2008; revised version received 29 February 2008; accepted 4 March 2008*)
DOI: 10.1002/jctb.1948

 J Chem Technol Biotechnol 0268–2575/2008/$30.00

offers many economic and engineering advantages over SmC, such as the possibility to use low-cost substrates and higher product concentrations. It has gained interest in recent years and has often been employed for the production of metabolites.[15,16] The advantages, however, have to be maintained during product recovery, since diluted extractions could dramatically increase product costs.[17] Therefore, in the development of industrial bioprocesses based on SSC, determination of the best conditions for enzyme recovery from the cultivated solids is of critical importance to obtain concentrated crude extracts, thus reducing downstream processing costs.[18] Unfortunately, only a few systematic studies are available dealing with the extraction of enzymes using SSC.[17–21]

Investigations of enzyme extraction that use conventional single factor optimization do not account for the interactions among these variables, are time consuming and require a large number of experiments to determine the optimal value of each factor.[22] The response surface methodology (RSM) is a time saving method, which reveals the interactions that occur among the different extraction conditions evaluated,[23] building models and evaluating the effects of several factors to obtain high productivity bioprocesses.

In this work, RSM was employed for planned statistical optimization to establish the optimal conditions for solvent, temperature, time of extraction, agitation, and solid:liquid ratio for TGase extraction. *Bacillus circulans* BL32 was recently isolated from the Amazon aquatic environment. Solid state cultivation studies were carried out using industrial fibrous soybean residue from soy isolated protein production as substrate. This material is a cheap agro-industrial residue that can be obtained in very large amounts and, if successfully applied as substrate in SSC, could reduce the overall production cost of many different enzymes.

MATERIAL AND METHODS

Microorganism

A strain of *B. circulans*, coded BL32 was used in this study. Its isolation and characterization have been described elsewhere.[11,13] Before culture, cells were recovered from frozen stocks in glycerol and were kept at 4 °C on Mueller–Hinton agar (Merck Darmstad, Germany) plates.

Substrate, medium, and inoculum

Industrial fibrous soy residue (IFSR), chemically defined elsewhere,[24] was used as solid substrate for cultures. Basic liquid medium, used as inoculum, had the following composition (g L^{-1}): 0.2 $MgSO_4$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.02 $CaCl_2$, and 0.05 $FeCl_2$. Inocula for all experiments on the SSC bioreactor were prepared in 1000 mL Erlenmeyer flasks filled with 160 mL of Luria–Bertani (LB) medium. Flasks were inoculated with a single colony from agar plates and incubated for 24 h at 37 °C and 150 rpm.

Bioreactor cultivation conditions

Bioreactor experiments were carried out in a 500 mL cylindrical bioreactor (60 mm diameter and 170 mm height) designed and constructed in the laboratory. Bioreactors were loaded with 20 g of dry IFSR soaked in 120 mL of basic liquid medium and inoculated with 20 mL of inoculum. Cultures were run at 33 °C, for 48 h. During cultivation, wet sterile air was supplied at a constant flow rate of 0.6 L min^{-1}.

Influence of extraction parameters on the recovery of transglutaminase

Five solvents (water; 6.06 g L^{-1} Tris–HCl buffer pH 8; 6.80 g L^{-1} acetate buffer pH 5; 10% solution of ethanol; or 10% solution of glycerol) pre-incubated at the tested extraction temperatures (7, 25 or 45 °C) were added to Erlenmeyer flasks containing the cultivated solids at a solid:liquid ratio of 2 g substrate (dry basis) per 20 mL solvent and mechanically stirred for 40 min at 150 rpm. Subsequently, solids were separated from the extract by centrifugation at 17 000 *g* for 15 min at 4 °C. The extract was filtered through Whatman No. 1 filter paper to obtain a clear extract and assayed for TGase activity. Data were analyzed using ANOVA procedure and comparisons of means were performed using Tukey's test.

In order to determine the influence of extraction parameters a central composite rotatable design[22,23] (CCRD), with $k = 3$, was used to generate 17 treatment combinations, with time of extraction, agitation, and solid:liquid ratio as independent variables. Five levels of each variable were chosen. The upper and lower limits were set in the range described in the literature and also based in previous experience. In the statistical model, Y denotes units of TGase specific activity (U mg^{-1} of protein). Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. This design is represented by a second-order polynomial regression model, Equation (1), to generate contour plots:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \qquad (1)$$

where Y = response variable, β_0 = constant, β_i = coefficient for the linear effect, β_{ii} = coefficient for the quadratic effect, β_{ij} = coefficient for the interaction effect, and x_i and x_j = the coded level of variable X_i and X_j. The above quadratic equation was used to plot surfaces for the variables.

The test factors were coded according to the following equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \qquad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the ith independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value.

Table 1. Process variables used in the CCRD, showing the treatment combinations and the mean experimental responses

	Coded setting levels			Actual levels			
Treatment	x_1	x_2	x_3	X_1	X_2	X_3	TGase specific activity (U mg^{-1} of protein)
1	−1	−1	−1	20	90	1:8	0.192 ± 0.011
2	−1	−1	+1	20	90	1:14	0.104 ± 0.008
3	−1	+1	−1	20	210	1:8	0.199 ± 0.015
4	−1	+1	+1	20	210	1:14	0.104 ± 0.005
5	+1	−1	−1	60	90	1:8	0.190 ± 0.009
6	+1	−1	+1	60	90	1:14	0.133 ± 0.007
7	+1	+1	−1	60	210	1:8	0.173 ± 0.008
8	+1	+1	+1	60	210	1:14	0.100 ± 0.002
9	−1.68	0	0	5	150	1:11	0.132 ± 0.009
10	+1.68	0	0	75	150	1:11	0.163 ± 0.010
11	0	−1.68	0	40	50	1:11	0.129 ± 0.006
12	0	+1.68	0	40	250	1:11	0.133 ± 0.006
13	0	0	−1.68	40	150	1:6	0.249 ± 0.019
14	0	0	+1.68	40	150	1:16	0.092 ± 0.005
15	0	0	0	40	150	1:11	0.136 ± 0.003
16	0	0	0	40	150	1:11	0.130 ± 0.008
17	0	0	0	40	150	1:11	0.128 ± 0.005

Results are the mean ± standard deviation of three experiments.
x_1, x_2 and x_3 are coded values.
X_1, X_2 and X_3 are the actual values.
X_1 = time (min); X_2 = agitation (rpm); X_3 = solid:liquid ratio.
$x_1 = \frac{X_1 - 40}{20}$; $x_2 = \frac{X_2 - 150}{60}$; $x_3 = \frac{X_3 - 1:11}{1:3}$.

All statistical experimental designs and results analyses were carried out using Statistica 7.0 software (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included Fisher's F-test (overall model significance), its associated probability $P(F)$, correlation coefficient R, determination coefficient R^2, which measures the goodness of fit of a regression model. It also includes the Student's t-value for the estimated coefficients and associated probabilities, $P(t)$. For each variable, the quadratic model was represented as contour plots. The error was calculated based upon the central point replications (pure error).

Transglutaminase activity assay

TGase activity was determined by the hydroxamate procedure.[25] A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One enzymatic unit (U) generates 1 μmol hydroxamic acid per minute at 37 °C. Enzyme was expressed as specific activity (U mg^{-1} of protein). For all experiments, the enzyme extract was obtained after 48 h of growth, which corresponds to the cultivation time with highest activity according to preliminary experiments (results not shown).

Protein determination

Protein concentration was determined according to the Lowry assay[26] against a standard curve of bovine serum albumin, fraction V (Sigma, USA).

RESULTS AND DISCUSSION

Solid-state cultivation is characterized by the absence of free water and by the fact that the recovery of its products requires their extraction from the solid cultivated medium.[16–18] Therefore, it is very important to study the operating conditions of the extraction. The first step of this work was to compare agitated and static (fixed bed) extractions to determine which would be best for enzyme recovery from the cultivated material. The static system recovered only 10% of enzyme specific activity compared with the agitated system (results not shown). It was also found that the recovery efficiency of the agitated system compared with that of static extraction was much higher than those reported by other authors. Ramakrishna *et al.*[27] found that amyloglucosidase recovery efficiency from moldy cultivations was only 8.5% higher in an agitated system when compared with a static bed. Heck *et al.*[28] showed that xylanase extraction produced by *B. circulans* BL53 with SSC using IFSR as substrate was only 20% higher in agitated systems compared with fixed bed systems. According to Ghildyal *et al.*[17] the higher leaching of enzymes in agitated system is probably related to a more efficient contact between solute and solvent, greater surface area of the solute for efficient mass transfer and enhanced diffusion of the solvent into the solid particles. Based on these considerations, stirred extraction was employed in the next steps of this work.

Influence of different solvents and temperatures on the transglutaminase extraction

Enzyme stability can be affected by both solvent and temperature during extraction and therefore it

Table 2. Selection of extraction solvent and temperature

Solvent	Transglutaminase specific activity (U mg^{-1} of protein)		
	7 °C	25 °C	45 °C
Water	0.124 ± 0.007^{a}	0.112 ± 0.006^{ab}	0.109 ± 0.008^{ab}
Tris buffer 6.06 g L^{-1} pH 8.0	0.113 ± 0.002^{ab}	0.113 ± 0.005^{ab}	0.114 ± 0.005^{ab}
Acetate buffer 6.80 g L^{-1} pH 5.0	0.110 ± 0.001^{ab}	0.092 ± 0.006^{c}	0.102 ± 0.001^{bc}
10% Ethanol	0.122 ± 0.005^{a}	0.104 ± 0.004^{bc}	0.099 ± 0.003^{bc}
10% Glycerol	0.104 ± 0.003^{bc}	0.101 ± 0.011^{bc}	0.105 ± 0.005^{bc}

Results are the mean ± standard deviation of three experiments.
Means followed by different letters are statistically different ($P < 0.05$).

is very important to define these two parameters.[20] Results for TGase extraction in different solvents and temperatures are shown in Table 2. Comparing the effects between temperatures of 25 and 45 °C for each solvent, Tukey's test showed that at 95% confidence level there was no significant difference. In most cases, transglutaminase extractions at 7 °C were higher than extractions at 25 and 45 °C, resulting in more concentrated extracts. The temperature can have two opposite effects on enzyme extraction: higher temperatures will increase protein solubility and diffusivity, producing higher activities in the extracts; on the other hand, enzymes are usually thermo inactivated, an effect that increases as temperature and contact time increase.[20] In relation to solvents, good results for enzyme recovery were obtained with water, 10% ethanol, and 6.06 g L^{-1} Tris buffer pH 8.0, probably due to the protective effect that this buffer has on enzyme stability.[13] Water and 10% ethanol, both at 7 °C, showed the best results for TGase extraction, 0.124 and 0.122 U mg^{-1} of protein, respectively. It has been suggested that the hydroxyl group of ethanol will form hydrogen bonds with protein molecules and this gives the stability of the enzyme molecule.[29] Glycerol produced low recovery activities, probably because this solvent has a higher viscosity than any other tested, which might have a negative effect on mass transfer from solid to the liquid phases.[20] The lowest TGase recovery was obtained with 6.80 g L^{-1} acetate buffer pH 5.0 at 25 °C (0.092 U mg^{-1} of protein), a result that contrasts with those presented by Heck *et al.*[28] and Castilho *et al.*,[20] who demonstrated that acetate buffer was a good solvent for xylanases and pectinases extraction on solid-state cultivation, respectively. This negative effect is probably due to the effect of low pH value (5.0), which has previously been shown to affect TGase, with extracts at this pH remaining with only 40% of its activity.[13] According to Castilho *et al.*[18] one important problem found in solid–liquid extractions is the loss of solute contained in the solvent that is retained in the solids. To reduce this, repeated extractions could be employed. However, the disadvantage of this method is that the resulting extract becomes too diluted. In order to minimize transglutaminase losses in the discarded solids, repeated extractions were performed with the five solvents but the extracts obtained from the second washing contained no TGase specific activity (results not shown).

Based on these results the extraction temperature was fixed at 7 °C, the solvent used was water, and only one extraction step was adopted for the subsequent experiments of transglutaminase recovery.

Optimization of extraction parameters on the recovery of transglutaminase

Many factors influence the efficiency of enzyme recovery from SSC, the most important being solid:liquid ratio, time and agitation.[16,20,28–32] A central composite rotatable design (CCRD) was used to investigate the best conditions of contact time, agitation and solid:liquid ratio for TGase recovery produced by *B. circulans* BL32 on solid-state cultivation.

The experimental design matrix and results obtained for enzyme specific activity are shown in Table 1. TGase specific activity varied in a range of 0.092 to 0.249 U mg^{-1} of protein according to the different levels of the extraction parameters. This variation reflected the importance of optimization for higher enzyme recovery.

The lowest values of TGase specific activity, 0.092 U mg^{-1} of protein (treatment 14), 0.100 U mg^{-1} of protein (treatment 8), and 0.104 U mg^{-1} of protein (treatment 2 and 4), were obtained for high solid:liquid ratio, suggesting that optimal enzyme extraction could be obtained in the lowest values of solid:liquid ratio. Treatment 13 showed the highest TGase specific activity (0.249 U mg^{-1} of protein). Treatment 13 (time, 40 min; agitation speed, 150 rpm; and solid:liquid ratio, 1:6) produced a specific activity approximately 2-fold higher than that obtained for treatment 11 (time, 40 min; agitation speed, 50 rpm; and solid:liquid ratio, 1:11), indicating that the increase in agitation during the extraction process and the decrease in solid:liquid ratio may favor transglutaminase extraction. Furthermore, the solid:liquid ratios used (1:6 or 1:11) promoted adequate enzyme solubilization without excessive liquid sorption to the fibers and was sufficient to penetrate throughout the cultivated solid matrix enabling the separation of enzyme from medium and microorganism. Contrasting with these results, Tunga *et al.*[29] reported that, when small solvent amounts

were used for alkaline protease extraction from wheat bran, the total activity decreased, while Singh *et al.*,[30] studying the extraction of pectinase from fermented bran cultivations of *Aspergillus carbonarius*, observed that low solvent volumes resulted in a marked decrease in extraction efficiency for this enzyme. It can be postulated that different enzymes, solid substrates for cultivation, and microorganisms, influence enzyme recovery, thus ideal conditions must be individually studied for each enzyme and system. In relation to the agitation effect, similar results were reported by Reddy *et al.*[33] studying the pullulanase extraction produced by *Clostridium thermosulfurogenes*.

The significance of each regression coefficient was determined by *t*-values and *P*-values, which are listed in Table 3. The *P*-value suggests that the negative linear effect of solid:liquid ratio is significant and this indicates that the volume of the solvent is the most influential factor for enhanced TGase recovery and confirms that specific activity decreases with elevated solid:liquid ratio. This observation is fundamental for downstream processing because it makes it possible to use smaller volumes of solvent in industrial processes. Inadequate lower ratios could lead to unsatisfactory recoveries, since a significant fraction of the solution could be retained in the cultivated mass,[30] but this was not observed in this work.

Second-order effects of time and solid:liquid ratio were also significant. The second-order effects of these variables indicate that they can act as limiting factors and even small variations in their values will impact transglutaminase extraction to a considerable extent.[34] The model clearly reveals significant interactions between extraction time and agitation and between extraction time and solid:liquid ratio (Table 3). Therefore, treating them separately may not reflect their real influence on the enzyme recovery. This information is essential and it would be difficult to obtain using the one-variable-at-a-time approach.

The quadratic model proposed is thus as follows:

$$Y = 0.1314 + 0.0036x_1 + 0.0054x_1.x_1 - 0.0027x_2 - 0.0004x_2.x_2 - 0.0423x_3 + 0.0135x_3.x_3 - 0.0071x_1.x_2 + 0.0066x_1.x_3 - 0.0029x_2.x_3 \quad (3)$$

where Y is the predicted response to the TGase specific activity (U mg^{-1} of protein), x_1 is the time (min), x_2 is the agitation (rpm), and x_3 is the solid:liquid ratio as coded settings.

Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA) and data is presented in Table 4. The computed F-value (31.51) was highly significant ($P < 0.0001$). The goodness of a model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient ($R^2 = 0.98$) implies that a sample variation of 98% for extracted enzyme activity is attributed to the independent variables, and can be explained by the model. The closer the value of R (correlation coefficient) to 1, the better the correlation between the experimental and predicted values. Here the value of R (0.99) suggests an excellent representation of the process model and a very good correlation between the experimental results and the theoretical values predicted by the model equation. This shows that the model, as expressed in Equation (3), is suitable to describe TGase extraction from SSC.

The contour shapes shown in Fig. 1 were plotted on the basis of the model equation and show the interaction among the variables for optimal TGase recovery. Each contour curve represents a number of combinations of two tested variables with the other maintained at their respective zero level.[35] Analysis of the contour shapes for extraction time, agitation, solid:liquid ratio and concentration of enzyme in the extract reveals that there are mutual

Table 4. Analysis of variance (ANOVA) for the model regression

Source	SS[a]	df[b]	MS[c]	F-value	*P*-value
Model	0.0279	9	0.0031	31.51	<0.0001
Residual	0.0007	7	0.0001		
Total	0.0286	16			

[a] SS: sum of squares.
[b] df: degrees of freedom.
[c] MS: mean square; R = 0.99; R^2 = 0.98.

Table 3. Effect and coefficient estimates by the regression model for optimization of TGase extraction on SSC

Independent variables (parameter)	Effect	Regression coefficient (β)	Standard error (β)	*t*-value	*P*-value
Intercept (β_0)	0.1314	0.1314	0.0024	54.78	0.0003
x_1	0.0072	0.0036	0.0011	3.19	0.0856
$x_1 x_1$*	0.0108	0.0054	0.0012	4.36	0.0488
x_2	−0.0053	−0.0027	0.0011	−2.36	0.1425
$x_2 x_2$	−0.0009	−0.0004	0.0012	−0.35	0.7621
x_3**	−0.0845	−0.0423	0.0011	−37.51	0.0007
$x_3 x_3$**	0.0271	0.0135	0.0012	10.92	0.0083
$X_1 x_2$*	−0.0143	−0.0071	0.0015	−4.84	0.0401
$x_1 x_3$*	0.0133	0.0066	0.0015	4.50	0.0460
$x_2 x_3$	−0.0058	−0.0029	0.0015	−1.95	0.1900

x_1, x_2 and x_3 are the coded values of variables time (min), agitation (rpm) and solid:liquid ratio, respectively.
* Statistically significant at 95% of confidence level.
** Statistically significant at 99% of confidence level.

interactions among the variables studied. Figure 1(a) shows the enzyme recovery in the extract with different contact time and agitation. Maximal enzyme extraction was obtained at low solid/solvent contact time and high agitation speed or at high solid/solvent contact time and low agitation speed. It was observed that increasing contact time and agitation also increased the viscosity of extract, probably due to exopolysaccharides produced by *B. circulans* BL32, as has been demonstrated elsewhere.[14] This phenomenon was also reported by Singh *et al*.[30] and is highly problematic in purification procedures because it can interfere with chromatographic columns. Figure 1(b) (shows the contour plot of time against solid:liquid ratio, demonstrating that the recovery of transglutaminase can be increased using low volumes of solvent with extraction times between 5 and 75 min. Tunga *et al*.[29] found the necessity for 3 h for total protease recovery produced by *Rhyzopus oryzae* cultivated in wheat bran. Figure 1(c) shows the contour for agitation speed against solid:liquid ratio, where the lowest solid:liquid ratio with agitation speed between 50 and 250 rpm led to increased TGase recovery. Analysis of the contour plots revealed that the optimal values for the different variables were the following: solid/solvent contact time 5 min, agitation speed 210–250 rpm, and solid:liquid ratio 1:6; or solid/solvent contact time 75 min, agitation speed 50–90 rpm, and solid:liquid ratio 1:6. Therefore, the system is flexible in terms of either extraction time or agitation speed, which might be useful for defining suitable industrial processes based on the cost of energy (agitation) or the equipment time used.

According to the optimized mathematical model, the optimal levels for the three extraction parameters obtained at the maximum point of the polynomial model were calculated to be: solid/solvent contact time, 5 min; agitation speed, 250 rpm and ratio solid:liquid, 1:6. Under these conditions the model predicts a maximum TGase activity response of 0.291 U mg^{-1} of protein. Other enzymes produced on SSC showed similar behavior during its extraction in relation to the low solid/solvent contact time. Singh *et al*.,[30] studying downstream processing parameters for pectinase recovery from SSC showed that solid/solvent contact times higher than 15 min resulted in decreased specific enzyme activity. According to Ghildyal *et al*.[17] longer extraction times may lead to loss in enzyme activity due to partial inactivation of the enzyme, extraction of denaturing agents, foam formation, or due to enzyme adsorption onto the finest particles of the solid medium. Another possible cause for TGase lower activities with longer extraction times might be explained by the fact that *B. circulans* BL32 produces some proteases on SSC (results not shown). The high agitation speed (250 rpm) for transglutaminase extraction is similar to the results obtained by Fernández-Lahore *et al*.[32] for acid protease recovery produced by *Mucor bacilliformis*, and by Singh *et al*.[30] for pectinase

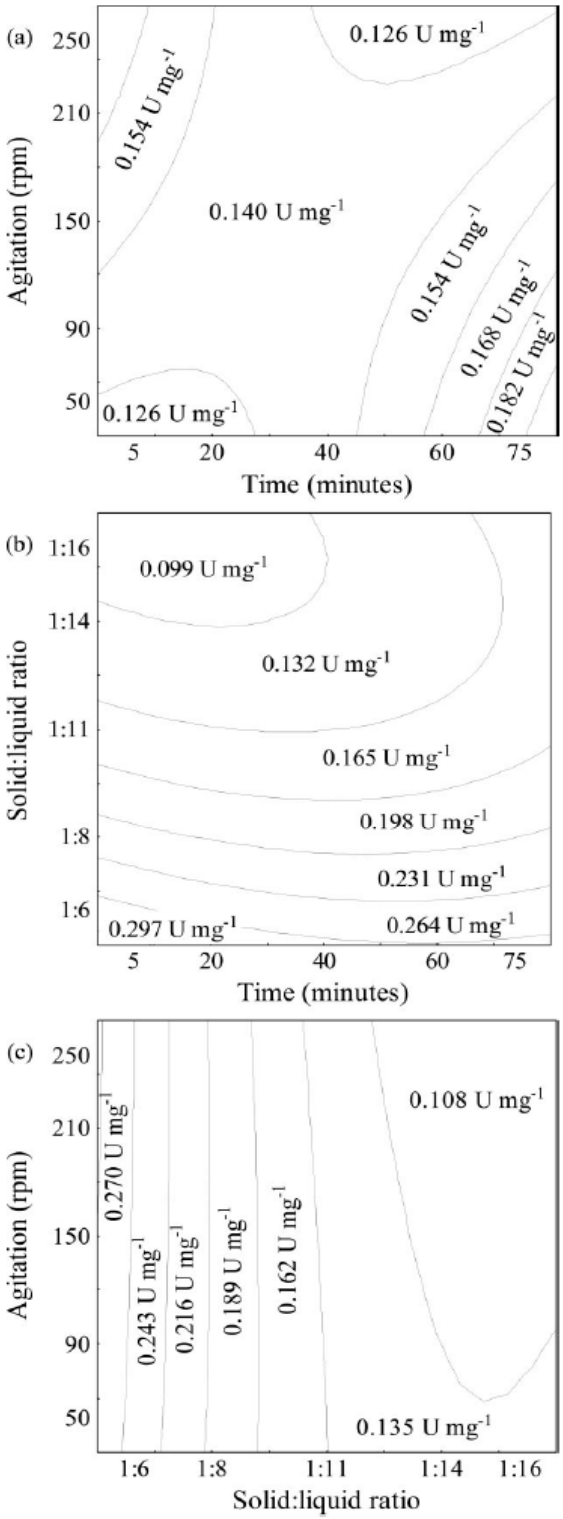

Figure 1. Contour plot for the effect of (a) time × agitation, (b) time × solid:liquid ratio, and (c) solid:liquid ratio × agitation on transglutaminase activity recovery. The variable not plotted is fixed at its zero level in all three graphs.

extraction from the fermented bran of *Aspergillus carbonarius*. In contrast, Heck *et al*.[28] reported that 150 rpm agitation speed was the best for xylanase extraction produced by *B. circulans* BL53 from cultivated solids.

In order to verify the predicted results, experiments were performed under the optimized process parameters levels, and the experimental value obtained was 0.285 U mg^{-1} of protein (mean of four runs). The excellent correlation between predicted and measured values in these experiments validates the response model and the existence of an optimal point. TGase recovery achieved under the optimized extraction conditions, according to the CCRD, was 2.5-fold higher than that obtained under previously non-optimized conditions employed (0.114 U mg^{-1} of protein at 30 min, 150 rpm and 1:8 solid:liquid ratio). This result is extremely important because concentrated crude extracts allows for easer downstream processing, reducing the time and cost of enzymes recovery.[20]

An interesting aspect of the research dealing with enzyme extraction produced on SSC is the great variation among results obtained by different authors.[16,20,28,30] Therefore, for each enzyme–substrate–microorganism combination, a very careful study of the process parameters that are involved in enzyme recovery is necessary.

CONCLUSIONS

Downstream recovery processes of enzymes with commercial applications are of paramount importance to improve productivity and to reduce costs. SSC is a promising technology for enzyme production, since it allows for controlled use of solvents, thus reducing volumes of enzyme extract prior to further steps of purification. In this work, the effects of different extraction conditions for TGase produced by *Bacillus circulans* BL32 under solid-state cultivation were studied Type of solvent, extraction temperature, solvent contact time with culture solids, agitation and solid:liquid ratio were evaluated. Water proved to be the best extractant for TGase, since it produced highly concentrated enzyme extracts. Being the cheapest possible solvent, this result is extremely interesting for the industry, significantly decreasing the final cost of purified TGase. Recovery conditions to obtain the most concentrated crude enzyme extracts were 7 °C, 5 min, 250 rpm, and 1:6 solid:liquid ratio, which are mild conditions regarding energy costs. The results obtained in this study will greatly facilitate further work on purification and characterization of TGase produced by *B. circulans* BL32 and possibly from other bacteria.

ACKNOWLEDGEMENTS

The authors wish to thank CNPq and CAPES for their financial support and scholarships.

J Chem Technol Biotechnol (2008)
DOI: 10.1002/jctb

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5.6. RESULTADOS VI. "Enzymatic properties of transglutaminase from a newly isolated *Bacillus circulans* BL32" - Journal of the Science of Food and Agriculture, submetido em julho de 2008.

Enzymatic properties of transglutaminase from a newly isolated *Bacillus circulans* BL32

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Abstract

BACKGROUND: We have recently reported the optimization of the production process of transglutaminase (TGase) from *Bacillus circulans* BL32, bacterium that was isolated from the Amazon basin region. An understanding of the properties of this enzyme may lead to better technological applications for it. Therefore, the purpose of this study was to characterize the TGase from *B. circulans* BL32.

RESULTS: Maximal activity was obtained over a range of pH 5.7-8.7 and temperature of 25-45 °C. This microbial TGase is stable at 50 °C, and over 90 % of its activity was kept after 120 min under this temperature. $CaCl_2$ and $MgCl_2$ exerted a positive effect on the enzyme activity and its thermal stability when in concentrations of up to 2 and 1 mol L^{-1}, respectively. Casein, isolated soy protein, and hydrolysed animal protein were treated with this TGase. The decrease in the amount of free amino groups, especially for the casein, demonstrated the cross-linking catalysed by this enzyme. The emulsifying properties of three proteins were improved after treatment with the enzyme.

CONCLUSION: The results demonstrated that this microbial TGase has a good potential to be used in food and non-food applications.

Keywords: Microbial Transglutaminase; *Bacillus circulans*; enzyme characterization; food proteins.

INTRODUCTION

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) is a family of enzymes that catalyze acyl transfer reactions using peptide-bonded glutamine residues as acyl donors and several primary amines as acceptors. These reactions introduce covalent cross-links between proteins, as well as peptides and various primary amines.[1] In the absence of amine substrates, TGase catalyses the deamidation of glutamyl residues during which water molecules are used as acyl acceptors. Substances having TGase activity are abundantly distributed in nature. Calcium-dependent TGases present in most animal or vegetable tissues and body fluids of vertebrates are involved in several biological processes.[2] TGase has been isolated from animal tissues, plants, and from microorganisms,[3] but microbial TGases are the most industrially important, mainly due to their properties, calcium independence, relatively low molecular weight, and because they are extracellular enzymes, which implies simpler separation and purification processes.[4] So far, the microbial TGase obtained from *Streptoverticillium mobaraense* remains the only commercial source of this enzyme.[5]

Interest in TGases has increased in recent years, mainly due to their applications in food industry. Much work has been carried out on the application of TGases in the processing of meat, fish, dairy, wheat, and soybean products, in order to improve texture, water-holding capacity, elasticity, nutritional value, and appearance.[2,4] This enzyme is also extensively used in biochemistry research, pharmaceutical, textile, and other chemical industries.[5]

The purification and preliminary characterization of this enzyme has been reported for bacteria and fungus, such as *Streptoverticillium ladakanum*,[6] *Streptoverticillium mobaraense*,[7] *Streptomyces hygroscopicus*,[8] *Bacillus subtilis*,[9] and *Physarum polycephalum*.[10] The utilization of microbial TGases has been limited by the high price of this enzyme. Moreover, as every industrial application may require specific properties of the biocatalysts, there is still an interest in finding new TGases that could create novel applications.

We have recently reported the purification and optimization of the production process of TGase from *Bacillus circulans* BL32, bacterium that was isolated from the Amazon basin region.[11,12] An understanding of the properties of this enzyme may lead to better technological applications for it. Therefore, the purpose of this study was to

characterize and partially purify TGase from *Bacillus circulans* BL32. In order to verify the potential use of this enzyme in food and non-food applications were obtained diverse information about its enzymatic properties. It was investigated the thermal stability, the influence of different salts and salt concentrations on both enzymatic activity and thermal stability of this TGase, the best temperature and pH conditions for enzyme activity, and the enzyme effect in the cross-linking process and emulsifying properties of casein, isolated soy protein, and hydrolysed animal protein.

MATERIALS AND METHODS

Materials

All the chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany), unless otherwise mentioned. N-carboxybenzoyl-L-glutaminyl-glycine (N-CBZ-Gln-Gly), bovine serum albumin (BSA), and trinitrobenzenesulphonic acid (TNBS) were obtained from Sigma-Aldrich Co. Ltd. (MO, USA). Q-Sepharose fast flow (FF) was from Pharmacia (Uppsala, Sweden). Casein was Spraynol produced by Coatec Industrial Ltd. (RS, Brazil). Isolated soy protein (ISP) was Supro 780 produced by The Solae Company S.A. (RS, Brazil). Hydrolysed animal protein (HAP) was obtained in our laboratory as mechanically deboned poultry meat treated with Flavourzyme protease from Novo Nordisk Bioindustrial do Brasil Ltd. (PR, Brazil) according to Soares *et al*..[13]

Microorganism and Inocula preparation

A strain of *Bacillus circulans*, coded BL32, which was isolated from the aquatic environment of the Amazon rain forest, was used in this study. Previously to culture, cells were recovered from frozen stocks in glycerol and were kept at 4 °C on Mueller-Hinton agar plates.

Erlenmeyer flasks (250 mL) containing 50 mL medium (M1), optimized in a previous work (Souza et al., 2006), composed of (g L^{-1}): glycerol 9.0, sucrose 2.0, peptone 7.0, tryptone 1.0, Na_2HPO_4 1.0, $MgSO_4.7H_2O$ 1.0 and $FeSO_4.7H_2O$ 0.1, were inoculated with a single colony from a stock culture and incubated at 30 °C in a rotatory shaker at 100 rpm and grown to optical density (OD) of 1.0 at 600 nm (approximately 20 h).

TGase production

Erlenmeyer flasks (1 L) containing 200 mL of M1 medium with pH adjusted to 8.5 were inoculated with 10 mL of inoculum. Cultivations were run at 30 ºC in a rotatory shaker at 100 rpm. After 8 days of cultivation the crude suspension was centrifuged at 4 ºC for 20 min at 17,000 *g* and the supernatant was used for the obtaining of the purified enzyme.

TGase purification

TGase from *B. circulans* BL32 was precipitated with 60-90 % ammonium sulfate (w/v) and, after dialysis, was applied to a Q-Sepharose fast flow (FF) ion-exchange column pre-equilibrated with 20 mmol L^{-1} Tris–HCl buffer, pH 8.0, followed by a linear gradient (0-1 mol L^{-1}) of NaCl.[11] Fractions containing the highest enzymatic activity were pooled, dialyzed against the 20 mmol L^{-1} Tris–HCl buffer pH 8.0 and lyophilized. This cleaned fraction of enzyme was then used for further analysis.

Determination of enzyme activity

TGase activity was determined by hydroxamate formation with the specific substrate, N-CBZ-Gln-Gly, described by Grossowicz *et al*..[14] A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One TGase unit (U) was defined as the amount of enzyme which causes the formation of 1 μmol L^{-1} glutamic acid γ-monohydroxamate per minute.

Protein determination

Protein concentration was determined according to the Lowry assay,[15] against a standard curve of bovine serum albumin, fraction V.

Thermal stability of the TGase

The temperature stability of enzyme was determined by incubating the enzyme solution at 30, 40, 50, 60, and 70 °C. Aliquots were withdrawn at intervals of 0, 1, 2, 5, 10, 20, 30, 60, and 120 min, placed in ice immediately and the enzyme residual activities were measured by using the method described above. Three replicates of each measurement were made. The enzymatic activity was expressed as percentage of relative activity. Activity without incubation was considered to be 100 %. The relative activity was calculated as:

Relative TGase activity, % = 100(C_t/C_0), (1)

where C_t is the activity at time t (min), and C_0 is the activity at time t = 0 min.

Effects of salts on stability of the TGase

NaCl, KCl, $CaCl_2$, and $MgCl_2$ were added to the enzyme solution at concentrations of 0, 0.25, 0.5, 1, 2, and 3 mol L^{-1}. After the incubation of salts with the enzyme for 30 min at 35, 40, 45, 50, and 55 °C, the samples were placed in ice immediately and TGase activity was determined as described above. Three replicates of each measurement were made. The enzymatic activity was expressed as percentage of relative activity. Activity without salts was considered to be 100 %.

Effects of pH and temperature on TGase activity

The effects of pH and temperature on the enzyme activity were determined by Central Composite Rotatable Design (CCRD), with k = 2, in order to generate 11 treatment combinations, with pH and temperature as independent variables. Five levels of each variable were chosen, the upper and lower limits of them set to be in the range described in the literature. In the statistical model, Y denotes units of TGase activity and the coded settings were defined as follows: x_1 = (pH − 6.5)/1.8; x_2 = (temperature − 40)/11.

Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and responses. Three replicates of each measurement were made. This design is represented by a second-order polynomial regression model (Eq. 2):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

Where: Y = response variable, β_0 = constant, β_i = coefficient for the linear effect, β_{ii} = coefficient for the quadratic effect, β_{ij} = coefficient for the interaction effect, and x_i and x_j = the coded level of variable X_i and X_j. The above quadratic equation was used to plot surfaces for the variables.

The test factors were coded according to the following equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \quad (3)$$

Where: x_i is the *coded* value and X_i is the *actual* value of the ith independent variable, X_0 is the actual value at the centre point, and ΔX_i is the step change value.

All statistical experimental designs and results analysis were carried out using Statistica 7.0 software (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's *F*-test (overall model significance), its associated probability *P*(*F*), correlation coefficient *R*, determination coefficient R^2, which measures the goodness of fit of regression model. It also includes the Student's *t*-value for the estimated coefficients and associated probabilities, *P*(*t*). For each variable, the quadratic model was represented as contour plots. The error was calculated based upon the central point replications (pure error).

Emulsifying and cross-linking properties

Polymerisation reaction

Casein, ISP and HAP were prepared in 20 mmol L^{-1} Tris–HCl buffer pH 7.2, centrifuged for 5 min at 10,000 *g* and soluble protein was determined according to the Lowry assay.[15] The concentration of soluble protein was fixed at 10 g L^{-1} and to these solutions enzyme was added at concentration of 0.5 U mL^{-1}. Reaction temperature was maintained at 32 °C for 60, 120 or 180 min. Enzyme reaction was stopped at 95 °C, 2 minutes. The emulsifying properties and the amount of free amino groups of these samples were determined according to the procedures described below.

Emulsifying properties

The emulsifying properties of samples were determined by the Pearce and Kinsella[16] method with the following modifications. Emulsions were prepared by mixing 3.0 mL of sample with 1.0 mL of maize oil and swirling for 1 min; after that time, 0.1 mL of this reaction was taken from the bottom of the tubes at different times and diluted with 4.9 mL of a 1 g L^{-1} solution of sodium dodecil sulfate (SDS). Absorbance was then measured at 500 nm. According to the method, emulsion stability (ES) is estimated by the permanence of the formed emulsion over 10 minutes, and expressed as ES (%) = ($Abs_{10\ min}$/ $Abs_{0\ min}$) x 100. Four replicates of each measurement were made.

Amino groups determination

The amount of free amino groups (N terminals or alpha-amines and ε-amino of lysine residues) of samples (incubated for 0, 60, 120 and 180 min with *B. circulans* BL32 TGase)

were determined by the trinitrobenzenesulphonic acid (TNBS) assay.[17] Protein samples of 0.25 mL were mixed with 1.75 mL of 0.2 mol L^{-1} phosphate buffer, pH 8.0, and 2.0 mL of a 0.5 g L^{-1} solution of TNBS. After 60 min of incubation at 50 °C in darkness, 4.0 mL of 0.1 mol L^{-1} HCl were added to stop the reaction. After cooling, absorbance was measured at 340 nm. Three replicates of each measurement were made. A calibration curve was prepared using l-leucine as standard. The values were expressed in amino equivalents of l-leucine/g of protein (μEq Leu-NH_2 g^{-1}).

RESULTS AND DISCUSSION

Thermal stability of the TGase

Thermal stability is a very important aspect when considering the industrial application of enzymes. Profiles of thermal stability of *B. circulans* BL32 TGase at 30, 40, 50, 60, and 70 °C are represented in Fig. 1.

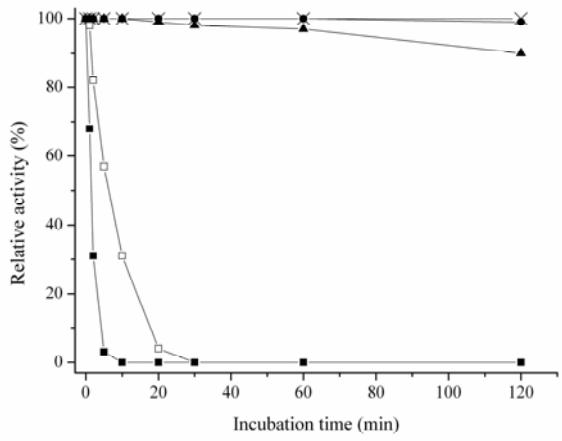

Fig. 1. Thermal stability of *B. circulans* BL32 TGase: (×) 30 °C, (●) 40 °C, (▲) 50 °C, (□) 60 °C, and (■) 70 °C.

The results shows that this TGase is well stable at 50 °C, even for incubation times up to 2 h. Activities will, however, sharply decrease at 60 and 70 °C, but still with 31 % of activity at 60 °C after 10 min incubation. The thermal stability of *B. circulans* BL32 TGase compared very well with other microbial TGases described in the literature. For instance, at 40 °C, its stability was similar as that demonstrated for *Streptoverticillium mobaraense*,[7] but it has shown higher stability than that from *Streptomyces hygroscopicus*, which kept 80 % of the initial activity when incubated at 40 °C for 30 min,[8] and that from

Streptoverticillium mobaraense WSH-Z2, which retained only 6 % of its activity at 60 °C for 10 min.[18] Under storage conditions of 4 °C for 30 days, and −18 °C for 6 months, no detectable loss of activity was observed for *B. circulans* BL32 TGase (results not shown), confirming its exceptionally good stability.

Effects of salts on stability of the TGase

The influence of different salts (NaCl, KCl, $CaCl_2$, and $MgCl_2$) and their concentrations on the enzymatic activity and thermal stability of *B. circulans* BL32 TGase are shown in Fig. 2. The enzymatic activity decreased in all samples for each salt concentration with the increasing of the temperature.

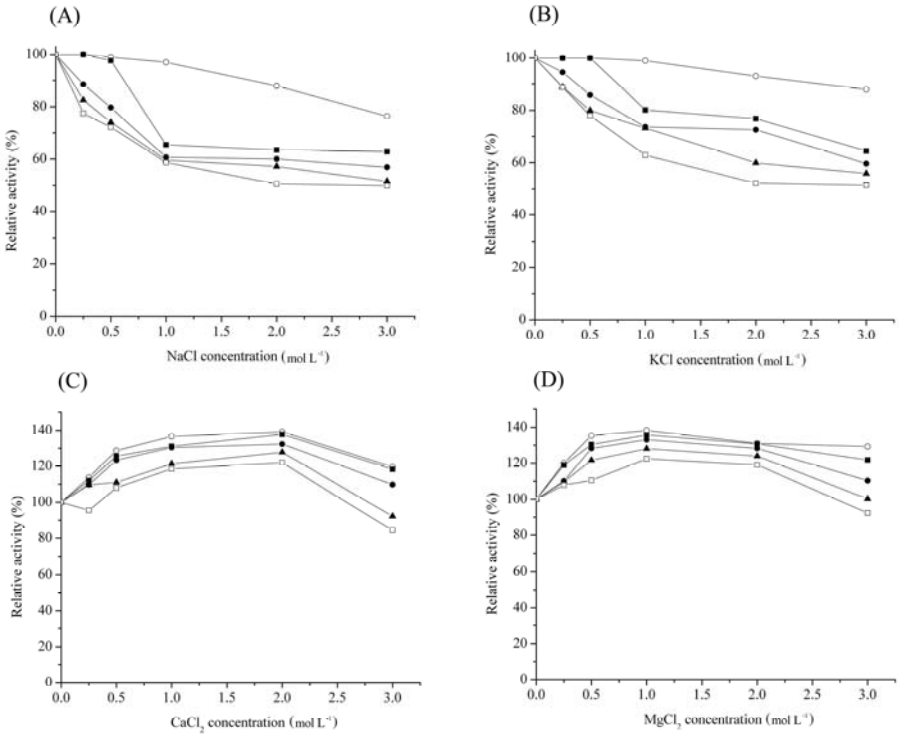

Fig. 2. Effects of the concentration of (A) NaCl, (B) KCl, (C) $CaCl_2$, and (D) $MgCl_2$ on the stability of *B. circulans* BL32 TGase at (○) 35 °C, (■) 40 °C, (●) 45 °C, (▲) 50 °C, and (□) 55 °C.

The TGase activity decreased with increasing NaCl (Fig. 2A) and KCl (Fig. 2B) concentrations for all tested temperatures. According to Kumazawa *et al*.[19] high concentrations of NaCl could induce conformational changes in the enzyme molecule, resulting in a decrease of TGase activity. Comparing with other TGases from different

sources, loss of activities when in presence of these salts, were reported by Worratao and Yongsawatdigul[20] for TGase from tilapia and by Ando *et al*.[7] for *Streptoverticillium mobaraense* TGase. On the other hand, the TGase activities from scallop, squid, and botan shrimp increased 11, 6, and 2-fold, respectively, by adding 0.5 mol L^{-1} of NaCl,[21] and Kütemeyer *et al*.[22] reported that addition of NaCl and KCl in concentrations of 5, 10, 15, and 20 %, lead to higher thermal stability of Activa WM microbial TGase, which is commercial preparation of the enzyme.

The activity of *B. circulans* BL32 TGase increased with Ca^{+2} and Mg^{+2} concentrations, and reached a plateau of maximum activity when the concentration of $CaCl_2$ (Fig. 2C) and $MgCl_2$ (Fig. 2D) were at 2 and 1 mol L^{-1}, respectively. The calcium-induced increase on TGase activity could be attributed to the conformational changes of the enzyme induced by this ion, which consequently exposed the cysteine located at the active site to a substrate.[23] As reported by Cui *et al*.[8] the activity from *Streptomyces hygroscopicus* TGase increased by 8 % by adding $CaCl_2$. In contrast, Activa WM microbial TGase showed a sharp decrease in its thermal stability due to presence of $CaCl_2$ but not for $MgCl_2$ at 50 and 55 °C.[22]

Effects of pH and temperature on TGase activity

CCRD was used to obtain the best conditions of both pH and temperature for TGase activity from *B. circulans* BL32. The experimental design and the results for enzyme activity are shown in Table 1.

Table 1. Process variables used in the CCRD, showing the treatment combinations and the mean experimental responses

Run No.	Coded setting levels		Actual levels		TGase activity
	x_1	x_2	X_1	X_2	(U mL^{-1})
1	-1	-1	4.7	29	0.46
2	-1	1	4.7	51	0.69
3	1	-1	8.3	29	1.74
4	1	1	8.3	51	0.68
5	-1.41	0	4.0	40	0.09
6	1.41	0	9.0	40	1.54
7	0	-1.41	6.5	25	2.54
8	0	1.41	6.5	55	0.98
9	0	0	6.5	40	2.33
10	0	0	6.5	40	2.26
11	0	0	6.5	40	2.20

x_1 and x_2 are coded values; X_1 (pH) and X_2 (temperature (^{0}C)) are the actual values.
$x_1 = (X_1 - 6.5)/1.8$; $x_2 = (X_2 - 40)/11$.

TGase activities varied from 0.09 to 2.54 U mL^{-1} according to different levels of the pH and temperature parameters. This variation reflects the importance of optimization for higher enzyme activity. Treatments 7 and 9–11 (central points) showed the highest levels of TGase activity (2.54, 2.33, 2.26, and 2.20 U mL^{-1}, respectively). These results show that the *B. circulans* BL32 TGase has a higher enzyme active at temperatures between 25 and 40 °C and pH around 6.5.

The significance of each regression coefficient was determined by *t*-values and *P*-values, which are listed in Table 2.

Table 2. Effect and coefficient estimates by the regression model for optimization of TGase activity

Independent variables (parameter)	Effect	Regression Coefficient	Standard error	*t*-Value	*P*-value
Intercept	2.2633	2.2633	0.0376	60.25	0.0003
x_1 *	0.8302	0.4151	0.0230	18.04	0.0031
$x_1.x_1$ *	-1.6458	-0.8229	0.0274	-30.06	0.0011
x_2 *	-0.7590	-0.3795	0.0230	-16.50	0.0037
$x_2.x_2$ *	-0.7008	-0.3504	0.0274	-12.80	0.0061
$x_1.x_2$ *	-0.6450	-0.3225	0.0325	-9.91	0.0100

* Statistically significant at 99 % of confidence level; x_1 and x_2 the coded values of variables pH and temperature (°C), respectively.

The *P*-values suggest that the positive linear effect of pH ($P\ x_1$ = 0.0031) and negative linear effect of temperature ($P\ x_2$ = 0.0037) were significant. The data indicate that increasing the pH and decreasing the temperature the TGase from *B. circulans* BL32 increases the activity for the catalytic reaction of CBZ-glutaminyl-glycine and hydroxylamine. Second-order effect of pH ($P\ x_1.x_1$ = 0.0011) and temperature ($P\ x_2.x_2$ = 0.0061) also were significant. The significance of variables on second-order model indicates that they can act as limiting factors and even small variations in their values will alter TGase activity to a considerable extent. The model clearly reveals significant interactions between pH and temperature ($P\ x_1.x_2$ = 0.0100). Therefore, treating them separately may not reflect their real influence on the TGase activity (e.g., optimum temperature activity changes along with pH). This information is essential and it would be of difficult solution using the *one-variable-at-a-time* approach.

We proposed then, that the quadratic model should be reduced to:

$$Y = 2.2633 + 0.4151\ x_1 - 0.8229\ x_1.\ x_1 - 0.3795\ x_2 - 0.3504\ x_2.\ x_2 - 0.3225\ x_1.\ x_2 \qquad (4)$$

Where: Y is the predicted response to the TGase activity (U mL^{-1}), x_1 is the pH and x_2 is the temperature (°C) as coded settings.

Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA) and data is presented in Table 3.

Table 3. Analysis of Variance (ANOVA) for the model regression*

Source	SS	Df	MS	F-value	*P*-value
Model	6.8434	5	1.3687	10.80	0.0103
Residual	0.6334	5	0.1267		
Total	7.4768	10			

* $R = 0.96$; $R^2 = 0.92$.

SS, sum of squares; Df, degrees of freedom; MS, mean square.

The computed F-value (10.80) was highly significant ($P = 0.0103$). The goodness of a model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient ($R^2 = 0.92$) implies that the sample variation of 92 % for enzyme activity is attributed to the independent variables, and can be explained by the model. The closer the value of R (correlation coefficient) to 1, better the correlation between the experimental and predicted values. Here the value of R (0.96) suggests an excellent representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation. This shows that the model, as expressed in Eq. 4, provides a suitable model to describe TGase activity.

The contour shapes are shown in Fig. 3. Contour plot indicates that the enzyme exhibited optimum activity for the catalytic reaction of CBZ-glutaminyl-glycine and hydroxylamine in the range of pH at 5.7–8.7 and temperature at 25-45 °C. The activity was found to decrease rapidly at acidic pH; however it decreased gradually at alkaline pH. The broad range of pH and temperature for activity suggests a good possibility of applying this enzyme in different food and non-food processes. The optimum pH of this enzyme was similar of that from *Streptoverticillium mobaraense*[7] and *Streptomyces hygroscopicus*.[8] However, it was different of TGase produced by *Streptoverticillium ladakanum* that has an optimal pH value in the range of 5.0 to 6.0.[6] In relation to the optimal temperature of this enzyme, similar value (40 °C) was observed by Ho *et al*.[6] for *Streptoverticillium ladakanum* TGase. In contrast, an optimal temperature of 60 °C was observed by Suzuki *et al*.[9] for *Bacillus subtilis* TGase.

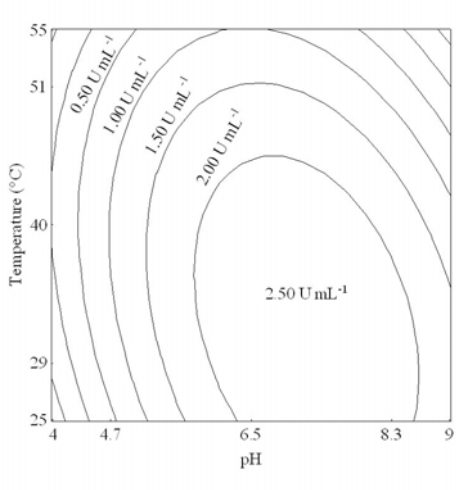

Fig. 3. Contour plot for the effects of pH and temperature on TGase activity from *B. circulans* BL32.

According to the optimized mathematical model, the optimal levels for the two TGase activity parameters obtained at the maximum point of the polynomial model were calculated to be: pH, 7.2 and temperature, 32 °C. Under these conditions the model predicts a maximum TGase activity response of 2.55 U mL^{-1}. In order to verify the predicted results, experimental validations were performed under the optimized process parameters levels, and the experimental value obtained was 2.48 U mL^{-1} (mean of three runs). The excellent correlation between predicted and measured values of these experiments confirms the response model and the existence of an optimal point.

The highest activity obtained according to the CCRD, was 25 % higher than the activity obtained under conditions usually described in the literature (2.04 U mL^{-1} at 37 °C and pH 6.0).[6,8]

Emulsifying and cross-linking properties

The effects of the *B. circulans* BL32 TGase treatment on the emulsifying properties of the casein, isolated soy protein (ISP), and hydrolysed animal protein (HAP) after 180 min of incubation are presented in Fig. 4.

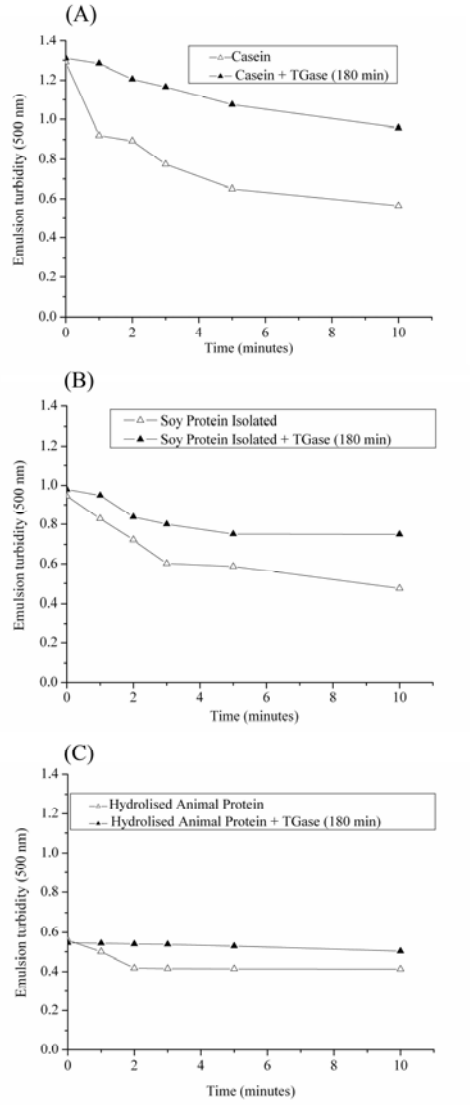

Fig. 4. Emulsifying properties of the (A) casein, (B) isolated soy protein (ISP), and (C) hydrolysed animal protein (HAP) without treatment and after 180 min of treatment with *B. circulans* BL32 TGase.

The emulsion stabilities (ES) for non-treated casein, ISP, and HAP were 43.7, 50.1, and 74.0 %, respectively. The formed emulsions of TGase treated casein, ISP, and HAP were more stable, ES of 73.1, 76.3, and 92.3 %, respectively, and presented higher turbidity values as compared to non-treated protein substrates. Soares *et al*.[24] also reported higher emulsion stability to casein and ISP treated with Activa TG-B TGase by 120 min in relation to protein without treatment, but HAP presented no considerable change in its emulsion stability after treatment with this commercial TGase. The ability of a protein to form an emulsion is related to its capacity of efficiently adsorbing and stabilizing the oil-

water interface. Dickinson *et al*.,[25] studying the ES of sodium-caseinate-stabilised emulsions, found that cross-linking by TGase prior to emulsification moderately improved the coalescence stability. According to Faergemand *et al*.[26] in emulsions stabilized by sodium caseinate and β-lactoglobulin, while extensive cross-linking by TGase reduced the emulsion stability, limited cross-linking was beneficial.

In order to evaluate the extent of the effect of *B. circulans* BL32 TGase in casein, ISP and HAP, the free amino groups of these proteins were quantified after 60, 120, and 180 min of the treatment (Fig. 5).

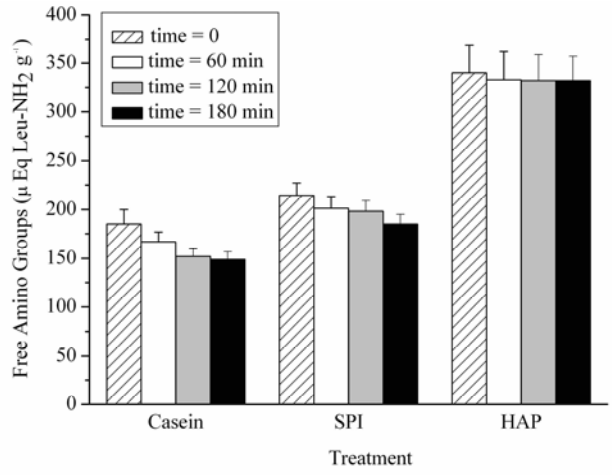

Fig. 5. Effect of treatment time with *B. circulans* BL32 TGase on the amount of free amino groups of casein, isolated soy protein (ISP), and hydrolysed animal protein (HAP).

TGase catalyses the reaction between an ε-amino group on protein-bound lysine residues and a γ-carboxyamide group on protein-bound glutamine residues, leading to covalent cross-linking of the proteins. Because of the involvement of the amino groups in the cross-linking reaction, a decrease in the amount of these groups would show that the enzyme is catalysing this reaction. Treatments of casein, ISP, and HAP with *B. circulans* BL32 TGase for 3 h, showed a reduction of free amino groups of 19.5, 13.6, and 2.4 %, respectively. The reduction in the amount of amino groups, especially for casein and ISP, confirmed the protein cross-linking catalysed by *B. circulans* BL32 TGase. A lower reduction in the amount of the free amino groups was reported by Soares *et al*.[24] when casein and ISP, 3 and 2 %, respectively, were treated with Activa TG-B TGase by 120 min.

However, a higher reduction in the amount of free amino groups was observed for the HAP of about 7 %.

The susceptibility of a protein to TGase-induced cross-linking depends on the macromolecular structure of the protein.[27] According to Flanagan and FitzGerald[28] the TNBS reagent may give an indication of the extent of TGase-catalysed cross-linking when cross-linking is the predominant reaction taking place. Probably, with the HAP protein the principal reactions catalyzed by *B. circulans* BL32 TGase were the deamidation and acyl-transfer reactions. Caseins is a good substrates for TGase, probably due to their low degree of tertiary structure, flexible, random-coil arrangement, and the absence of any disulphide bonds in the α_{s1}- and β-caseins, leaving the reactive groups exposed to the enzyme.[29] According to Siu *et al*.[30] the protein cross-linking process by TGase can result in the decreasing of the emulsifying activity and emulsion stability. The increase in the molecular weight of polypeptide chains may lead to some loss of flexibility and reduces the protein ability to unfold at the oil-water interface. In spite of the fact that the cross-linking process was higher for casein (Fig. 5), it did not cause reduction in the emulsion stability of this food protein; quite opposite, it showed the highest ES after the treatment with the *B. circulans* BL32 TGase (Fig. 4).

CONCLUSIONS

Microbial TGases can be employed in many attractive food and non-food applications. However, the expanded utilization of this enzyme has been limited by its high price, especially on cheap protein sources such as ISP. Cheaper sources of TGases are essential if industry is to benefit from the use of this enzyme in general processes. Although many reports on production and purification of microbial TGases have been published, little information about enzymatic characteristics and optimization of this enzyme activity is available. In this work, it was determined the best temperature and pH conditions for TGase activity of *B. circulans* BL32, a newly isolated strain, by the CCRD and regression analysis methods. Maximal activity was obtained over a range of pH at 5.7–8.7 and temperature at 25-45 °C. The broad range of pH and temperature for activity suggests the high possibility for applying this enzyme in different types and processes of food. This microbial TGase is stable at 50 °C and its good storage stability showed the possibility of using this microbial TGase in order to obtain commercial preparations of it. The addition

of bivalent ions increased the enzymatic activity and its thermal stability, and the emulsifying properties of the casein and ISP were improved after treatment by this enzyme. The results presented in this work demonstrated that the microbial TGase from *B. circulans* BL32 has a great potential for use in food and non-food applications. Further studies are granted to apply this enzyme in other food protein sources and to optimize and scale-up its bioreactor production.

ACKNOWLEDGEMENTS

This research was partially financed by grants from CNPq and CAPES.

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5.7. RESULTADOS VII. “Toxicological evaluation of transglutaminase from a newly isolated *Bacillus circulans* BL32” - Food Control, submetido em julho de 2008.

Toxicological evaluation of transglutaminase from a newly isolated *Bacillus circulans* BL32

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Abstract

Transglutaminase (TGase) from a *Bacillus circulans* strain isolated from the Amazon environment was submitted to short term *in vivo* and *in vitro* toxicological evaluations. No evidence of short term *in vivo* toxicity was found in the study subacute 14-day oral toxicity. There were no differences between the groups for relative weight gain of the rats, hematology and clinical chemistry values, and histopathological examination of organs in this study. It was not verified any evidence of cytotoxicity, genotoxicity and mutagenic effects on Chinese hamster lung fibroblasts cultured cells. The results suggest that the microbial TGase from *B. circulans* BL32 has a potential for use in the food industry.

Keywords: Microbial Transglutaminase; *Bacillus circulans*; toxicity.

1. Introduction

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) is a family of enzymes that catalyze acyl transfer reactions using peptide-bonded glutamine residues as acyl donors and several primary amines as acceptors. These reactions introduce covalent cross-links between proteins, as well as peptides and various primary amines (Nonaka et al., 1989). In the absence of amine substrates, TGase catalyses the deamidation of glutamyl residues during which water molecules are used as acyl acceptors. Substances having TGase activity are abundantly distributed in nature. Calcium-dependent TGases present in most animal or vegetable tissues and body fluids of vertebrates are involved in several biological processes (Nielsen, 1995). TGase has been isolated from animal tissues, plants, and from microorganisms (Kuraishi, Yamazaki, & Susa, 2001), but microbial TGases are the most industrially important, mainly due to their properties, calcium independence, relatively low molecular weight, and because they are extracellular enzymes, which implies simpler separation and purification processes (Zhu, Rinzema, Tramper, & Bol, 1995; Téllez-Luis, Ramírez, & Vázquez, 2004). So far, the microbial TGase obtained from *Streptoverticillium mobaraense* remains the only commercial source of this enzyme. The utilization of this microbial TGase has been limited by the high price of this enzyme. Moreover, as every industrial application may require specific properties of the biocatalysts, there is still an interest in finding new TGases that could create novel applications (Arrizubieta, 2007).

Interest in TGases has increased in recent years, mainly due to their applications in food industry. Much work has been carried out on the application of TGases in the processing of meat, fish, dairy, wheat, and soybean products, in order to improve texture, water-holding capacity, elasticity, nutritional value, and appearance (Nielsen, 1995). This enzyme is also extensively used in biochemistry research, pharmaceutical, textile, and other chemical industries (Bernard, Tsubuku, & Shioya, 1998; Jülicher, Haalck, Meusel, Cammann, & Spener, 1998; Collighan, Cortez, & Griffin, 2002; Cortez, Bonner, & Griffin, 2004).

We have recently reported the purification and optimization of the production process of TGase from *Bacillus circulans* BL32, bacterium that was isolated from the Amazon basin region (Soares, Assmann, & Ayub, 2003; Souza, Flôres, & Ayub, 2006). An understanding of the characteristics of this enzyme may lead to better technological

applications for it. Therefore, the purpose of this study was to evaluate some toxicity aspects as short term *in vivo* in a subacute toxicity study in rats of the partially purify TGase from *Bacillus circulans* BL32. We also determined its cytotoxicity, genotoxicity, using comet assay, and mutagenic effects, employing micronucleus test, in cultured mammalian cells to evaluate the safety of this enzyme in terms of genetic toxicity.

2. Materials and Methods

2.1. Materials

All the chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany), unless otherwise mentioned. N-carboxybenzoyl-L-glutaminyl-glycine (N-CBZ-Gln-Gly), Cytochalasin-B (Cyt-b), and methyl methanesulphonate (MMS) were obtained from Sigma-Aldrich Co. Ltd. (MO, USA). Q-Sepharose fast flow (FF) was from Pharmacia (Uppsala, Sweden). Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, L-glutamine, and antibiotics were purchased from Gibco BRL (NY, USA). Low-melting point agarose and agarose were obtained from Invitrogen (CA, USA).

2.2. Microorganism and Inocula preparation

A strain of *Bacillus circulans*, coded BL32, which was isolated from the aquatic environment of the Amazon rain forest, was used in this study. Previously to culture, cells were recovered from frozen stocks in glycerol and were kept at 4 °C on Mueller-Hinton agar plates.

Erlenmeyer flasks (250 mL) containing 50 mL medium (M1), optimized in a previous work (Souza et al., 2006), composed of (g/L): glycerol 9.0, sucrose 2.0, peptone 7.0, tryptone 1.0, Na_2HPO_4 1.0, $MgSO_4.7H_2O$ 1.0 and $FeSO_4.7H_2O$ 0.1, were inoculated with a single colony from a stock culture and incubated at 30 °C in a rotatory shaker at 100 rpm and grown to optical density (OD) of 1.0 at 600 nm (approximately 20 h).

2.3. TGase production

Erlenmeyer flasks (1 L) containing 200 mL of M1 medium with pH adjusted to 8.5 were inoculated with 10 mL of inoculum. Cultivations were run at 30 ºC in a rotatory shaker at 100 rpm. After 8 days of cultivation the crude suspension was centrifuged at 4 ºC

for 20 min at 17,000 *g* and the supernatant was used for the obtaining of the purified enzyme.

2.4. TGase purification

TGase from *B. circulans* BL32 was precipitated with 60-90 % ammonium sulfate (w/v) and, after dialysis, was applied to a Q-Sepharose fast flow (FF) ion-exchange column pre-equilibrated with 20 mM Tris–HCl buffer, pH 8.0, followed by a linear gradient (0 - 1 M) of NaCl (Soares et al., 2003). Fractions containing the highest enzymatic activity were pooled, dialyzed against the 20 mM Tris–HCl buffer pH 8.0 and lyophilized. This cleaned fraction of enzyme was then used for further analysis.

2.5. Determination of enzyme activity

TGase activity was determined by hydroxamate formation with the specific substrate, N-CBZ-Gln-Gly, described by Grossowicz, Wainfan, Borek, & Waelsch (1950). A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One TGase unit (U) was defined as the amount of enzyme which causes the formation of 1 μmol L-glutamic acid γ-monohydroxamate/min.

2.6. Toxicity study

2.6.1. In vivo assay

In order to study the toxicity from microbial TGase produced by *B. circulans* BL32, a subacute 14-day oral toxicity in rats was performed. The test substance used for the toxicology study described in this paper is a liquid enzyme preparation. In order to obtain a sufficiently high TGase concentration for this study, enzyme preparations were lyophilized to the desired concentration and administered to 150 U/kg body weight/day.

2.6.1.1. Animal tests

Adult, 90 days old (200 – 280 g), male Wistar rats, obtained from the Central Animal House, Pelotas Federal University, Rio Grande do Sul, Brazil, were used in this study. The rats were randomly assigned into 2 groups, each consisting of 10 individuals. The animals were housed individually in stainless steel metabolic cages designed for separate collection of faeces and urine. Cages were located in a room with a 12 h light/dark

cycle, at a temperature of 21 ± 2 °C, fitted with an appropriate ventilation system. Food and water were given *ad libitum* during the experimental period. The control group received distilled water, by daily oral gavage (a volume of 10 mL/kg bw/day). The experimental group (*B. circulans* BL32 TGase group) received the liquid enzyme preparation, by daily oral gavage, at dose of 150 U/kg bw/day. The animals were observed daily for clinical signs. Feed consumption and body weights were measured daily throughout the study period. At the end of 14th day, final body weights of individual animal were recorded. The animals were anesthetized with a combination of 5 mg/kg xylazine (2 % xylazine chloride) and 90 mg/kg ketamine (5 % ketamine chloride) injected intramuscularly (Allen, Pringle, Smith, & Pasloske, 1998) and after abdominal incision, blood samples were collected of the cava vein from each rat. Hematology and serum clinical chemistry were carried out on all animals from each group using standard analytical methods at Laboratory of Veterinary Clinical Analysis (Faculdade de Veterinária, UFRGS, Porto Alegre, Brazil). Hematology parameters included red blood cell count (RBC), white blood cell count (WBC), hemoglobin concentration (Hb), hematocrit concentration (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and differential leukocyte count. Clinical chemistry parameters included total protein concentration, creatinine concentration (CRE), and alanine aminotransferase activity (ALT). Under anaesthesia, the diaphragm was incised to kill the animal. After sacrifices, liver, kidneys, spleen, thymus, adrenal glands, heart, lungs, and brain were carefully dissected and immediately weighed. The organ weight was related to body weight. This parameter was expressed as relative systemic organ weight.

2.6.1.2. Histological processing

All the animals in the TGase and control groups were taken for histopathology studies. The internal organs (liver, kidneys, spleen, thymus, adrenal glands, heart, lungs, and brain) from each rat were dissected. Representative fragments of each organ were fixed in 10 % formalin, embedded in paraffin, in order to obtain serial sections of approximately 4 μm, fixed on poly-lysine-coated glass slides, and de-paraffinised. The sections were stained with hematoxylin-eosin (H&E) and inspected on an Olympus AX70 Routine microscope adapted with a Nikon E 4500 camera (Zeiss) for any morphological changes in the tissues due to the consumption of *B. circulans* BL32 TGase preparation.

Photographs of representative organs from each group of rats were taken at Laboratory of Veterinary Pathology (Faculdade de Veterinária, UFRGS, Porto Alegre, Brazil). All stained slides and paraffin blocks were archived.

2.6.1.3. Statistical analysis

The data were expressed as mean ± standard error (SE). Statistical analysis of the data was carried out by Student's *t*-test and by repeated measure ANOVA. Differences were considered to be statistically significant when $P < 0.05$.

2.6.2. Evaluation of cytotoxicity, genotoxicity and mutagenic effects in mammalian cultured cells

2.6.2.1. Cell culture and treatment

V79 cells (Chinese hamster lung fibroblasts) were cultured under standard conditions in DMEM supplemented with 10 % heat-inactivated-FBS, 0.2 mg/mL L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were kept in tissue-culture flasks at 37 °C in a humidified atmosphere containing 5 % CO_2 in air, and were harvested by treatment with 0.15 % trypsin-0.08 % EDTA in phosphate-buffered saline solution (PBS). Cells were seeded (3 x 10^6 cells) in 5 mL complete medium in a 25-cm^2 flask, and grown for 2 days up to 60-70 % confluence before the treatment with the test substance. *B. circulans* BL32 TGase was added to FBS-free medium to achieve 150 U/treatment while the control group was exposed to solvent.

2.6.2.2. Cytotoxicity evaluation by colony-forming ability (Clonal survival)

Exponentially growing V79 cells were treated according to the experimental protocol. Thereafter, they were trypsinized, and 500 cells/60 mm dish were seeded in triplicates to determine colony-forming ability. An aliquot *B. circulans* BL32 TGase of 30 U/mL solution was thawed, diluted, and added to cells after attachment (4 h) to a final concentration of 150 U/treatment. After 5 days of incubation, colonies were fixed with ethanol, stained with Giemsa, counted, and their survival calculated as a percentage relative to control treatment.

2.6.2.3. Genotoxic evaluation using comet assay

Alkaline comet assay was performed as described by Singh, McCoy, Tice, & Schneider (1988) with minor modifications (Collins, 2004; Hartmann & Speit, 1997). V79 cells were incubated with *B. circulans* BL32 TGase for 3 h in FBS-free medium. After treatment, cells were washed with ice-cold PBS, trypsinized, and ressuspended in complete medium. Then, 20 μL of cell suspension (3 x 10^6 cells/mL) were mixed with 0.75 % low-melting point agarose, and immediately spread onto a glass microscope slide pre-coated with a layer of 1 % normal melting point agarose. Agarose was allowed to set at 4 °C for 5 min. Slides were incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1 % Triton X-100, and 10 % DMSO, pH 10.0) at 4 °C for at least 1 h to remove cell proteins, leaving DNA as "nucleoids". After the lysis procedure, slides were placed on a horizontal electrophoresis unit, covered with fresh buffer (300 M NaOH, 1 M EDTA, pH 13.0) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was performed for 20 min at 25 V (300 mA). All the above steps were conducted under yellow light or in the dark in order to prevent additional DNA damage. Slides were then neutralized (0.4 M Tris, pH 7.5), washed in bi-distilled water, and stained using a silver staining protocol. After drying at room temperature overnight, gels were analyzed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected, and analyzed for the concentration of each test substance. When selecting cells, the edges and cells around air bubbles were avoided (Collins, 2004). Cells were visually scored according to tail length into five classes: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length 1–2x the diameter of the head; (4) class 3: with a tail longer than 2x the diameter of the head and (5) class 4: comets with no heads. A value (damage index) was assigned to each comet according to its class. International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it is highly correlated with computer-based image analysis. The damage index is based on the length of migration and on the amount of DNA in the tail, and it is considered a sensitive DNA measurement. The other parameters, damage frequency (DF) and image length (IL), although considered in the analysis, were only used as complementary DNA damage parameters. Damage index ranged from 0 (completely undamaged: 100 cells x 0) to 400 (with maximum damage: 100 cells x 4) (Collins, 2004;

Hartmann & Speit, 1997; Tice et al., 2000; Burlinson et al., 2007). The damage frequency (%) was calculated based on the number of cells with tails versus those without tails. The vehicle was used as negative control.

2.6.2.4. Clastogenic evaluation using micronucleus test

The micronucleus assay in binucleated cells was performed according to Bonacker et al. (2004), with modifications by Fenech (2000). V79 cells were incubated with *B. circulans* BL32 TGase for 3 h in FBS-free medium. Cultures were then washed twice with medium, and Cyt-B was added to a final concentration of 2 μg/mL. Cultures were harvested 21 h after Cyt-B addition. Cells were separated from the bottle by trypsinization, and the cell suspension was centrifuged at 1,000 *g* for 5 min. Cells were ressuspended in 0.075 M KCl, and maintained at 4 °C for 3 min (mild hypotonic treatment). They were then centrifuged, and a methanol/acetic acid (3:1) solution was slowly added. This fixation step was repeated twice, and cells were finally ressuspended in a small volume of methanol/acetic acid, dropped on clean slides, and stained with 10 % Giemsa (pH 6.8) for 3 - 4 min. Slides were mounted, and codified prior to microscopic analysis. The vehicle was used as negative control. Micronuclei were counted in 2,000 binucleated cells (BNC) with well-preserved cytoplasm. The identification of micronuclei was carried out according to Fenech (2000).

2.6.2.5. Statistical analysis

All experiments were independently performed in triplicates, and the results are expressed as mean ± SE. Data were analyzed by one-way analysis of variance (ANOVA), followed by test of Tukey at $P < 0.05$, which was considered as statistically significant.

3. Results and discussion

According to Bernard et al. (1998) enzymes are generally considered to be of low toxic potential, but microbial enzymes produced by biotechnological process are enzymatic preparations that contain not only a desired enzyme activity but also other metabolites produced during the cultivation process of microorganism and enzyme production (Pariza & Johnson, 2001). Therefore, it is necessary to evaluate the toxicological aspects of these preparations before to use in food industry or in others applications (Pariza & Foster, 1983;

Kessler, Taylor, Maryanski, Flamm, & Kahl, 1992). In the present study we evaluated some biologic characteristics and safety of microbial TGase produced by *B. circulans* BL32.

3.1. In vivo assay

No fatalities were observed among rats of the control or test groups during the 14-day study. Furthermore, all rats were normal in appearance throughout the test period and no clinical signs related to the treatment with *B. circulans* BL32 TGase (test group) were observed. The total weight gain (mean ± SE) was 50.27 ± 3.06 and 48.98 ± 3.71 g for the control and test groups, respectively (P = 0.125, one-way ANOVA). The relative weight gain of the rats treated with distilled water and *B. circulans* BL32 TGase are illustrated in Fig. 1.

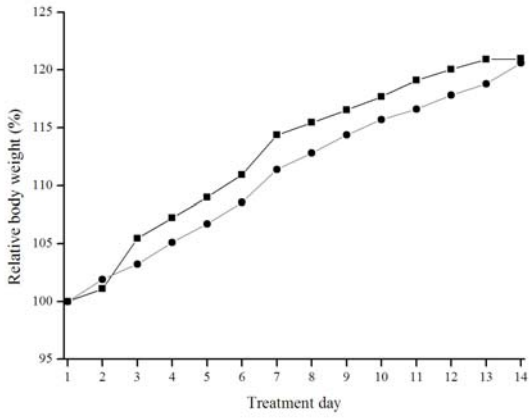

Fig. 1. Relative weight gain of the rats (with respect to day 1 which represented 100 %) treated with water (control, ■) or *B. circulans* BL32 TGase (●).

According to Jahn & Günzel (1997), in all toxicity studies, clinical observations, body weights, and body weight changes can provide useful indicators of the general health status of the animal. Therefore, interpretation of toxic effects of enzymatic preparations should always consider the presence or absence of body weight alterations. Depression in body weight or reduction in weight gain may reflect a variety of responses including treatment-induced anorexia or systemic toxicity. Promotion of body weight or body weight gain should also be considered as a toxic response. The results obtained in this work show that there were no treatment-related changes in mean body weight or body weight gain in

the test group that received *B. circulans* BL32 TGase preparation at dose of 150 U/kg bw/day.

The weight of individual organs was measured and the ratio of organ weight per final body weight was calculated. Table 1 shows the relative weight of the organs of animals treated with water (control group) and *B. circulans* BL32 TGase preparation for 14 days. There was significant difference ($P < 0.05$, Student's *t*-test) only in relation to weight of the lungs between the groups studied.

Table 1

Relative organs weight (%) of rats treated with water or *B. circulans* BL32 TGase for 14 days[a]

Organs	Control group	*B. circulans* BL32 TGase group
Liver	3.01 ± 0.035	3.10 ± 0.043
Right kidney	0.42 ± 0.010	0.42 ± 0.011
Left kidney	0.40 ± 0.008	0.41 ± 0.010
Spleen	0.27 ± 0.008	0.29 ± 0.004
Thymus	0.13 ± 0.009	0.15 ± 0.011
Right adrenal gland	0.01 ± 0.001	0.01 ± 0.001
Left adrenal gland	0.01 ± 0.001	0.01 ± 0.001
Heart	0.34 ± 0.010	0.35 ± 0.009
Lungs	0.42 ± 0.008	0.55 ± 0.020[b]
Brain	0.68 ± 0.019	0.71 ± 0.015

[a] Data are reported as mean ± SE ($n = 10$) based on the percentage of the organ weight in relation to total body weight.

[b] Significantly different at $P < 0.05$ by Student's *t*-test.

Table 2 shows the results of the hematological and blood chemistry evaluation of rats of the control and test groups.

Table 2

Hematological[a] and blood chemistry evaluation of rats treated with water or *B. circulans* BL32 TGase for 14 days [b]

Parameters[c]	Control group	*B. circulans* BL32 TGase group
Hematocrit (%)	47.89 ± 0.77	47.33 ± 0.50
Hemoglobin (g/dL)	15.14 ± 0.36	15.34 ± 0.22
RBC (10^6/μL)	8.33 ± 0.22	8.22 ± 0.20
WBC (10^6/μL)	6.31 ± 0.59	5.59 ± 0.39
MCV (fL)	57.67 ± 1.29	57.74 ± 1.07
MCHC (%)	31.60 ± 0.29	32.42 ± 0.37
Neutrophils (10^6/mL)	1.07 ± 0.13	1.03 ± 0.10
Eosinophils (10^6/mL)	0.0 ± 0.0	0.0 ± 0.0
Basophils (10^6/mL)	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes (10^6/mL)	5.08 ± 0.53	4.39 ± 0.33
Monocytes (10^6/mL)	0.16 ± 0.04	0.05 ± 0.02
Alanine aminotransferase (U/L)	65.16 ± 10.52	59.27 ± 11.07
Creatinine (mg/dL)	0.52 ± 0.02	0.47 ± 0.01
Total protein (mg/mL)	61.33 ± 0.82	62.22 ± 0.85

[a] RBC, red blood cells; WBC, white blood cells; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration.

[b] Data are reported as mean ± SE (n = 10).

[c] There was no significant difference between control and test groups ($P > 0.05$, Student's t-test).

There was no significant difference ($P > 0.05$) in the serum levels of total protein, CRE, and ALT between the two groups. Hematological findings did not show any effects related to treatment with *B. circulans* BL32 TGase. No significant differences were seen between rats of the control and test group in hemoglobin, hematocrit, erythrocyte count, mean corpuscular volume, and mean corpuscular hemoglobin concentration. Similarly, total and differential leukocyte counts in the rats treated with TGase were not significantly

different from those in the control group. Moreover, all values remained within the normal range of historical data for rats of this age and strain.

Histopathological examination of liver, kidneys, spleen, thymus, adrenal glands, heart, lungs, and brain revealed no differences between rats received *B. circulans* BL32 TGase preparation at dose of 150 U/kg bw/day and those received distilled water. The bigger lungs of rats treated with TGase presented normal histological appearances (Fig. 2B) when compared with the control group (Fig. 2A).

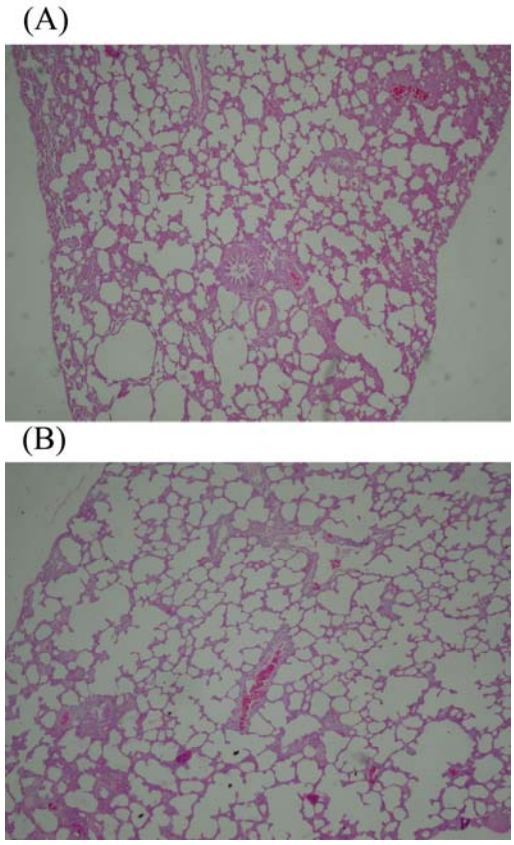

Fig. 2. Hystologic sections of the lungs of the animals of control group (Fig. 2A) and *B. circulans* BL32 TGase (Fig. 2B). The sections were stained with hematoxylin-eosin (H&E). Magnification is 4X.

The microscopic analysis of the lungs from animals receiving *B. circulans* BL32 TGase preparation showed no changes on the lung cells in comparison with the control. Cell morphology and tissue characteristics were typical of lung cells. In addition, there were no microscopic changes observed in any of the organs evaluated to indicate any toxicity as a result of the exposure to the liquid enzyme preparation, by daily oral gavage,

at dose of 150 U/kg bw/day. Therefore, it was concluded that enzymatic preparation of *B. circulans* BL32 TGase did not cause any toxic effects in the tissues examined when the animals were exposed to the enzyme in a short term period.

Based upon these data, it was observed that there were no treatment-related changes in the test group suggestive of toxicity in rats, after daily oral doses of 150 U/kg bw/day for 14 days. Therefore, the results obtained in the study of subacute 14-day oral toxicity in rats show that the impurities no removed after the chromatographic purification of this enzymatic preparation no present toxic potential.

Similar results were obtained by Bernard et al. (1998) in relation to microbial TGase from *Streptoverticillium* spp.. The toxicological studies performed on this TGase preparation did not demonstrate any significant toxicological effects in the rats within 14 days after a single oral dose of microbial TGase from *Streptoverticillium* spp..

3.2. Genetic toxicity of TGase in mammalian cultured cells

A balance between desirable versus toxicological effects of a substance is an important parameter when evaluating its usefulness as a pharmacological drug or biotechnological product. Cell culture can be used to evaluate basal cytotoxicity and target organ toxicity (Ekwall & Ekwall, 1988). In this study, V79 fibroblasts were used because this cell line is well characterized and commonly used in mutagenicity and cytotoxicity studies (Bradley, Bhuyan, Francis, Langenbach, Peterson, & Huberman, 1981). The results shown in Table 3 indicate that *B. circulans* BL32 TGase preparation at 150 U had not toxic effects in V79 cells. These concentrations should be used in future studies evaluating its industrial application.

Table 3

Genotoxic evaluation of *B. circulans* BL32 TGase during 3 h in FBS-free medium in V79 cells

Treatment	Cytotoxicity	Comet assay		Micronuclei	
	Clonal survival (%)	Damage index	Damage frequency	Cell proliferation (% binucleated cells)	Micronuclei/2000 binucleated cells
NC	100.0 ± 0.0	21.3 ± 8.7	20.3 ± 6.9	93.0 ± 2.1	11.0 ± 1.1
PC[a]	51.4 ± 2.2*	143 ± 19.9*	88.3 ± 9.7*	62.1 ± 0.9*	52.4 ± 8.0*
B. circulans BL32 TGase	94.1 ± 3.5	17.2 ± 2.0	21.3 ± 1.4	95.9 ± 3.0	9.2 ± 0.5

[a] The symbol * represents $P < 0.05$, as tested by one way ANOVA (Tukey's test): *B. circulans* BL32 TGase treatment was compared to negative control; Mutagen treatment was compared against negative control. NC: negative control (distilled water). PC: positive control (4×10^{-5} mol/L MMS).

Considering the need of a complete toxicological evaluation, we investigated whether this enzyme preparation could induce DNA damage under these experimental conditions. The *in vitro* alkaline (pH >13) comet test and micronucleus test are the most frequently used assays for routine screening of potential genotoxic agents (Hartmann et al., 2001), and can be performed with a variety of cell types, including V79 cell lines. V79 cells were selected in our study because they have stable karyotype, short generation time, and are easy to maintain. While the alkaline version of the Comet assay (Singh et al., 1988) detects primary (repairable) DNA single and double strand breaks and alkali-labile sites, the micronucleus test detects DNA lesions that lead to chromosome mutations. As can be seen in Table 3, this enzyme preparation did not generate significant DNA damage at concentration 150 U/treatment.

The results of the micronucleus test are shown in Table 3. Micronuclei are the results of acentric fragments or lagging chromosomes that fail to incorporate into either of the daughter nuclei during telophase of the mitotic cells (Albertini et al., 2000). The treatment with this enzymatic preparation did not alter the percentage of binucleated cells, reinforcing the absence of cytotoxic effect of this enzyme on cell proliferation. Again, the concentration of 150 U caused no significant increase in the incidence of micronuclei, suggesting that the treatment with *B. circulans* BL32 TGase is safe.

Since the commercial use of microbial TGases ensure the increased likelihood of human exposure to these preparations, this study is relevant to determine health hazards, to evaluate the safety of possible future applications to several industrial and biotechnological fields, and to explore new biological properties of these preparations. However, little research has been carried out to test aspects of genetic toxicity of microbial TGase preparations. In this sense, Bernard et al. (1998) showed that *Streptoverticillium* spp. TGase was not mutagenic in bacteria, in V79 cells, using the approach of chromosomal aberrations, and micronucleus assay in male mouse. In our study, the *B. circulans* BL32 TGase preparation did not show cytotoxic, genotoxic or mutagenic effects in V79 cells, reinforcing the fact that these enzymes can be considered to be of low toxic potential. Moreover, the impurities profile of this preparation does not promotes undesired cellular changes, as residual DNA inducing mutagenic potential, as requested in the safety principles for biotechnological products (Bass, Purves, & Papaluca Amati, 2000).

4. Conclusions

Microbial TGases can be employed in many attractive food and non-food applications. Although many reports on production and purification of microbial TGases have been published, little information about toxicity aspects is available. In this work, the microbial TGase from *B. circulans* BL32 was submitted to toxicological evaluation. No evidence of toxicity was found in enzymatic preparation in the study subacute 14-day oral toxicity in rats and this preparation was not either cytotoxic, genotoxic or mutagenic to V79 cultured cells, showing its safety. It can be concluded that no safety concerns were identified in the studies conducted with this TGase preparation derived from *B. circulans* and produced under controlled cultivation conditions. The results presented in this work suggest that the microbial TGase from *B. circulans* BL32 has a potential for use in the food industry. Further studies are granted to evaluate the safety of this TGase in other dose levels and toxicological model systems.

Acknowledgements

This research was partially financed by grants from CNPq and CAPES. The animal experimentation was authorized by Resolution 01/2007 of the Research Committee of the Food and Science Institute of Technology of The Federal University of Rio Grande do Sul State. One of the authors (JGV) has a formal authorization to humanely manipulate experimental animals. Our group supports the ethically and morally justified use of animals in experimentation, but we also support the search for proper models to completely replace animal experimentation in the near future.

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5.8. RESULTADOS VIII. "Kinetics of thermal inactivation of transglutaminase from a newly isolated *Bacillus circulans* BL32" – Journal of Biotechnology, submetido em julho de 2008.

Kinetics of thermal inactivation of transglutaminase from a newly isolated *Bacillus circulans* BL32

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Abstract

In this research it is described the modeling of the kinetics of thermal inactivation of transglutaminase (TGase) from a newly isolated *Bacillus circulans* BL32, isolated from the Amazon environment. The purified enzyme was incubated at temperatures ranging from 30 to 70 °C and values of the thermodynamic inactivation parameters activation energy (ΔE), activation enthalpy (ΔH), activation entropy (ΔS), and free energy (ΔG) for thermal inactivation, were calculated. The kinetics of TGase thermo-inactivation followed a Lumry-Eyring model. The enzyme was very stable up to 50 °C, with approximately 50 % of activity remaining after heating for 12 h. It was completely inactivated by incubation at 70 °C for 2 min. The ΔE of the TGase was 350.5 kJ mol^{-1}. The ΔH and ΔS for thermo-inactivation of the TGase were 347.8 kJ mol^{-1} and 744 J mol^{-1} K^{-1} at 50 °C, respectively. Dynamic light scattering measurements suggest that the thermal inactivation of this microbial TGase can be partially attributed to the formation of aggregates. These results provide useful information about the thermal characteristics of the microbial TGase from *B. circulans* BL32 and indicate that this enzyme could be a good candidate for industrial applications.

Keywords: Transglutaminase; *Bacillus circulans* BL32; Enzyme inactivation; Thermal stability; Thermodynamic parameters.

1. Introduction

Transglutaminases (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) are a family of enzymes that catalyses acyl transfer reactions between γ-carboxyamide groups of glutamine residues and the ε-amino group of lysines in proteins, leading to the covalent inter or intramolecular crosslinking of the proteins (Nonaka et al., 1989). Calcium-dependent TGases present in most animal or vegetable tissues and body fluids of vertebrates are involved in several biological processes. In addition to its biochemical interest, this enzyme has a number of important commercial applications. The most important applications of TGase are in the food industry. TGase catalyses the formation of homologous and heterologous polymers within meat, soybean, milk, and wheat proteins, promoting the polymerization, texture improvement, restructuring, and gel formation in foods (Motoki and Seguro, 1998). Researches have shown that the TGase can be used in many others attractive non-food applications such as pharmaceuticals (Bernard et al., 1998), textiles (Cortez et al., 2004), immobilization of enzymes (Josten et al., 1999), and tissue engineering (Orban et al., 2004).

Guinea-pig liver used to be the only source of commercial TGase, but the practical application of this enzyme was limited due to the scarce source, and the complicated separation and purification processes for obtaining this TGase (Zheng et al., 2001). Recently, efforts have been made aiming at the biotechnological production of microbial TGases (Ando et al., 1989). The purification and preliminary characterization of this enzyme from various bacterial and fungal sources have been reported such as for *Streptomyces hygroscopicus* (Cui et al., 2008); *Bacillus subtilis* (Suzuki et al., 2000), and *Physarum polycephalum* (Klein et al., 1992). However, the only industrially important and best studied TGase is from *Streptoverticillium mobaraense* (Ando et al., 1989). Therefore, the optimization of cultivation processes and the study of properties from a wide variety of microbial TGases would offer new possibilities in improving the potential and effective use of this enzyme.

The physicochemical stability of an enzyme, as in the case of any other catalyst, is a major issue from both technical and scientific viewpoints. Several agents are able to promote the denaturation and inactivation of an enzyme such as temperature, pH, chemical

agents, autolysis (proteases) or ionic strength, among others (Ladero et al., 2005). The use of an enzyme in industrial processes depends on its stability during preparation, storage, and application under some harsh conditions (Cui et al., 2008). For efficient application of exogenous enzymes in industrial processes, knowledge on their processing stability is essential for adequate development since rapid deactivation determines the usefulness of the application (Ladero et al., 2005). Studies concerning the kinetics of thermal inactivation of TGase from *Streptoverticillium mobaraense* and *Streptomyces hygroscopicus* were reported by Menéndez et al. (2006) and Cui et al. (2008), respectively, but there are no reports on the literature of the thermal stability of TGase from *Bacillus.*

We have recently reported the purification, preliminary characterization (Soares et al., 2003), and optimization of production process (Souza et al., 2006) of TGase from *Bacillus circulans* BL32, originated from the Amazon basin region. An understanding of the stability of this enzyme may contribute to the technological applications of the enzyme in the food and non-food industries. The purpose of this study was to evaluate the kinetics of the inactivation of TGase from *Bacillus circulans* BL32 over a wide range of inactivation temperatures and propose a mathematical modeling of the processes. It was also determined the thermodynamic parameters for the inactivation of TGase, and the effects of the sodium and calcium chloride, two commonly used salts in processed foods that are known to influence protein stability, on the thermal inactivation of TGase.

2. Materials and Methods

2.1. Materials

All chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany), unless otherwise mentioned. N-carboxybenzoyl-L-glutaminyl-glycine (N-CBZ-Gln-Gly) was obtained from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). Q-Sepharose fast flow (FF) and Octyl-Sepharose 4 FF were from Pharmacia (Uppsala, Sweden).

2.2. Microorganism and Inocula preparation

It was used a strain of *Bacillus circulans*, coded BL32, isolated from the aquatic environment of the Amazon rain forest and described elsewhere (Soares et al., 2003; Souza et al., 2006). Prior to use, cells were recovered from frozen stocks in glycerol (50 % v/v) and were kept at 4 °C on Mueller-Hinton agar plates.

Erlenmeyer flasks (250 mL) containing 50 mL medium (M1), optimized in a previous work (Souza et al., 2006), composed of (g L^{-1}): glycerol 9.0, sucrose 2.0, peptone 7.0, tryptone 1.0, Na_2HPO_4 1.0, $MgSO_4.7H_2O$ 1.0 and $FeSO_4.7H_2O$ 0.1, were inoculated with a single colony from stock cultures and incubated at 30 °C in a rotatory shaker at 100 rpm and were grown to optical density (OD) of 1.0 at 600 nm (approximately 20 h).

2.3. Transglutaminase production

Erlenmeyer flasks (1 L) containing 200 mL of M1 medium, initial pH 8.5, were inoculated with 10 mL of preinoculum. Cultivations were run at 30 ºC in a rotatory shaker at 100 rpm. After 8 days of cultivation, the crude suspension was centrifuged at 4 ºC for 20 min at 17,000 *g* and the supernatant was used for enzyme purification.

2.4. Transglutaminase purification

TGase was precipitated with 60-90 % ammonium sulfate (w/v) and after dialysis was applied to a Q-Sepharose fast flow (FF) ion-exchange column pre-equilibrated with 20 mM Tris–HCl buffer pH 8.0, following a linear gradient of elution (0 - 1 M) of NaCl (Soares et al., 2003). Fractions showing highest TGase activity were pooled, again dialyzed, and applied to an Octyl-Sepharose 4 FF column and eluted with a linear gradient (3.5 - 0 M) of $(NH_4)_2SO_4$. Fractions containing the highest enzymatic activity were pooled and dialyzed against 20 mM Tris–HCl buffer pH 8.0. Purity was checked by SDS-gel electrophoresis. This purified enzyme was then used in further analysis.

2.5. Determination of enzyme activity

TGase activity was determined by hydroxamate formation with the specific substrate, N-CBZ-Gln-Gly, described by Grossowicz et al. (1950). A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One TGase unit (U) was defined as the amount of enzyme that causes the formation of 1 μmol L-glutamic acid γ-monohydroxamate per minute at 37 °C.

2.6. Kinetics of thermo-inactivation of transglutaminase

Thermal stability of the TGase was studied at pH 8.0 in 20 mM Tris–HCl buffer with an enzyme activity of 2.0 U mL^{-1} at different temperature: 30, 40, 50, 60, and 70 °C.

Periodically, the samples were withdrawn from the water bath and immediately cooled on ice water. The residual enzyme activities were determined by using the method described above, taking the activity of a sample without incubation as 100 %. The relative activity was calculated as shown in equation 1.

$$a = \frac{C_t}{C_0} \tag{1}$$

Where a is the relative TGase activity; C_t is the activity at time t (s), and C_0 is the activity at time $t = 0$ s.

The effect of NaCl and $CaCl_2$ on thermal stability of the TGase was examined by incubating the enzyme preparation at 50 °C for appropriate periods of time in the presence of these salts at concentration of 1, 3, and 5 % (w/v). The residual enzyme activities were determined by using the method described above, taking the activity of a sample without incubation as 100 %. The relative activity was calculated as equation 1.

All experiments were conducted in triplicate and results shown are mean values.

2.7. Kinetic data analysis

Kinetics was investigated using the mechanism proposed by Lumry-Eyring (Lumry and Eyring, 1954; Polakovic and Bryjak, 2002). This mechanism can be represented by de following reaction:

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} D \xrightarrow{k_2} I ,$$

Where the native form N is reversibly transformed to the denatured form, D, which further reacts irreversibly to the inactive form I. The integrated form of the Lumry–Eyring model can be derived from the mechanism by applying the mass action principle, described by the following set of differential equations:

$$\frac{d[N]}{dt} = -k_1 \cdot [D] + k_{-1} \cdot [N] \tag{2}$$

$$\frac{d[D]}{dt} = +k_1 \cdot [N] - k_{-1} \cdot [D] - k_2 \cdot [D] \tag{3}$$

$$t = 0 \qquad [N] = [N]_0 \qquad [D] = 0 \tag{4}$$

Where k_1, k_{-1}, and k_2 are the rate constants of individual reactions (temperature dependent) and $[N]$ and $[D]$ are enzyme concentrations. The above model, derived from the Lumry-Eyring mechanism, was used to fit the experimental data.

The relative activity, a, is then simply calculated from the equation 5,

$$a = \frac{[N]+[D]}{[N]_0} \tag{5}$$

The model parameters were estimated using the software MATLAB®, with a hybrid deterministic-stochastic method joining a nonlinear regression technique based on the Levenberg-Marquardt algorithm (lsqnonlin) and the particle swarm optimization algorithm (PSO) (Levenberg, 1944; Marquardt, 1963; Kennedy and Eberhart, 1995).

2.8. Calculation of activation parameters

The temperature dependence of the rate constants for inactivation was analyzed according to an Arrhenius plot (Ortega et al., 2004a). The activation energy (ΔE) was obtained from the slope of the Arrhenius plot (regression of semi-logarithm of reaction rate constants versus reciprocal of absolute temperature).

Activation enthalpy (ΔH) for each temperature was calculated according to equation 6:

$$\Delta H = \Delta E - R \cdot T \tag{6}$$

Where R is the universal gas constant and T is the absolute temperature.

The values for free energy of inactivation (ΔG) at different temperatures are calculated from the rate constant of the inactivation process by equation.7:

$$\Delta G = -R \cdot T \cdot \ln\left(\frac{k \cdot h}{K \cdot T}\right) \tag{7}$$

Where h is the Planck constant (6.6262×10^{-34} J·s); K is the Boltzmann constant (1.3806×10^{-23} J·K^{-1}).

Activation entropy (ΔS) of inactivation was calculated from equation 8:

$$\Delta S = \frac{\Delta H - \Delta G}{T} \tag{8}$$

Estimated rate constants for the inactivation of TGase gave values for the enzyme half-life ($t_{1/2}$), calculated as equation 9:

$$t_{1/2} = \frac{\ln(2)}{k} \quad (9)$$

2.9. Light scattering measurements

2.9. 1. Sample preparation

TGase solution (2.0 $U.mL^{-1}$, in 20 mM Tris–HCl, pH 8.0) was filtered through 0.2 μm pore diameter membranes (Millipore) into dust-free cells.

2.9. 2. Experimental setup and data analysis

Light scattering was performed on a Brookhaven Instruments standard setup (BI-200M goniometer, BI-9000AT digital correlator) with a He–Ne laser (λ = 632.8 nm) as light source. An interference filter was used before detecting the signal on the photomultiplier. The samples were placed into dust free cells for light scattering measurements that were placed in the index-matching liquid decahydronaphthalene (decalin, Aldrich) and heated from 30 to 70 °C in a rate of 0.5 $^{o}C.min^{-1}$ with intervals of 15 min at each 5 ^{o}C when light scattering was measured.

The apparent values of hydrodynamic radius R_h, the polydispersity index and the size distributions of the enzyme organizations in solution where determined in this work by dynamic light scattering at 90° scattering angle as described elsewhere (Mertins et al., 2006). The time correlation functions were measured in the multi-τ mode using 224 channels and data treatment was preliminary performed by means of the cumulants method of the Provencher Software (CONTIN) where the R_h is determined by the Stokes–Einstein relation, applying the diffusion coefficient obtained at 90°.

A more careful analysis of light scattering data was performed analyzing the relaxation times of the correlation functions (τ) through the GENDIST program using the constrained regularization calculation algorithm REPES. In this way, $\tau A(\tau)$ is obtained by the regularized inverse Laplace transformation of the dynamic light scattering data (Pereira-Lachataignerais et al., 2006).

3. Results and discussion

3.1. Inactivation kinetic

Enzyme inactivation is defined as the process where the secondary, tertiary or quaternary structures of a protein change without breaking covalent bonds (Dogan and Tari, 2008). Various behaviors can be found when the inactivation kinetic of enzymes is observed. Some enzymes show exponential decay of activity with time, while others follow different non-exponential kinetics. A number of models have been proposed to explain the mechanism of enzyme thermal inactivation (Polakovic and Vrabel, 1996; Vrabel et al., 1997). The Lumry-Eyring was selected in this study because TGase inactivation experimental data showed the best fit using this model and in Fig. 1 are depicted the kinetics at different temperatures.

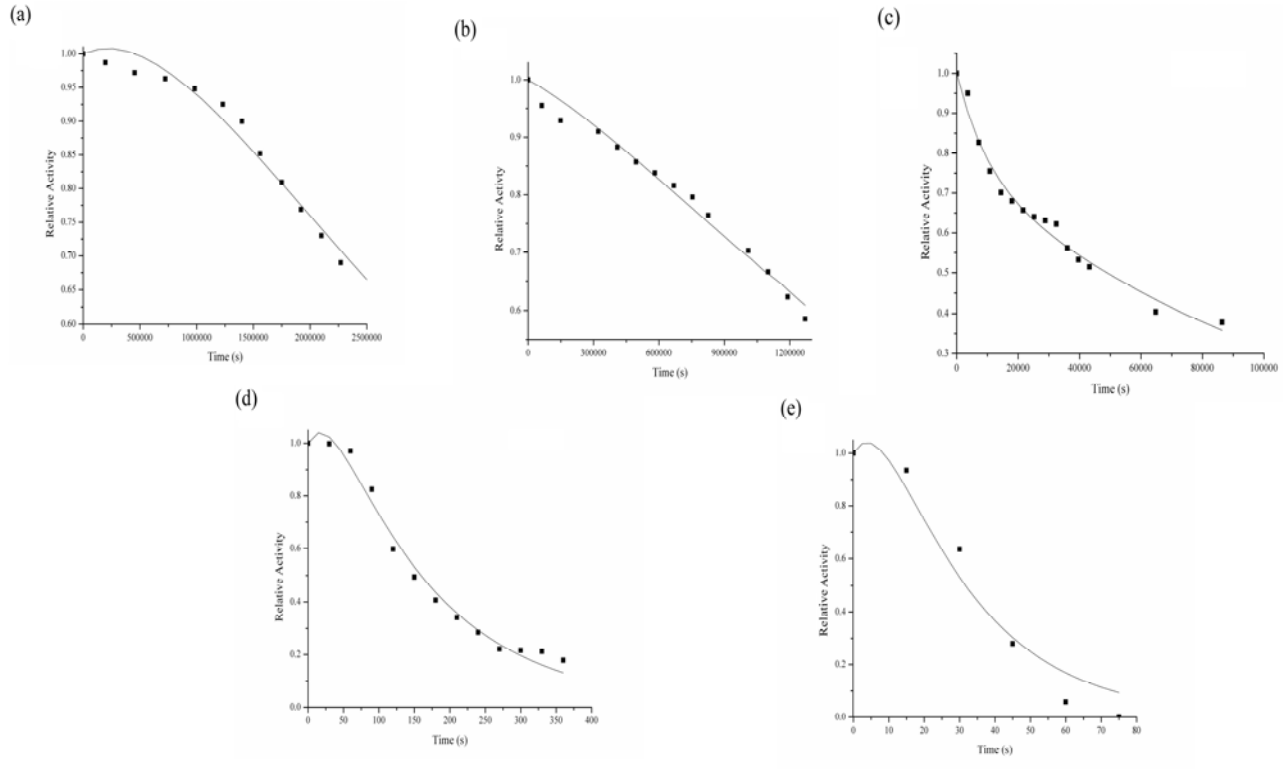

Fig. 1. Kinetics of thermal inactivation of TGase from *B. circulans* BL32: (a) 30 °C, (b) 40 °C, (c) 50 °C, (d) 60 °C, and (e) 70 °C. The model fitting is represented as lines.

Table 1 shows the kinetic parameters and determination coefficient for thermal inactivation of TGase from *B. circulans* BL32 at different temperatures.

Table 1

Effect of increasing temperature on thermal inactivation kinetic parameters and determination coefficient for microbial TGase from *B. circulans* BL32

Inactivation rate constant (s^{-1})	Temperature (°C)				
	30	40	50	60	70
k_1	5.533×10^{-7}	7.283×10^{-7}	3.001×10^{-5}	1.287×10^{-2}	7.641×10^{-2}
k_{-1}	1.157×10^{-7}	4.459×10^{-8}	5.296×10^{-5}	9.844×10^{-3}	1.979×10^{-2}
k_2	5.502×10^{-7}	1.087×10^{-6}	3.090×10^{-5}	1.731×10^{-2}	6.093×10^{-2}
R^2	0.973	0.982	0.984	0.989	0.974

The determination coefficients ($0.973 < R^2 < 0.989$) of the curves drawn in Fig. 1 show that the mechanism is mathematically fit to explain the inactivation process and that inactivation of the TGase from *B. circulans* BL32 is not a simple first-order process, commonly observed for other enzymes. Menéndez et al. (2006) and Cui et al. (2008) reported that the inactivation of TGases from *S. mobaraense* and *Streptomyces hygroscopicus* followed first-order kinetic models.

The results of the *B. circulans* BL32 TGase inactivation at 60 and 70 °C demonstrated grace-period inactivation (Fig. 1d and Fig. 1e). An initial, slow phase followed by the acceleration of inactivation rate characterizes the grace-period. Inactivation described by the Lumry-Eyring mechanism exhibits a grace period when the reversible reaction occurs mainly during the initial phase of inactivation (Polakovic and Vrabel, 1996). According to Tomazic and Klibanov (1988), enzymes undergo partial unfolding when temperature rises above certain level, resulting in a change in native conformation and a loss of activity. The activity is fully reversible after short period of treatment at high temperatures, but after longer periods only a small fraction of activity is regained, configuring a process of irreversible thermo-inactivation.

The thermal stability profile for *B. circulans* BL32 TGase showed that about 50 % of the relative activity still remained after 12 h at 50 °C. However, after thermal treatment by 5 min at 60 °C, the relative activity was only 22 %, and it was completely inactivated by incubation at 70 °C during 2 min, indicating its inactivation. According to Menéndez et

al. (2006), after thermal treatment relative activity of the TGase from *S. mobaraense* was 11 % after 5 min at 60 °C and it was complete inactivated after 2 min at 80 °C. It seems to be that TGase from *B. circulans* BL32 is more stable at 60 °C than TGase from *S. mobaraense.*

To determine the activation energies (ΔE), the semi-logarithmic plots of the thermo-inactivation constants (k_1, k_{-1}, k_2) obtained at 40, 50, 60 and 70 °C versus the reciprocal of the absolute temperature were obtained and are depicted in Fig. 2.

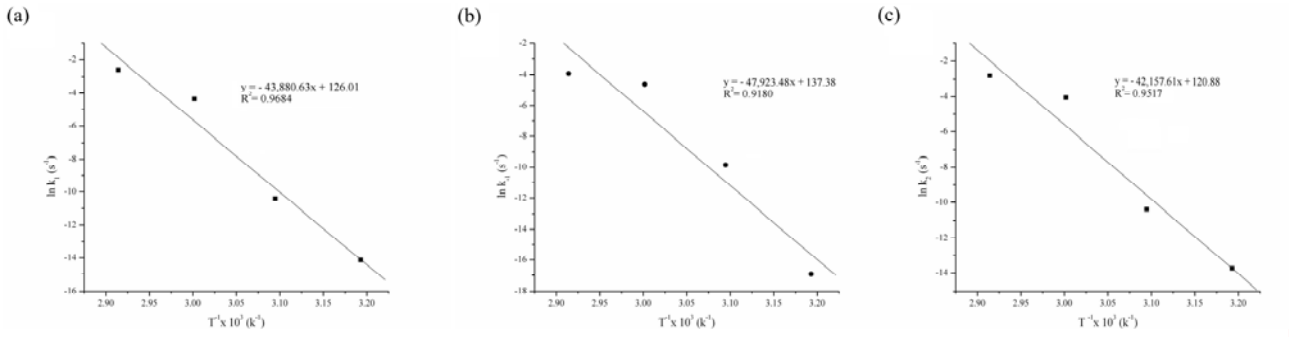

Fig. 2. Arrhenius plot of the inactivation rate constants (a) k_1, (b) k_{-1}, and (c) k_2 obtained from the Lumry-Eyring model for the kinetics of thermal inactivation of TGase from *B. circulans* BL32.

The fitting of the data by linear regression to an Arrhenius-type equation allowed calculating the activation energies. According to Cobos and Estrada (2003), the ΔE value is the energy necessary to change the enzyme conformation. These values for the thermal inactivation of the microbial TGase from *B. circulans* BL32 (Table 2) were 364.8 kJ mol^{-1} (for k_1, Fig. 2a), and 350.5 kJ mol^{-1} (for k_2, Fig. 2c), which are in the range of activation energies (140 - 629 kJ mol^{-1}) reported for the general inactivation reactions (Whitaker, 1972). The higher value of ΔE for k_{-1} (398.4 kJ mol^{-1}, Fig. 2b) indicates the existence of grace-period at high temperatures, as shown in Fig. 1. According to Klibanov (1983), these are high values, uncharacteristic of a covalent reaction, and suggest the existence of protein unfolding followed by refolding into new thermodynamically stable structure but otherwise catalytically inactive.

Table 2

Kinetic and thermodynamic parameters for thermal inactivation of TGase from *B. circulans* BL32 at different temperatures

Parameter	Temperature (ºC)	k_1	k_{-1}	k_2
ΔE (kJ mol^{-1})		364.8	398.4	350.5
$t_{1/2}$ (s)	40	9.517 x 10^5	1.554 x 10^7	6.377 x 10^5
	50	2.309 x 10^4	1.309 x 10^4	2.243 x 10^4
	60	5.386 x 10^1	7.041 x 10^1	4.004 x 10^1
	70	9.071	3.502 x 10^1	1.138 x 10^1
ΔH (kJ mol^{-1})	40	362.2	395.8	347.9
	50	362.1	395.7	347.8
	60	362.1	395.7	347.7
	70	362.0	395.6	347.6
ΔG (kJ mol^{-1})	40	113.6	120.9	112.6
	50	107.3	105.8	107.3
	60	94.0	94.7	93.1
	70	91.8	95.6	92.4
ΔS (J mol^{-1} K^{-1})	40	794	878	752
	50	789	897	744
	60	805	903	764
	70	787	874	744

The half-life times ($t_{1/2}$) for TGase from *B. circulans* BL32 are summarized in Table 2. According to Busto et al. (1999), it is assumed that $t_{1/2}$ values are additive. The TGase produced by *B. circulans* BL32 was very stable until 50 °C. The $t_{1/2}$ for TGase

from *S. hygroscopicus* were 433, 96, and 20 min at 30, 40, and 50 °C, respectively (Cui et al., 2008). These results showed that TGase obtained from the *B. circulans* BL32 is more stable in these temperatures than that obtained from the *S. hygroscopicus*. However, at 60 °C the $t_{1/2}$ for microbial TGase from *S. hygroscopicus* was 8 min, indicating that in this temperature the TGase from *B. circulans* BL32 is less stable.

The thermodynamic parameters of inactivation provide information on enzyme thermal stability for each step of the heat-induced denaturation process. This could help to detect any secondary stabilization or destabilization effects that would go unnoticed if only half-life times were considered (Longo and Combes, 1999). The thermodynamic parameters, ΔH (enthalpy), ΔG (free energy), and ΔS (entropy) of the thermal inactivation of the microbial TGase from *B. circulans* BL32 were calculated in the temperature range of 40 to 70 °C from experimental data using Eqs. (6) - (8). The values of these parameters are listed in Table 2.

Results for ΔH show that the enthalpy is practically independent of temperature, thus there is no change in enzyme heat capacity (Cobos and Estrada, 2003). According to Ortega et al. (2004b), the ΔH value can be correlated to the number of non-covalent broken bonds during the process of protein denaturation. Therefore, the higher ΔH is, the bigger will be the number of non-covalent bonds present in the protein molecule, which is going to be more stable. In fact, the stability of a protein is the result of a delicate balance between stabilizing and destabilizing forces, which may be influenced by several factors, e.g., the number of hydrogen and disulphide bridges, the folding degree and hydrophobicity of the molecule, the amount of ionic and other interactions (Bruins et al., 2001).

The ΔG value is directly related with the protein stability: the higher ΔG is, the higher will be the enzyme stability (Longo and Combes, 1999). When the incubation temperature was elevated from 40 to 70 °C there was a significant reduction of ΔG values for this microbial TGase, indicating that the destabilization of this protein followed the rise in temperature. According to Busto et al. (1999), all ΔG values in Table 2 are of the order of magnitude expected for protein denaturation.

All ΔS values for thermal inactivation of the microbial TGase from *B. circulans* BL32 in the temperature range of 40 to 70 °C were positives. This suggests the increase in disorder, or randomness, of the system upon denaturation. According to Marangoni (2003),

usually an increase in the randomness of the system is associated with enzyme denaturation. The most common cause of the heat inactivation of enzymes is the loss of the native conformation (unfolding of the active tertiary protein structure to a disordered polypeptide), a process identified as thermodenaturation, which takes place as a result of increased molecular mobility at elevated temperature (Klibanov, 1983).

The effect of salts on thermal stability of the TGase from *B. circulans* BL32 was examined by heating the enzyme preparation at 50 °C in the presence of NaCl and $CaCl_2$ (Fig. 3).

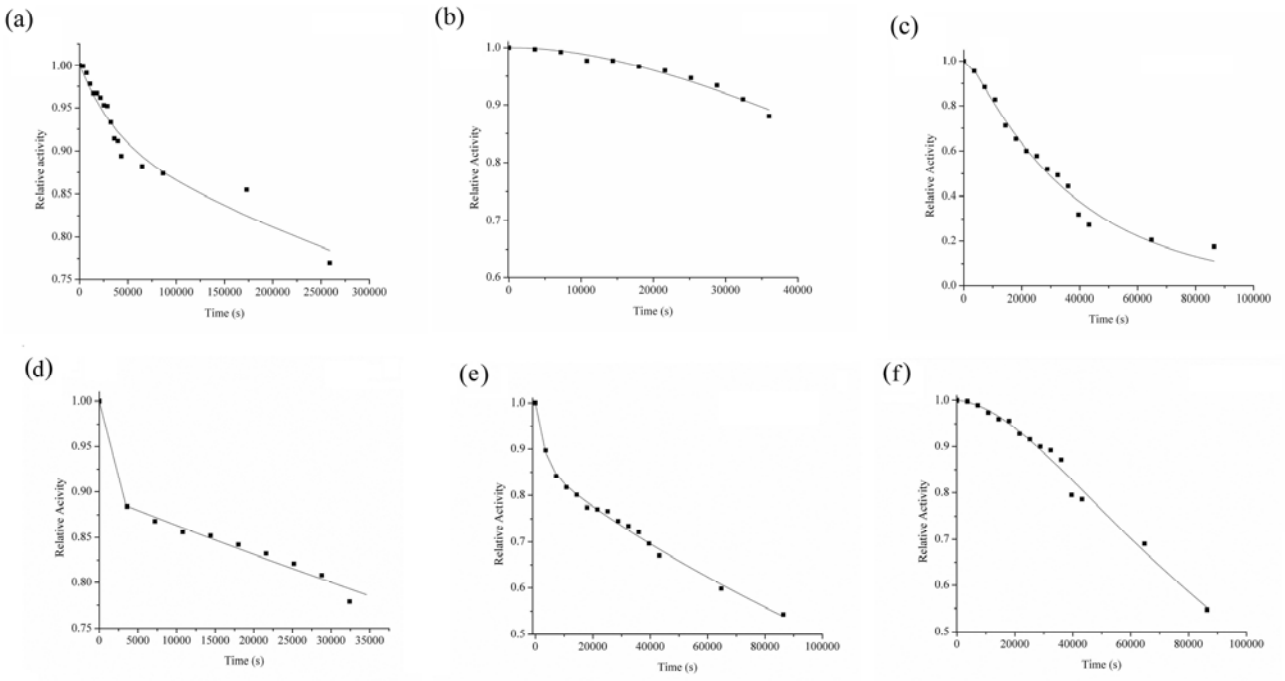

Fig. 3. Effects of NaCl and $CaCl_2$ concentrations on the kinetics of thermal inactivation of TGase from *B. circulans* BL32 at 50 °C. NaCl, (a) 1 %, (b) 3 %, (c) 5 %; $CaCl_2$, (d) 1 %, (e) 3 %, (f) 5 %. The model fitting is represented as lines.

In presence of both salts the thermal inactivation of the TGase from *B. circulans* BL32 could be described by a Lumry-Eyring model, with high determination coefficients ($0.953 < R^2 < 0.995$). The kinetic parameters and determination coefficients are given in Table 3.

Table 3

Effect of NaCl and $CaCl_2$ concentration on thermal inactivation kinetic parameters and determination coefficient for TGase from *B. circulans* BL32 at 50 °C

Inactivation rate constant (s^{-1})	NaCl (%)			$CaCl_2$ (%)		
	1	3	5	1	3	5
k_1	1.251×10^{-5}	1.933×10^{-5}	1.735×10^{-4}	7.797×10^{-6}	1.250×10^{-5}	1.309×10^{-5}
k_{-1}	1.300×10^{-5}	1.934×10^{-6}	2.437×10^{-4}	2.000×10^{-3}	1.713×10^{-4}	5.974×10^{-6}
k_2	1.199×10^{-6}	1.300×10^{-5}	6.928×10^{-5}	1.902×10^{-3}	1.398×10^{-4}	2.979×10^{-5}
R^2	0.953	0.972	0.983	0.989	0.995	0.988

Higher NaCl concentrations resulted in faster rates of inactivation and lower thermal stability (Table 3). However, it is very important to note that TGase thermal stability actually increased in concentrations of NaCl between 1 (Fig. 3a) and 3 % (Fig. 3b) compared to its stability in the absence of this salt (Fig. 1c). This fact is of foremost importance because most meat-processed products contain sodium chloride concentrations in this range. The thermal stability of the enzyme increased for concentrations of $CaCl_2$ between 1 and 5 % (Table 3).

3.2. Conformational changes during heat treatment of the TGase

Dynamic light scattering measurements were used to determine the behavior of TGase in aqueous buffer solution during heat treatment. Fig. 4 shows the plots of $\tau A(\tau)$ versus $\log(\tau)$ for the enzyme at increasing temperatures.

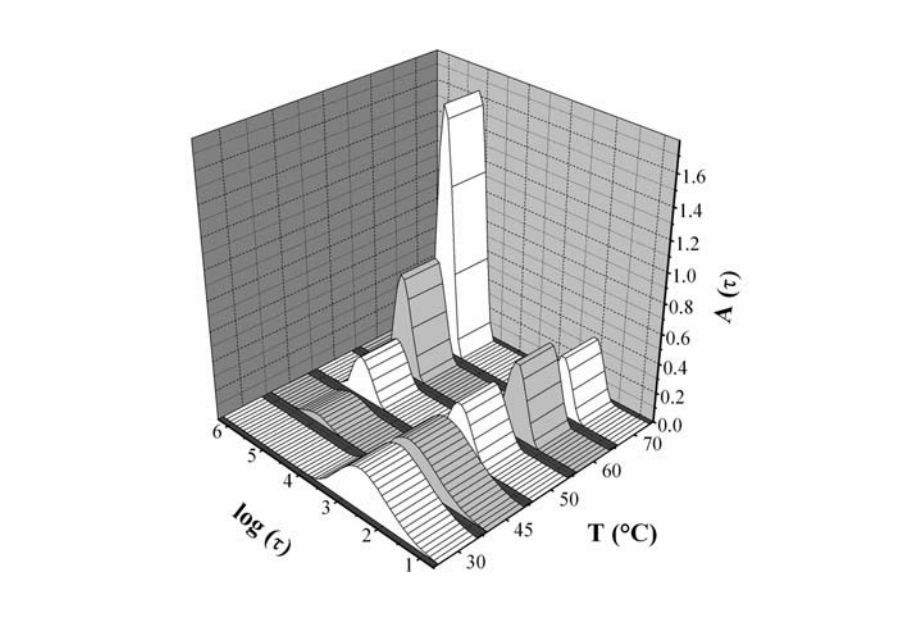

Fig. 4. Plots of log(τ) versus $\tau A(\tau)$ obtained from dynamic light scattering for microbial TGase from *B. circulans* BL32 in buffer TGase solution (2.0 U.mL^{-1}, in 20 mM Tris–HCl, pH 8.0) at different temperatures (T).

At 30 °C, it can be observed a modal distribution of particles corresponding to a single relaxation mode. This result evidences the presence of only one population with a relatively narrow size distribution (Fig. 5b). The results of light scattering showed the same behavior up to 40 °C. At 45 °C, the correlation functions performed two relaxation times: the same as for 30 °C and one that corresponds to higher log(τ) values. With increasing temperatures, this second relaxation also increased in importance relatively to the first one. At 60 °C, the importance of the second relaxation overcame the first one and this behavior followed up to the end of the experiment. It is clear that at 70 °C the second relaxation represents a much larger quantity of structures than the first one.

In order to compare sizes for the system under heating, the values of hydrodynamic radius distributions (Rh) versus temperature are plotted in Fig. 5.

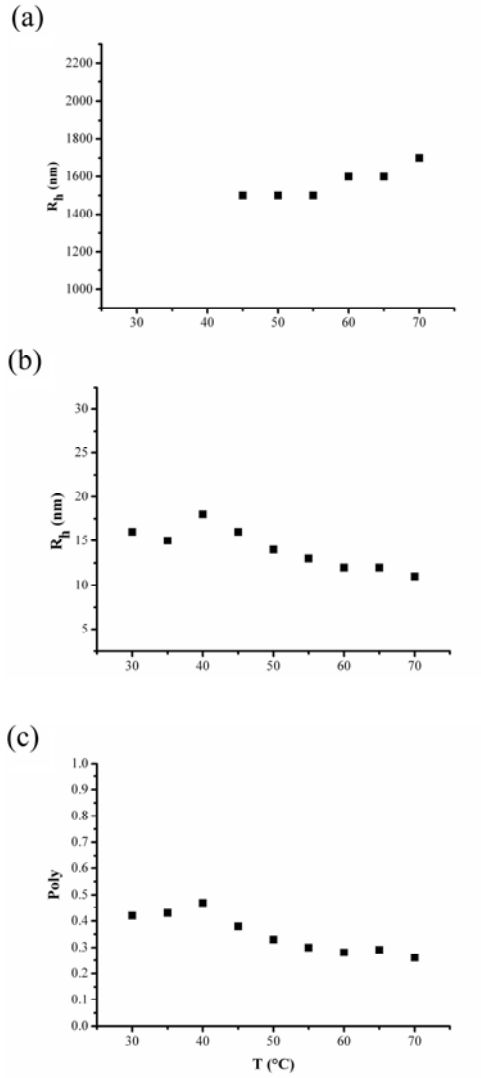

Fig. 5. Hydrodynamic radius (Rh ± 10 %) as a function of temperature (T) for TGase from *B. circulans* BL32 in buffer solution (2.0 $U.mL^{-1,}$ in 20 mM Tris–HCl, pH 8.0) obtained by dynamic light scattering: (a) slow dynamics gives the big structures dimension and (b) fast dynamics gives the small structures dimension. (c) Polydispersity index (Poly) is presented for the small particles radius.

In Fig. 5b can be seen the population of structures having a distribution of Rh greater than 10 and smaller than 20 nm for all measured temperatures. The size distribution of this population starts narrowing after 45 °C (Fig. 4), which is the temperature when the high size population appears in the system (Fig. 5a and Fig. 4). Polydispersity index for the low size particles also drops at this temperature and higher. This profile suggests that part of the small size population is being transferred to the large size population in an

aggregation process. The quantity of large size structures increases in relation to the small structures (Fig. 4) along the temperature increases. Thus, these results suggest that the thermal inactivation of TGase from *B. circulans* BL32 can be partially attributed to the formation of aggregates. According to Clark et al. (2001), in thermally induced aggregation, the compact native form of protein, with its well defined secondary and tertiary structures, becomes more flexible on heating and more reactive towards its neighbors. The degree to which it happens is temperature- and time-dependent, and is itself a multi-stages process. Increasing the temperature, some molecular regions of proteins, like hydrophobic regions or free SH-groups, become accessible to new intermolecular interactions, forming soluble aggregates through non-covalent and disulfide bonds (Honda et al., 2000). Moreover, intermolecular β-sheet formation can take place and contribute to intermolecular association of the partially unfolded protein molecules (Stokkum et al., 1995).

Although many reports on production and purification of microbial TGases have been published, little information about thermal characteristics of this enzyme is available. In this work the kinetic of thermal inactivation of the TGase from *B. circulans* BL32 was studied in a broad temperature range of 30 – 70 °C. The kinetics of inactivation followed a Lumry-Eyring model and the enzyme was very stable until 50 °C. The activation energies for the thermal inactivation of this enzyme were high, indicating a good thermal stability. High NaCl concentrations resulted in faster rates of inactivation and lower thermal stability, whereas low NaCl concentrations and high $CaCl_2$ concentrations increased the thermal stability when compared with the absence of these salts. Dynamic light scattering measurements showed that due to the temperature increasing the enzyme tends to form aggregates and the formation of these probably resulted in the loss of TGase activity. An understanding of the kinetic of thermal inactivation of this TGase produced by a newly isolated *B. circulans* BL32 will be important to better explore the catalytic activity of this enzymatic preparation at an industrial level for different applications.

Acknowledgments

The authors wish to thanks CNPq and CAPES for their financial support and scholarships.

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6. DISCUSSÃO GERAL

O interesse na produção de TGases microbianas tem aumentado significativamente nos últimos anos, devido ao seu amplo e crescente potencial de aplicação industrial em inúmeras áreas e a facilidade de produção e purificação a partir de bioprocessos (ANDO *et al.*, 1989; ZHU *et al.*, 1995; NIELSEN, 1995; MOTOKI & SEGURO, 1998; COLLIGHAN *et al*., 2002; CORTEZ *et al*., 2004; TÉLLEZ-LUIS *et al.*, 2004a; ARRIZUBIETA, 2007).

Neste trabalho foram realizados diversos estudos sobre a enzima TGase de origem microbiana produzida pelo isolado Amazônico *Bacillus circulans*, principalmente, em relação à produção, através do cultivo clássico em meio líquido e do cultivo em estado sólido, e à caracterização da enzima, purificada e parcialmente purificada, visando aplicações na indústria alimentícia e não alimentícia.

6.1. Produção da transglutaminase em cultivo submerso

O cultivo submerso é caracterizado pela utilização de um meio de cultura líquido, com nutrientes solúveis, para crescimento do microrganismo e produção de bioprodutos. Este processo tem como vantagens a facilidade de controle de parâmetros como aeração, agitação, pH, temperatura e a possibilidade de automação. Este tipo de cultivo pode ser realizado em frascos agitados, biorreatores de bancada ou biorreatores em escala industrial (SCHMIDELL *et al*., 2001).

A produção de TGase em cultivo submerso pode ser influenciada por diferentes variáveis como: o microrganismo produtor da enzima; a composição do meio de cultura, principalmente, em relação à concentração e tipo de fontes de carbono, nitrogênio e sais minerais; e as condições de cultivo, tais como, pH do meio de cultura, temperatura, aeração e agitação (ZHU *et al*., 1996; JUNQUA *et al*., 1997; ZHU *et al*., 1998a; ZHU *et al.,* 1998b; ZHENG *et al*., 2001; ZHENG *et al.,* 2002a; ZHENG *et al.,* 2002b; TÉLLEZ-LUIS *et al.*, 2004a; TÉLLEZ-LUIS *et al.*, 2004b; YAN *et al*., 2005). Dessa forma, a otimização desses parâmetros para a determinação das condições ótimas de produção de TGases microbianas é de grande interesse.

Atualmente, a otimização de bioprocessos tem como objetivos maximizar o rendimento do produto e minimizar os custos de produção. Em processos biotecnológicos geralmente se observa a influência de inúmeras variáveis, cuja importância deve ser determinada. Portanto, a otimização baseada no planejamento experimental e análise de superfície de resposta é uma ferramenta muito útil para uma melhor compreensão do sistema, permitindo a análise individual do efeito de cada variável e de suas interações nas respostas desejadas (RODRIGUES & IEMMA, 2005). A metodologia de superfície de resposta baseia-se no método do planejamento fatorial consistindo num grupo de técnicas utilizadas para estudar as relações entre uma ou mais respostas medidas analiticamente e um número de variáveis de entrada independentes que podem ser controladas (BOX *et al.,* 1978). Segundo KALIL *et al.* (2000) e RODRIGUES & IEMMA (2005), a importância do uso da técnica de planejamento experimental e análise de superfície de resposta é o fato de esta metodologia possibilitar a análise dos efeitos sinérgicos ou antagônicos entre as variáveis, que só podem ser observados através da determinação dos efeitos de interação entre as mesmas com o auxílio do planejamento fatorial.

A maioria dos trabalhos sobre a produção de TGase utilizam um meio de cultura contendo amido, 20 g/L; peptona, 20 g/L; extrato de levedura, 2 g/L; $MgSO_4.7H_2O$, 2 g/L; KH_2PO_4, 2 g/L e K_2HPO_4, 2 g/L (ZHU *et al*., 1996; ZHU *et al.,* 1998a; ZHU *et al.,* 1998b; ZHENG *et al*., 2001; ZHENG *et al.,* 2002a; ZHENG *et al.,* 2002b; YAN *et al*., 2005). Esse meio de cultivo também foi usado anteriormente, conforme descrito por SOARES *et al*. (2003b) para a produção de TGase pelo *B. circulans* BL32, porém apresentou o problema de grande formação de espuma.

Assim, o estudo da otimização do meio de cultura (Resultados I) para a produção da enzima TGase pelo *B. circulans* BL32 em sistema submerso, em frascos agitados, foi realizado empregando metodologias de planejamento experimental e análise de superfície de resposta. Na primeira etapa do trabalho visando a escolha de fontes de carbono, nitrogênio orgânico e inorgânico e sais minerais, inúmeros nutrientes foram avaliados através do método clássico de uma-variável-de-cada-vez. Numa segunda etapa, com o auxílio do PB, foram estudados os efeitos das concentrações de glicerol, sacarose, peptona, triptona, Na_2HPO_4, $MgSO_4.7H_2O$ e $FeSO_4.7H_2O$, em dois níveis (+1 e -1). Na terceira etapa, a otimização das concentrações dos dois nutrientes determinados como significativos (sacarose e peptona) foi realizada através da MSR, com 12 experimentos, sendo quatro replicatas do ponto central. A partir dos resultados do planejamento fatorial completo foi possível determinar um meio de cultura para a produção de TGase pelo microrganismo *B. circulans* BL32. A composição desse meio é: glicerol, 9 g/L; sacarose, 2 g/L; peptona, 7 g/L; triptona, 1 g/L; Na_2HPO_4, 1 g/L; $MgSO_4.7H_2O$, 1 g/L e $FeSO_4.7H_2O$, 0,1 g/L. A otimização do meio de cultura resultou em um aumento de 60 % na produção de transglutaminase pelo *B. circulans* BL32, comparando com a máxima atividade obtida, nas mesmas condições de cultivo, empregando um meio previamente citado na literatura, e acima descrito, para a produção dessa enzima, além de redução nos custos do mesmo.

JUNQUA *et al.* (1997) otimizaram a composição de um meio de cultura para a produção de TGase microbiana de *Streptoverticillium cinnamoneum* CBS 683.68. Inicialmente, utilizando o método clássico de uma-variável-de-cada-vez, inúmeras fontes de carbono e nitrogênio foram avaliadas. A seguir, os autores estudaram a significância dos nutrientes previamente selecionados, caseína, glicerol, peptona e extrato de levedura. Na terceira etapa, a otimização da concentração dos dois nutrientes previamente determinados como significativos (caseína e glicerol), foi realizada através do planejamento fatorial completo e da MSR, com 13 experimentos, sendo cinco replicatas do ponto central. A composição do meio de cultivo ótimo foi: glicerol, 31,2 g/L; caseína, 38,4 g/L; peptona, 10,5 g/L; extrato de levedura, 2,5 g/L; $MgSO_4$, 0,5 g/L; KH_2PO_4, 2 g/L e Na_2HPO_4, 5 g/L.

TÉLLEZ-LUIS *et al.* (2004a) também relataram a otimização de um meio de cultura contendo glicerol para produção de TGase microbiana de *Streptoverticillium ladakanum* NRRL-3191.

A determinação dos parâmetros de cultivo é um aspecto relevante que deve ser considerado no desenvolvimento de bioprocessos. Sequencialmente, com o meio de cultura otimizado, foi verificada a influência do pH inicial e da temperatura de cultivo (Resultados II) sobre a produção da enzima TGase pelo microrganismo *B. circulans* BL32 em sistemas submersos. Um planejamento experimental fatorial completo 2^2 foi realizado com as variáveis independentes, pH do meio de cultura e temperatura de produção da enzima, na faixa de valores encontrados na literatura.

Os resultados desse estudo de otimização das condições de cultivo mostraram que a produção de TGase pelo *B. circulans* BL32 é dependente do pH e da temperatura de cultivo. As melhores condições para a produção da TGase pelo *B. circulans* BL32 em cultivo submerso foram 30 °C e pH 8.5. Além disso, observou-se uma correlação entre a produção da enzima e a esporulação desse microrganismo. Assim, a máxima atividade de TGase em cultivos submersos é obtida após vários dias de cultivo, o que torna o bioprocesso longo.

Ainda, em relação à produção da enzima em cultivo submerso, estudou-se o efeito das condições de aeração e agitação (Resultados III), avaliadas através do coeficiente de transferência volumétrica de oxigênio (k_La), em biorreatores de bancada com capacidade de 2 L, sendo a temperatura e o pH inicial fixados em 30 °C e 8,5, respectivamente. Os resultados demonstraram que as condições ótimas de processo para a formação de biomassa e esporulação são diferentes. Portanto, adotando-se uma estratégia de cultivo em biorreator com dois estágios de aeração, primeiramente favorecendo a formação de biomassa (2 vvm e 500 rpm) e após a esporulação e produção de enzima (sem aeração e 500 rpm), foi possível reduzir de forma significativa, de 192 h para 50 h, o tempo de cultivo para a obtenção de máxima atividade enzimática em sistema submerso.

6.2. Produção da transglutaminase em cultivo no estado sólido

Em um segundo momento foi realizada uma série de estudos sobre a produção (Resultados IV) e recuperação (Resultados V) dessa TGase microbiana a partir de cultivos no estado sólido em Biorreatores Cilíndricos Verticais Estáticos.

Primeiramente, vários resíduos agroindustriais (resíduo fibroso de soja, bagaço de malte, casca de soja, gritz de milho e quirera de arroz) foram avaliados como substrato para crescimento do microrganismo e produção da enzima TGase pelo *B. circulans* BL32. A seguir, utilizando o substrato selecionado (resíduo fibroso de soja) um planejamento experimental fatorial completo 2^3 foi realizado com as variáveis independentes, aeração, temperatura e concentração celular do inóculo. Os resultados desse estudo mostraram que as melhores condições para a produção de TGase pelo *B. circulans* BL32 em cultivo no estado sólido são, 0,6 L/min ar, 33 °C e 10 $\log_{10}$ UFC/g substrato para a concentração celular do inóculo, em resíduo fibroso de soja. Os resultados obtidos foram extremamente satisfatórios e possibilitaram uma produção da enzima de 1,25 U/g substrato, em apenas 48 h de cultivo.

A determinação das condições de extração, para a efetiva recuperação da TGase produzida em cultivo no estado sólido, foi realizada através do emprego de ferramentas de planejamento experimental. A partir da otimização dos parâmetros de extração da TGase produzida foi possível determinar as melhores condições de temperatura, agitação, tempo de extração, relação sólido:líquido e o solvente empregado. A melhor condição de extração da enzima foi quando se utilizou água a 7 ºC como solvente, por 5 minutos, 250 rpm e uma relação de sólidos/líquidos de 1:6.

6.3. Caracterização da transglutaminase de *B. circulans* BL32

Como enzimas microbianas utilizadas no processamento de alimentos são tipicamente comercializadas como preparações enzimáticas que contém não somente a enzima desejada, mas também outros metabólitos produzidos pelo microrganismo, em uma terceira etapa desse trabalho (Resultados VI e VII) realizaram-se estudos de caracterização da enzima parcialmente purificada.

Utilizando as condições otimizadas em cultivo submerso, a TGase do *B. circulans* BL32 foi produzida, parcialmente purificada, conforme descrito em SOARES *et al.* (2003a), e caracterizada quanto a pH ótimo, temperatura ótima e de estabilidade e a influência de sais minerais na atividade enzimática (Resultados VI). Os resultados mostraram que a mesma apresenta atividade ótima em pH entre 5,7 a 8,7 e temperatura

entre 25 a 45 °C. Além disso, essa TGase microbiana é relativamente estável a 50 °C. A temperatura ótima para a reação enzimática da TGase de *B. circulans* BL32 é menor que a de *Streptoverticillium mobaraense* (52 °C) (LU *et al*., 2003) e de *Streptoverticillium ladakanum* (50 °C) (TSAI *et al*., 1996).

Além disso, três sistemas protéicos alimentares, caseína, proteína isolada de soja e proteína hidrolisada de carne, foram submetidas a tratamento com TGase de *B. circulans* BL32. Os resultados demonstraram que, após 3 h de incubação com a enzima, as propriedades emulsificantes das proteínas foram melhoradas. Já, a formação de ligações cruzadas ocorreu, principalmente, na caseína e na proteína isolada de soja, indicando a polimerização das cadeias protéicas, como conseqüência da formação de ligações ε-(γ-glutamil)lisina, catalisada por essa enzima.

A literatura relata principalmente o tratamento das proteínas da carne (actina e miosina), do trigo (gliadina e glutenina), da soja (globulinas) e do leite (caseína) com a enzima TGase.

SAKAMOTO *et al.* (1994) sugerem que pedaços de carne podem ser unidos pela aplicação de TGase microbiana, sem a necessidade de aquecimento, adição de NaCl, ou de fosfatos, resultando em um produto cárneo fresco reestruturado. NIELSEN *et al.* (1995) aplicaram a TGase do fator XIII da coagulação sanguínea no músculo *M. longissimus dorsi* e observaram que essa enzima afetou de forma negativa a coloração dos produtos cárneos reestruturados.

As proteínas da soja têm sido estudadas como substratos para a TGase por diversos pesquisadores (IKURA *et al*., 1980; MOTOKI & NIO, 1983; KURTH & ROGERS, 1984; NIO *et al*., 1985; NONAKA *et al*., 1989). O tratamento dessas proteínas com a TGase resultou na formação de ligações cruzadas, melhorando inúmeras propriedades funcionais, tais como, a capacidade de retenção de água, de gelatinização e de textura (NONAKA *et al.,* 1994; ZHU *et al*., 1995; KURAISHI *et al*., 1997; KURAISHI *et al*., 2001).

A caseína também é um bom substrato para a TGase (IKURA *et al*., 1980). A eficiência desta proteína, segundo KURTH & ROGERS (1984), é devido a sua estrutura ser similar a do fibrinogênio. As frações da caseína reagem de forma diferente com a TGase. A β-caseína e κ-caseína são mais reativas que a α-caseína (ÖZRENK, 2006). Segundo FAERGEMAND *et al.* (1999), isso ocorre não somente devido ao conteúdo de resíduos de glutamina e lisina, mas também em função da especificidade da TGase. Já, as

proteínas do soro, devido às suas estruturas globulares compactas, são muito menos reativas à formação de ligações cruzadas catalisadas por essa enzima (SHARMA *et al.*, 2002). FAERGEMAND *et al.* (1998) estudando as propriedades emulsificantes das proteínas do leite, observaram que até um determinado grau de reticulação a estabilidade da emulsão aumenta após tratamento com a TGase microbiana de *Streptoverticillium mobaraense*, obtida da indústria Ajinomoto.

PORTA *et al.* (1990) avaliando a reatividade de diferentes proteínas de origem vegetal, verificaram que as globulinas, gluteninas e gliadinas são mais reativas que as prolaminas como substratos acil doadores. KURTH & ROGERS (1984) estudaram a susceptibilidade de diferentes proteínas à reação de reticulação com a miosina catalisada pela TGase. Os resultados demonstraram que, a caseína é mais reativa que as proteínas do glúten que, por sua vez, são mais reativas que as da soja.

Segundo JONG & KOPPELMAN (2002), uma condição fundamental para que a enzima TGase catalise a formação de ligações cruzadas entre cadeias protéicas é a exposição suficiente dos resíduos de lisina e glutamina do substrato protéico. Algumas proteínas, tais como, gelatina e caseína, são facilmente ligadas pela TGase, pois as lisinas e glutaminas estão facilmente disponíveis. Enquanto que, outras proteínas, como a ovoalbumina, têm estruturas mais rígidas que previnem a formação de ligações cruzadas.

A formação de ligações cruzadas em uma proteína catalisada pela TGase pode ser acompanhada utilizando diferentes metodologias, tais como, avaliação das propriedades funcionais, desaparecimento de grupos amino através do método do ácido trinitrobenzenosulfônico (TNBS) ou produção de NH_3 (NIELSEN, 1995). Um importante ensaio utilizando a alteração das propriedades funcionais das proteínas devido à reação de reticulação é a determinação da força do gel (SAKAMOTO *et al.,* 1994). Neste trabalho, as medidas de resistência das amostras de géis foram realizadas em placas de *microtiter* que permitiram analisar um grande número de amostras em um curto tempo. NONAKA *et al.* (1994) mostraram que a resistência de géis de proteína de soja poderiam ser melhoradas significativamente pelo tratamento com a TGase microbiana.

Ainda, em relação à enzima parcialmente purificada foram realizados estudos relacionados à sua toxicidade *in vivo* e *in vitro* (Resultados VII).

Nos testes *in vivo*, os sinais de intoxicação sistêmica são avaliados a partir da redução na massa corporal dos animais experimentais; da redução do consumo de água e

ração; de alterações comportamentais e apatia; e de outros que podem indicar, por exemplo, injúria hepática (RAUBERT *et al*., 2006).

A preparação enzimática da TGase de *B. circulans* BL32 foi avaliada quanto ao seu potencial toxicológico através de ensaio *in vivo* de toxicidade oral aguda de 14 dias. Os resultados mostraram que a administração oral de TGase microbiana de *B. circulans* BL32 (150 U/kg/dia) por gavagem, por 14 dias, não produziu efeitos tóxicos em ratos Wistar adultos machos. Durante o tratamento nenhum sinal clínico visível de toxicidade foi detectado. Além disso, comparando os resultados, dos grupos controle e teste, de ganho de peso corporal, hematológicos e bioquímicos do sangue e os histológicos dos órgãos (fígado, rins, baço, timo, adrenais, coração, pulmão e cérebro) não foram verificadas quaisquer evidências de toxicidade na preparação enzimática da TGase microbiana de *B. circulans* BL32.

Por outro lado, os ensaios de toxicidade *in vitro* investigam os efeitos tóxicos e os mecanismos moleculares envolvidos nesses processos usando células em cultura. Essa área do conhecimento desenvolveu diversas estratégias para abordagem do efeito de substâncias em células cultivadas, o que melhorou consideravelmente os testes e os procedimentos de validação toxicológicos de diversas substâncias, explorando a base celular e molecular da toxicidade (ZUCCO & VIGNOLI, 1998). No campo da genética toxicológica, os ensaios de células em cultura permitem avaliar as lesões ao DNA assim como o potencial mutagênico e recombinogênico de qualquer substância com facilidade, rapidez, segurança e boa correlação aos resultados de estudos *in vivo* (ZUCCO *et al.,* 2004). Os ensaios de toxicidade *in vitro* empregando células de mamíferos em cultura datam de 1968 e as primeiras linhagens celulares utilizadas para esse propósito foram os fibroblastos de pulmão de hamster chinês, conhecidas como V79. Essas células possuem interessantes propriedades para ensaios de toxicologia genética: fácil cultivo e manutenção; crescimento rápido e curto período de adaptação; tempo de geração entre 12 e 16 horas e capacidade de iniciar um rápido crescimento exponencial a partir de um pequeno inóculo (BRADLEY *et al.,* 1981).

As anormalidades na estrutura do cromossomo são uma conseqüência direta do dano em nível de DNA. Por exemplo, as quebras cromossômicas podem resultar de quebras duplas de DNA não reparadas e os rearranjos cromossômicos, do reparo incorreto de quebras na fita de DNA. A perda de cromossomos e os erros de segregação são eventos

importantes na carcinogênese, causados principalmente por defeitos na formação do fuso, centrômero e alterações na condensação da cromatina antes da metáfase. O ensaio de micronúcleo é hoje um dos testes citogenéticos melhor estabelecido, com validação internacional e aplicável a qualquer população celular nucleada (FENECH, 2000).

O ensaio cometa, também conhecido como eletroforese de célula única em gel, é amplamente empregado para avaliar dano ao DNA e reparação em células eucarióticas. A popularidade desse teste deve-se a sua sensibilidade, custo relativamente baixo e simplicidade. Além disso, o teste pode ser realizado em uma suspensão celular contendo uma população pequena de células, em proliferação ou não, e corresponde a um teste citogenético (COLLINS, 2004; HARTMANN *et al.,* 2001). O princípio desse ensaio leva em conta o comportamento do DNA em células individualizadas e sua organização no núcleo celular. Para a realização do ensaio, as células são embebidas em agarose, suas membranas são rompidas com uso de detergentes em pH alcalino e as proteínas nucleares são removidas com uso de altas concentrações salinas. Dessa maneira, na lâmina permanece apenas o nucleóide íntegro. Quando a lâmina é submetida ao campo elétrico, o DNA do nucleóide migra no gel de acordo com seu tamanho. Os fragmentos pequenos migram com uma velocidade maior que a matriz nuclear. Assim sendo, células com DNA danificado formam, após a migração, o aspecto de um cometa e a extensão da migração correlaciona-se diretamente com a quantidade de dano ocorrido (BURLINSON *et al*., 2007; COLLINS*,* 2004; TICE *et al.*, 2000). As lesões detectadas por esse teste podem ser reparadas. A análise dos resultados pode ser realizada visualmente em microscópio ótico quando as células forem coradas com nitrato de prata ou em microscópio de fluorescência, quando coradas com brometo de etídeo, laranja de acridina ou iodeto de propídeo. As células são classificadas de acordo com o tamanho da cauda em relação a cabeça (núcleo) em 4 classes de danos: classe 0: sem cauda (sem dano); classe 1: com uma pequena cauda menor que o diâmetro da cabeça; classe 2: com o comprimento da cauda entre uma e duas vezes o diâmetro da cabeça; classe 3: com uma cauda longa, superior a duas vezes o diâmetro da cabeça; classe 4: cauda longa e espalhada em forma de leque (TICE *et al.,* 2000).

Nesse contexto, a preparação enzimática da TGase de *B. circulans* BL32 também foi submetida a ensaios de toxicidade *in vitro* utilizando células V79. Os seus efeitos citotóxicos e genotóxicos foram estudados através do ensaio cometa e seu potencial

mutagênico foi avaliado pelo ensaio de micronúcleo. Não foram verificadas quaisquer evidências de efeitos citotóxicos, genotóxicos e mutagênicos devido à aplicação de 150 U/tratamento da TGase microbiana de *B. circulans* BL32 em cultura de células de fibroblastos de pulmão de hamster chinês.

Finalmente, a enzima purificada foi caracterizada quanto a sua cinética de inativação térmica (Resultados VIII).

A entalpia de inativação térmica (ΔH) é a quantidade de energia (J/mol) necessária para desnaturar termicamente a enzima. Um valor alto e positivo para o termo ΔH pode ser associado com uma alta estabilidade térmica da enzima, uma vez que maiores quantidades de energia são necessárias para que o processo de desnaturação térmica ocorra. A entropia de inativação térmica (ΔS), por sua vez, é a quantidade de energia (J/mol.K) envolvida na transição da forma ativa para uma forma desnaturada termicamente da enzima. Um valor positivo para o termo ΔS é indicativo de aumento na desordem do sistema (proteína-solvente) durante o processo de desnaturação térmica. Por outro lado, um valor negativo para o termo ΔS é indicativo de decréscimo na desordem do sistema (proteína-solvente) durante esse processo. Usualmente, um aumento na desordem desse sistema é associado com a desnaturação térmica de enzimas. Então, quanto maior a variação na entropia do sistema durante o processo de desnaturação, menos estável é a enzima. Já, a energia livre de inativação térmica (ΔG) é a quantidade de energia (J/mol) que inclui a contribuição de ambos os termos, entálpico e entrópico, e é um indicador mais confiável da estabilidade térmica de uma enzima. Quanto menor, ou mais negativo, o valor de ΔG mais espontâneo é o processo. Então, quanto menor, ou mais negativo, o termo ΔG mais facilmente a enzima sofre desnaturação, ou seja, menor será a sua estabilidade térmica (MARANGONI, 2003).

Assim, a fim de investigar o mecanismo de inativação térmica, incubou-se a enzima por diferentes períodos de tempo em temperaturas entre 30 e 70 °C e após determinou-se a atividade enzimática residual. As cinéticas de termoinativação desta TGase seguiram o modelo de Lumry-Eyring e a enzima mostrou-se estável até 50 °C, sendo que após 12 h nessa temperatura a mesma ainda mantém 50 % da sua atividade enzimática. Os valores dos parâmetros termodinâmicos de entalpia de inativação térmica (ΔH) e de energia livre de inativação térmica (ΔG) da TGase de *B. circulans* BL32 indicam que essa enzima apresenta uma boa estabilidade térmica no intervalo de temperatura estudado. Além disso, os resultados mostraram que essa enzima se agrega quando submetida a aquecimento, o

que, provavelmente, causa redução da sua atividade enzimática, principalmente em temperaturas acima de 60 °C.

Os resultados apresentados nesse trabalho sugerem que o *B. circulans* BL32 é um sistema biológico promissor para a produção de TGase em processos em larga escala, além disso, a enzima apresenta um grande potencial de uso em aplicações alimentícias e não alimentícias.

7. PERSPECTIVAS

Este trabalho apresentou a possibilidade de produção biotecnológica, tanto em sistema de cultivo submerso quanto em estado sólido, da transglutaminase microbiana, enzima que apresenta grandes perspectivas em função de suas inúmeras aplicações. Portanto, é de fundamental importância a realização de trabalhos futuros visando o escalonamento dos processos biotecnológicos utilizados.

Outra perspectiva que deve ser considerada é o estudo do efeito de diferentes formas de condução dos cultivos submersos, por exemplo, batelada alimentada, sobre a produção da transglutaminase por esse microrganismo.

Além disso, seria importante avaliar a possibilidade de emprego de outros resíduos agroindustriais como substratos para a produção dessa enzima em sistema de cultivo em estado sólido.

Cabe salientar a importância da realização de estudos de manipulação genética, a fim de caracterizar o gene e expressar em sistemas mais eficientes.

Além disso, com base nos resultados dessa pesquisa, os trabalhos futuros com esta enzima podem incluir um estudo mais aprofundado sobre a cinética de inativação térmica da mesma, principalmente em relação ao efeito de diferentes pHs.

E por último, é importante aprofundar os estudos em relação ao potencial de aplicação da transglutaminase produzida pelo isolado *B. circulans* BL32 sobre outras proteínas alimentícias e não alimentícias.

8. REFERÊNCIAS BIBLIOGRÁFICAS

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9. ANEXOS

Em anexo são apresentados dois trabalhos realizados durante o doutorado sobre as alterações nutricionais de diferentes proteínas alimentares devido à reticulação pela aplicação da transglutaminase comercial.

No artigo do Anexo A foram estudadas as características nutricionais das proteínas da carne e da soja devido ao processo de reticulação.

O artigo do Anexo B mostra os aspectos nutricionais das proteínas do trigo e do leite tratadas com a transglutaminase comercial.

9.1 ANEXO A. "Nutritional effects of mechanically deboned chicken meat and soybean proteins cross-linking by microbial transglutaminase" – Food Science and Technology International, submetido em março de 2008.

Nutritional effects of mechanically deboned chicken meat and soybean proteins cross-linking by microbial transglutaminase

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Abstract

In this work it was studied the nutritional effects of protein cross-linking as mediated by microbial transglutaminase (TGase) using rats as biological models. These rats were divided into six groups that received diets containing casein (standard protein diet), cross-linked meat protein (CMP), meat protein (MP), cross-linked soy protein (CSP) or soy protein (SP) as the protein sources of their diets, which were compared against an aproteic diet (basal diet). The cross-linking process showed a significant ($P < 0.05$) impact on the true digestibility (TD) of meat and soy proteins, decreasing it for the first and increasing it for the second. As a consequence, the TD of modified soy protein became similar to that observed for cross-linked meat protein, although, the biological value (BV), net protein utilization (NPU), net protein ratio (NPR), and protein retention efficiency (PRE) values of meat and soy proteins were not affected in the process. These results suggest that the use of TGase for the reticulation of isolated soy protein can improve its nutritional quality.

Keywords: Nutritional quality of protein; meat protein, isolated soy protein, protein cross-linking, transglutaminase.

INTRODUCTION

Proteins play a major role in the quality of many foods. They determine the nutritional value and many of the functional properties of these systems. The nutritional quality of a protein is related mainly to its essential amino acid composition and digestibility (FAO/WHO, 1973), while its functional properties, such as solubility, water-holding capacity, viscosity, among others, will have strong influence in the food characteristics and industrial applications (Panyam and Kilara, 1996).

The modification of proteins by enzymes and chemicals has been extensively studied and has been shown to be important for improving functional properties of the final product (Gerrard, 2002). The use of enzymes to replace synthetic chemicals for these modifications is being preferred by industry due to the growing concerns about the possible undesirable side-effects of chemicals in foods (Gujral and Rosell, 2004). Protein functionality can be modified by forming intramolecular or intermolecular cross-links (Jong and Koppelman, 2002). The cross-linking of food proteins by enzymatic reactions produces substantial changes in the structure of proteins and can influence many properties of food, including texture, viscosity, solubility, water-holding capacity, thermal stability, emulsification, and gelation (Kuraishi, 2000; Motoki and Kumazawa, 2000). Transglutaminase (TGase) is an enzyme that has received extensive recent attention for its ability to cross-link proteins (Kuraishi et al., 2001).

Transglutaminases (protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) are a family of enzymes that catalyses acyl transfer reactions between γ-carboxyamide groups of glutamine residues and the ε-amino group of lysines in proteins, leading to the covalent inter or intramolecular cross-linking of the proteins (Nonaka et al., 1989). TGase has been found in animal and plant tissues and microorganisms (Zhu et al., 1995). The microbial enzyme has widespread and growing applications in the food processing industry (Kuraishi et al., 2001). TGase catalyses the formation of homologous and heterologous polymers of meat, soybean, milk, and wheat proteins (Zhu et al., 1995; Motoki and Seguro, 1998; Kuraishi et al., 2001). For instance, the structural meat proteins actin and myosin can be cross-linked by TGase. Kuraishi et al. (1997) produced restructured meat using microbial transglutaminase. According to Muguruma et al. (2003) the texture of sausages made from chicken meat was improved by the formation of ε-(γ-glutamyl)lysine cross-links by the addition of TGase, even at reduced levels of phosphate. Some researches have also shown

that it is possible to polymerize the soy protein using microbial TGases and that the use of this enzyme allows manufacturers to work with greater degrees of texture control (Motoki and Seguro, 1998). Babiker et al. (1996) showed that the polymerization with TGase improves the solubility and surface functional properties of soy protein peptides. This enzymatic cross-linking showed to increase the temperatures of thermal denaturation of proteins of isolated soy protein (Tang et al., 2006). Ramírez-Suárez and Xiong (2003) have shown that microbial TGase catalyzed the interaction and gelation of mixed myofibrillar and isolated soy protein.

The introduction of covalent cross-links between proteins in food produces substantial changes in the structure of proteins, and therefore might affect the nutritional quality of the final product (Gerrard, 2002). The formation of unnatural covalent cross-linking of amino acids, either intra or extra molecular, may decrease the digestibility and biological availability of essential amino acids that are involved in the cross-linking of the food proteins (Erbersdobler, 1989).

Although the use of TGase by the food industry has been extensively explored, the nutritional effects of protein cross-linking by this enzyme have not been reported for *in vivo* models. Since modern diets are designed considering their nutritional values and sensory properties, the present study was undertaken to evaluate the nutritional effects of the cross-linking by microbial TGase. It was employed meat of low economical value, but otherwise nutritionally rich, the mechanically deboned chicken meat (MDCM), and isolated soy protein as a vegetal protein. These two materials were chosen because they are important sources of protein, largely used by the food industry, but lack some functional properties that may be improved by cross-linking.

MATERIALS AND METHODS

Materials

Mechanically deboned chicken meat (MDCM) was obtained from Eleva S.A. (Rio Grande do Sul, Brazil). The isolated soy protein (ISP) was kindly provided by The SOLAE Company (Rio Grande do Sul, Brazil). Microbial TGase was from Ajinomoto (Japan) and had a specific activity of 100 U/g of product. All reagents were of analytical grade.

Mechanical strength evaluation

A study was performed in order to determine the effect of reaction time of the TGase on the mechanical strength of samples. Different MDCM and ISP samples were incubated with 1 % (w/w) of TGase at 37 °C for an incubation time of 0, 1, 2 and 3 h. MDCM, which is in the form of minced meat, was readily mixed with enzyme by manual homogenization for 5 minutes. ISP, which is in the form of powder, was previously hydrated with 3.5 times its weight with distilled water and this gel was then mixed with enzyme as described above. Control samples were incubated without enzyme. The mechanical strength of samples was determined using a Texture Analyzer TA-XT2 (Stable Micro Systems Ltd., New York, USA). Breaking force (g) was determined using a cylindrical stainless steel probe with a pressing surface diameter of 35 mm at room temperature. The instrument was adjusted to the following conditions: probe area, 9.62 cm^2; measurement time, 14 s; penetration speed, 0.5 mm/s; penetration distance, 7 mm into surface. Gel strength was carried out in triplicate and expressed as g/cm^2 of probe area. Results were statistically analyzed using one-way ANOVA (Analysis of Variance) within the groups with and without enzymatic treatment.

Sample preparation

The cross-linking of both meat and soy proteins was prepared by mixing either MDCM or ISP with 1 % (w/w) of TGase, as described above. The mixtures were incubated at 37 °C for 2 h. These samples were designated cross-linked meat protein (CMP) and cross-linked soy protein (CSP), respectively. MDCM and ISP were incubated at 37 °C for 2 h, without TGase, and these samples were designated, meat protein (MP) and soy protein (SP) and served as controls. All four samples were dried in an air circulation oven at 45 °C, for 4 hours, and powdered in a food micro homogenizer to pass through a 60-mesh sieve. These flours were stored at 4 ^oC for the subsequent chemical analysis and for preparation of the respective diets.

Basic chemical composition

The percentage of moisture, protein, lipid, and ash of all samples were determined by following the respective AOAC methods (1996).

Composition of diets

Four test protein diets (CMP, MP, CSP, SP), one standard protein diet (casein) and one non-protein diet, used as controls to estimate the endogenous nitrogen excretion of the rats, were prepared by mixing the protein source and others ingredients and their exact compositions are shown in Table 1. Standard and test protein diets were adjusted to 10 % protein, whereas the non-protein diet was devoid of any protein. In addition to the protein sources, the diets contained vitamins and minerals mix (AIN-93G) according to Reeves et al. (1993). For the preparation of the diets, ingredients were homogenized and passed through a 60-mesh sieve to ensure uniform distribution of minerals and vitamins. All the diets were analyzed for their moisture and protein contents (AOAC, 1996).

Table 1. Composition of the experimental diets

Ingredients (%)	Basal diet	Casein diet	Test diets			
			CMP diet	MP diet	CSP diet	SP diet
Casein	-	12.20	-	-	-	-
CMP flour	-	-	20.83	-	-	-
MP flour	-	-	-	21.15	-	-
CSP flour	-	-	-	-	12.75	-
SP flour	-	-	-	-	-	12.82
Sucrose	10.00	10.00	10.00	10.00	10.00	10.00
Soybean oil	7.00	7.00	-	-	7.00	7.00
Cellulose	5.00	5.00	5.00	5.00	5.00	5.00
Salt mixture*	3.50	3.50	3.50	3.50	3.50	3.50
Vitamin mixture*	1.00	1.00	1.00	1.00	1.00	1.00
L-cystine	-	0.30	-	-	-	-
Choline bitartrate	-	0.25	-	-	-	-
Corn starch	73.50	60.75	59.67	59.35	60.75	60.68

Basal diet = non-protein diet; Casein diet (control diet) = standard protein diet.

CMP, cross-linked meat protein; MP, meat protein; CSP, cross-linked soy protein; SP, soy protein.

* According to Reeves et al. (1993).

Animal tests

Young 21–25 days old, white male Wistar rats with average body weight 45.0 ± 10.0 g, were obtained from the Central Animal House, Pelotas Federal University, Rio Grande do Sul, Brazil. The rats were randomly divided into six groups, each consisting of six rats. The animals were housed individually in stainless steel metabolic cages designed for separate collection of faeces and urine. Cages were located in a room with a 12 h light/dark cycle, at a temperature of 21 ± 2 °C, fitted with an appropriate ventilation system. Food and water were given *ad libitum*.

Growth experiments

For growth studies, the rats were housed individually in stainless steel metabolic cages. One group of rats was fed on a casein diet (control diet) and four groups of rats were fed on test diets (containing flours of either CMP, MP, CSP, or SP). The rats were initially weighed and then weighed on every alternate day. Food and water were given *ad libitum*. The weighed diet was given daily and the unconsumed diet was collected and weighed. The rats fed on different experimental and control diets were weighed for four weeks and the gain in weight during this period was recorded. The consumed protein was calculated from the consumed nitrogen, based on the nitrogen content of the diet. Protein efficiency ratio (PER), food efficiency ratio (FER), and food transformation index (FTI) were calculated by the following formulas, as described by FAO/WHO (1989):

$$PER = \frac{[Gain\ in\ body\ weight\ (g)]}{[\mathrm{Pr}otein\ consumed\ (g)]} \qquad (1)$$

$$FER = \frac{[Gain\ in\ body\ weight\ (g)]}{[Food\ consumed\ (g)]} \qquad (2)$$

$$FTI = \frac{[Food\ consumed\ (g)]}{[Gain\ in\ body\ weight\ (g)]} \qquad (3)$$

The corrected protein efficiency ratio (C-PER) was calculated according to Chapman et al. (1959), where 2.5 as standard value for casein:

$$C - PER = \frac{PER \times 2.5}{[Deter\,\mathrm{min}ed\ PER\ for\ reference\ casein]} \qquad (4)$$

Nitrogen balance experiments

For nitrogen balance studies, the rats were housed individually in stainless steel metabolic cages. One group of rats was fed on a protein-free diet (basal diet) and another on a casein diet (control diet). Four groups of rats were fed on test diets (containing flours of either CMP, MP, CSP, or SP). Experiments were conducted for fourteen days, which included an initial conditioning period of four days. During the last ten days, urine and faeces of each rat were collected separately. Food and water were given *ad libitum* and the change in body weight was recorded. The faeces were oven-dried at 100 °C for 24 h. The dried samples were ground to 20 meshes. The concentration of nitrogen in the urine and faecal powder was estimated by the micro Kjeldhal method (AOAC, 1996). The data obtained from these experiments were used to calculate nitrogen absorbed (NA), nitrogen retention (NR), apparent nitrogen digestibility (AND), true digestibility (TD), biological value (BV), net protein utilization (NPU) and net protein retention (NPR), as described by FAO/WHO (1989); and protein retention efficiency (PRE), as described by Bender and Doell (1957), by employing the following formulas:

$$NA = NI - NF_1 \tag{5}$$

$$NR = NI - (NF_1 + NU_1) \tag{6}$$

$$AND = \frac{[NI - NF_1]}{NI} \times 100 \tag{7}$$

$$TD = \frac{NI - (NF_1 - NF_2)}{NI} \times 100 \tag{8}$$

$$BV = \frac{NI - (NF_1 - NF_2) - (NU_1 - NU_2)}{NI - (NF_1 - NF_2)} \times 100 \tag{9}$$

$$NPU = \frac{BV \times TD}{100} \tag{10}$$

$$NPR = \frac{[Weight\ gain\ of\ test\ group + Weight\ loss\ of\ protein - free\ group]}{[Weight\ of\ test\ protein\ consumed]} \tag{11}$$

$$PRE = NPR \times 16 \tag{12}$$

where, NI is nitrogen intake of animals fed test diet; NF_1 the nitrogen excreted in faeces of animals fed test diet; NF_2 the nitrogen excreted in faeces of animals fed protein-free diet

(basal diet); NU_1 the nitrogen excreted in urine of animals fed test diet; NU_2 the nitrogen excreted in urine of animals fed protein-free diet (basal diet).

Statistical analysis

All the parameters were calculated for each rat and means were calculated within each group. Analysis of variance (ANOVA) was used to evaluate the mean differences between the dietary treatments. The least significant different (LSD) values were computed in case the F-test showed significant differences. A significant difference was considered at a level of $P < 0.05$.

RESULTS AND DISCUSSION

Mechanical strength evaluation

The effect of reaction time of TGase on the mechanical strength of samples of meat and soy proteins, cross-linked and non-cross-linked, are shown in Figure 1. The maximal gel strength of meat and soy proteins was obtained at 2 h. Results show that gel strength for the MDCM (Fig.1A) and ISP (Fig. 1B) samples are similar ($P > 0.05$) for incubation times of either 2 or 3 h with the enzyme. Therefore, in further experiments, it was used the reaction time of 2 hours for the completion of enzymatic cross-linking.

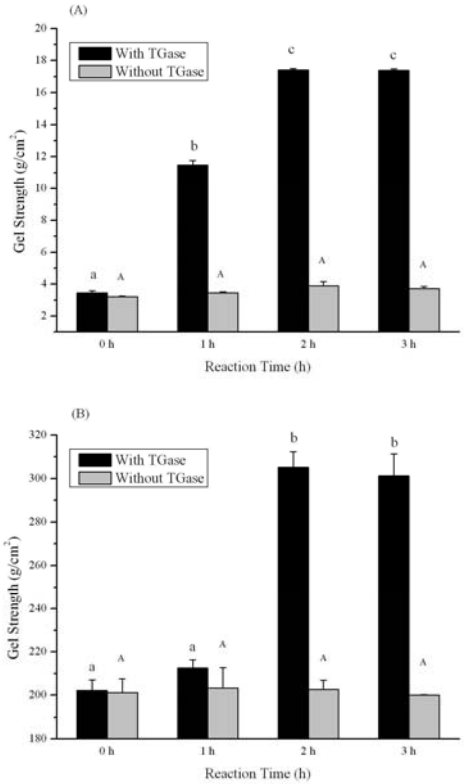

Figure 1. Mechanical strength of non-crosslinked and cross-linked samples of MDCM and ISP modified by TGase as measured by gel strength. (A) meat protein; (B) soy protein. Equal letters indicate that samples do not significantly differ ($P > 0.05$) (lower caps: samples with enzymatic treatment; capital: untreated samples).

Proximate chemical composition

The chemical composition of the flour of cross-linked meat protein (CMP), meat protein (MP), cross-linked soy protein (CSP), and soy protein (SP) is presented in Table 2. Chemical compositions were similar for both samples of meat protein (CMP and MP) and soy protein (CSP and SP) with a small increase of the protein fraction in the cross-linked samples. Some authors have shown that there is a slight increase in protein content of samples after cross-linking. Ahn et al. (2005), observed that after the treatment with TGase, the protein contents of wheat, barley, and soy flours increased 0.6 %, 0.8 %, and 1.6 %, respectively. Rosell et al. (2003) reported that the wet gluten content of wheat flours was slightly increased with TGase treatment due to a polymerization of proteins.

Table 2. Proximate chemical composition (%) of the flours of cross-linked meat protein, meat protein, cross-linked soy protein and soy protein [*]

Samples	Moisture	Protein	Lipids	Ash	Total carbohydrate[**]
CMP flour	7.11 ± 0.01	48.00 ± 0.02	37.01 ± 0.12	2.85 ± 0.01	5.03 ± 0.10
MP flour	7.41 ± 0.01	47.29 ± 0.18	37.46 ± 0.39	2.98 ± 0.09	4.86 ± 0.27
CSP flour	9.52 ± 0.01	78.45 ± 0.31	1.10 ± 0.06	4.04 ± 0.07	6.89 ± 0.25
SP flour	9.48 ± 0.02	78.00 ± 0.27	0.72 ± 0.04	3.86 ± 0.08	7.94 ± 0.18

CMP, cross-linked meat protein; MP, meat protein; CSP, cross-linked soy protein; SP, soy protein.

[*] Values are means ± S.D. of triplicate analysis.

[**] Total carbohydrate = 100 - (sum of percentages of moisture, protein, lipids, and ash).

Growth experiments

Results of the effects of cross-linked and non-cross-linked meat and soy proteins on animal growth are shown in Table 3. Food intake after 28 days on the CSP and casein (standard protein) diets were the highest, 418.27 and 415.32 g per rat, respectively, but these did not significantly differ ($P > 0.05$) from the other diets where food intake ranged from 398.39 to 403.28 g per rat (Table 3). The results of food intake for the rats fed with CMP and CSP diets, 401.91 and 418.27 g, respectively, were similar to values obtained by Seguro et al. (1996) for rats fed with diets containing cross-linked casein. Protein intake of the control group was 42.24 g per rat, which did not significantly differ ($P > 0.05$) from protein intake of the other test groups that ranged from 40.83 to 44.13 g per rat (Table 3). Body weight gain on the control group was the highest (117.75 g per rat) after 28 days, but the difference was not significant ($P > 0.05$) in comparison to CMP and MP diets, 104.59 and 102.77 g per rat (Table 3). However, the body weight gain was significantly lower ($P < 0.05$) in the groups fed on isolated soy protein and cross-linked isolated soy protein. The cross-linking reaction of the meat and soy proteins had no effect on the food and protein intake by the rats and body weight gain of the animals after 28 days. These results show that cross-linked meat and soy proteins support the growth of young rats similarly to the non-cross-linked proteins. Moreover, the cross-linked meat protein supports the growth of the test animals similarly to the standard protein (casein).

Table 3. Rat growth assay values[*] of cross-linked meat protein, meat protein, cross-linked soy protein and soy protein

Parameters	Casein diet	Test diets			
		CMP diet	MP diet	CSP diet	SP diet
Food intake (g)	415.32 ± 66.58[a]	401.91 ± 32.81[a]	403.28 ± 32.03[a]	418.27 ± 42.93[a]	398.39 ± 33.73[a]
Protein intake (g)	42.24 ± 6.77[a]	40.83 ± 3.33[a]	41.70 ± 3.31[a]	44.13 ± 4.53[a]	41.71 ± 4.53[a]
Body weight gain (g)	117.75 ± 26.89[a]	104.59 ± 10.66[a]	102.77 ± 11.93[a]	74.86 ± 8.23[b]	73.06 ± 8.23[b]
PER	2.77 ± 0.34[a]	2.57 ± 0.23[a]	2.46 ± 0.20[a]	1.70 ± 0.09[b]	1.75 ± 0.06[b]
C-PER[**]	2.50 ± 0.00[a]	2.32 ± 0.20[ab]	2.22 ± 0.16[b]	1.53 ± 0.08[c]	1.58 ± 0.06[c]
FER	0.28 ± 0.03[a]	0.26 ± 0.02[a]	0.25 ± 0.02[a]	0.18 ± 0.01[b]	0.18 ± 0.01[b]
FTI	3.59 ± 0.44[a]	3.86 ± 0.34[a]	3.95 ± 0.36[a]	5.60 ± 0.31[b]	5.46 ± 0.20[b]

Casein diet (control diet) = standard protein diet.

CMP, cross-linked meat protein; MP, meat protein; CSP, cross-linked soy protein; SP, soy protein.

Means with different superscripts in the same horizontal row are significantly different ($P < 0.05$).

[*] Values are means ± S.D. of six rats in each group throughout 28 days of experimental period.

[**] Based on values of 2.5 as standard for casein.

Casein diet produced a PER of 2.77 (Table 3). Although not significant ($P > 0.05$), this value was higher than cross-linked and non-cross-linked meat protein diets (2.57 and 2.46, respectively), and the PER of these three formulations were significantly higher ($P < 0.05$) than the PER of the cross-linked and non-cross-linked soy protein (1.70 and 1.75, respectively), probably due to the limiting amino acids contents of soy, such as sulfur-containing amino acids (Sikka et al., 1978). The protein availability of cross-linked and non-cross-linked meat was similar ($P > 0.05$). The C-PER values of the four test diets - 1.53 (CSP), 1.58 (SP), 2.22 (MP) and 2.32 (CMP) - were lower than that of the standard diet (2.50), and differences were not significant ($P > 0.05$) only between CMP and the standard protein diet. The differences between the PER and C-PER of the cross-linked and non-cross-linked proteins were not significant, suggesting that the formation of ε-(γ-glutamyl)lysine cross-links in meat and soy proteins have no influence in the body weight gain per unit of protein intake. These results are similar to those obtained by Seguro et al. (1996) who studied the bioavailability of the ε-(γ-glutamyl)lysine moiety in cross-linked casein in rats and obtained the C-PER value of 2.47 for the diet containing cross-linked casein, which is very close to the 2.50 value of non-treated casein.

The FER was highest for casein diet (0.28). This value did not significantly differ ($P > 0.05$) from cross-linked and no cross-linked meat protein (0.26 and 0.25, respectively), but does from the isolated soy protein diets (0.18). The food transformation index (FIT) was better for rats fed with casein diet, with this group requiring lower feeding to increase one gram of weight (3.59) compared to the rats fed with either CMP (3.86), MP (3.95), SP (5.46), or CSP (5.60) diets. Statistical analysis showed a similar trend to the food efficiency ratio. The polymerization of meat and soy proteins with TGase did not affect the FER or FTI of the animals.

The biological evaluation of protein provides useful information regarding the overall nutritional quality of a protein when tested *in vivo* (Sogi et al., 2005). The body weight gain, PER, FER, and FTI values, especially for meat proteins, show the good nutritional quality of the cross-linked proteins by TGase. According to Sgarbieri (1996), when experimental animals gain weight and other indexes as FER and PER are good, it is an excellent indicator that the fed diets are of high quality.

Nitrogen balance experiments

Results of nitrogen balance studies of meat and soy proteins cross-linked by microbial TGase are shown in Table 4. Results indicate that the consumed, absorbed, and retained nitrogen were similar for casein and the test group diets. Although their values were not significantly different ($P > 0.05$), nitrogen consumed and nitrogen absorbed of the CSP diet were slightly higher than the other protein sources, 3.33 g and 3.19 g per rat, respectively. In relation to retained nitrogen, the highest value ($P > 0.05$) was obtained for the standard diet, 2.63 g per rat. The retained nitrogen was positive in all groups, showing that nitrogen intake was larger than the faecal and urinary excretion of nitrogen. Nitrogen excreted in faeces was the lowest ($P < 0.05$) for the animals on MP and CSP diets (Table 4), while excreted nitrogen in urine was higher ($P < 0.05$) for the animals on both soy proteins diets, probably due to the limiting amino acids contents of soy, leading to the great portions of protein being deaminated for energy production (Sikka et al., 1978).

Table 4. Nitrogen balance evaluation values* of cross-linked meat protein, meat protein, cross-linked soy protein and soy protein

Parameters	Casein diet	Test diets			
		CMP diet	MP diet	CSP diet	SP diet
Daily food intake (g)	14.32 ± 1.46^{a}	13.09 ± 1.46^{a}	13.21 ± 1.52^{a}	14.10 ± 1.42^{a}	14.09 ± 1.19^{a}
Daily protein intake (g)	1.46 ± 0.15^{a}	1.33 ± 0.15^{a}	1.37 ± 0.16^{a}	1.49 ± 0.15^{a}	1.48 ± 0.12^{a}
Daily body weight gain (g)	5.20 ± 0.97^{a}	3.38 ± 0.55^{b}	3.02 ± 0.55^{b}	2.60 ± 0.37^{b}	3.12 ± 0.24^{b}
Body weight loss** (g)	11.09 ± 2.86	11.09 ± 2.86	11.09 ± 2.86	11.09 ± 2.86	11.09 ± 2.86
Nitrogen consumed (g)	3.26 ± 0.33^{a}	2.98 ± 0.33^{a}	3.06 ± 0.35^{a}	3.33 ± 0.33^{a}	3.30 ± 0.28^{a}
Nitrogen absorbed (g)	3.02 ± 0.28^{a}	2.69 ± 0.32^{a}	2.91 ± 0.35^{a}	3.19 ± 0.32^{a}	2.99 ± 0.24^{a}
Nitrogen retained (g)	2.63 ± 0.21^{a}	2.24 ± 0.29^{a}	2.43 ± 0.35^{a}	2.46 ± 0.27^{a}	2.29 ± 0.20^{a}
Nitrogen faecal (g)	0.24 ± 0.06^{b}	0.29 ± 0.04^{ab}	0.15 ± 0.01^{c}	0.14 ± 0.03^{c}	0.31 ± 0.05^{a}
Nitrogen urinary (g)	0.39 ± 0.11^{b}	0.45 ± 0.07^{b}	0.48 ± 0.09^{b}	0.73 ± 0.08^{a}	0.70 ± 0.07^{a}
AND (%)	92.54 ± 1.21^{b}	90.08 ± 1.23^{c}	95.16 ± 0.59^{a}	95.64 ± 0.61^{a}	90.52 ± 0.88^{c}
TD (%)	94.38 ± 1.34^{b}	92.09 ± 1.17^{c}	97.12 ± 0.39^{a}	97.44 ± 0.65^{a}	92.32 ± 0.98^{c}
BV (%)	88.16 ± 2.85^{a}	84.18 ± 2.30^{a}	84.25 ± 3.13^{a}	78.06 ± 1.84^{b}	77.62 ± 1.64^{b}
NPU (%)	83.22 ± 3.26^{a}	77.54 ± 2.73^{bc}	81.83 ± 3.23^{ab}	76.08 ± 1.81^{cd}	71.67 ± 2.08^{d}
NPR	4.13 ± 0.57^{a}	3.14 ± 0.29^{b}	2.80 ± 0.30^{bc}	2.29 ± 0.21^{c}	2.66 ± 0.22^{bc}
PRE	66.06 ± 9.19^{a}	50.18 ± 4.58^{b}	44.78 ± 4.74^{bc}	36.61 ± 3.43^{c}	42.60 ± 3.54^{bc}

Casein diet (control diet) = standard protein diet.
CMP, cross-linked meat protein; MP, meat protein; CSP, cross-linked soy protein; SP, soy protein.
Means with different superscripts in the same horizontal row are significantly different ($P < 0.05$).
* Values are means ± S.D. of six rats in each group throughout 10 days of experimental period.
** Group the rats fed with protein-free diet.

Results in Table 4 indicate that the true digestibility (TD) was significantly lower ($P < 0.05$) for the cross-linked meat protein (92.09 %) and significantly higher ($P < 0.05$) for the cross-linked soy protein (97.44 %) than the TD value obtained for rats on casein diet. TD and apparent nitrogen digestibility (AND) followed a similar trend. Therefore, cross-linking decreases the percentage of nitrogen intake absorbed for meat proteins, while it increases this absorption for soy proteins. The results obtained in the present study showed that cross-linking by microbial TGase had a different effect on the digestibility of the meat proteins and isolated soy protein, probably due to different conformational changes of these proteins, which, in turn, might result in alterations towards the action of proteases over these proteins in the digestive tract (Sgarbieri, 1996). Ahhmed et al. (2007), studying gel improvement of sausage texture of chicken and beef induced by TGase, suggested that this enzyme restructures the protein conformation of these sources. Researches on the degradation of *in vitro* ε-(γ-glutamyl)lysine show that after ingestion of cross-linked proteins, normal mammalian gastrointestinal digestive enzymes cleave them into amino acids but leave the ε-(γ-glutamyl)lysine dipeptide intact. The resistant ε-(γ-glutamyl)lysine dipeptide may be absorbed through the intestinal brush-border and transported to the kidney (Fink et al., 1980). Generally, the digestibility of animal protein is higher than vegetal proteins. However, in this research, the cross-linking process modified the soy protein digestibility to an extent that the TD was up 97.44 % and became very similar to TD of the meat protein (97.12 %).

The biological value (BV) for rats on the standard diet (88.16 %) was similar ($P > 0.05$) to those for rats on the CMP (84.18 %) and MP diets (84.25 %) (Table 4). The VB values obtained for rats on isolated soy protein diets (77.62 % to SP and 78.06 % to CSP) were lower ($P < 0.05$) compared to the other proteic sources. The VB and PER (Table 3) values also followed a similar trend. The action of TGase involves cross-linking of free primary amino groups of lysine with glutamine residues, but the results indicate that the biological value of the meat and soy protein was not reduced by this process. According to Seguro et al. (1996), the cross-linking by TGase is thought to protect nutritionally valuable lysine residues in food from various deteriorative reactions. Furthermore, the use of TGase potentially allows production of food proteins of higher nutritional quality by cross-linking of different proteins containing complementary amino acids (Zhu et al., 1995). Ikura et al. (1981) introduced methionine into soybean globulins and lysine into wheat gluten using

the guinea pig liver enzyme. Their results suggested that the TGase reaction can also be used to improve the nutritional value of food proteins through the covalent attachment of limiting essential amino acids.

The NPU value was higher (83.22 %) (Table 4) for rats on the control diet but similar ($P > 0.05$) to the values obtained for rats on the MP diet (81.83 %). The NPU values obtained for rats on diets of CMP and CSP, 77.54 % and 76.08 %, respectively, were similar ($P > 0.05$), but lower ($P < 0.05$) than the standard protein diet. The cross-linking process decreased the net protein utilization of the meat and soy protein in relation to the casein diet. However, there are no significant differences between NPU values of the rats fed on cross-linked and non-cross-linked meat protein diets, and between the NPU values of the rats fed on cross-linked and non-cross-linked soy protein diets. The results suggest that the enzymatic cross-linking of these proteins did not change the amount of nitrogen intake.

Daily protein intakes of animals on the casein control diet and the test diets were not significantly different ($P > 0.05$) (Table 4). However, animals on the casein diet gained body weight rapidly and had significantly ($P < 0.05$) higher NPR (4.13) than animals on test diets**.** The differences between the NPR values of the cross-linked proteins and non-cross-linked were non-significant *(*$P > 0.05$), so the cross-linking of meat and soy proteins had no influence on the ability of proteins to support both maintenance and growth.

Table 4 indicates the distinct superiority of the casein diet over the other diets, with respect to protein retention efficiency (PRE). The PRE value of casein diet was 66.06, and for test diets this ranged from 36.61 to 50.18. This was expected since casein is a pure protein source with well balanced amino acid profile and hence the choice as a standard protein diet (control diet) with which other diets can be compared. The highest value of PRE (50.18) among test diets was for cross-linked meat protein. The results suggest that the protein retention efficiency of meat and soy proteins is not affected by the cross-linking process.

CONCLUSIONS

The food industry is constantly seeking novel ingredients to improve existing products or to allow for the introduction of new ones. Transglutaminase has been used to promote polymerization, texture improvement, restructuring and gel formation in foods. This

enzyme has found applications in a diverse range of food processing such as meat, milk, wheat and soy products. Although some works showed the physicochemical and functional changes of diverse food proteins treated with transglutaminases, the nutritional quality of these modifications has only being described for casein. Since cross-linking has a profound effect on the structure and function of proteins in foods, it could affect their nutritional quality (Friedman, 1999). Our results suggest that cross-linked meat and soy proteins support the growth of young rats, and the transglutaminase-mediated cross-linking process of these proteins does not affect some nutritional parameters, such as body weight gain, protein efficiency ratio (PER), food efficiency ratio (FER), food transformation index (FTI), biological value (BV), net protein utilization (NPU), net protein ratio (NPR), and protein retention efficiency (PRE). On the hand, protein digestibility of meat and soy were modified by the covalent cross-linking, probably due to the action of this enzyme in the conformational structure of the tested proteins. Since transglutaminase is been applied to many types of foods, it would be important to study its effects on the nutritional qualities of other protein-containing food systems such as milk and milk-derived products, wheat flour, beans and peas.

ACKNOLEDGEMENT

The authors wish to thank CNPq and CAPES for their financial support and scholarships. The animal experimentation was authorized by Resolution 01/2007 of the Research Committee of the Food and Science Institute of Technology of The Federal University of Rio Grande do Sul State. One of the authors (JGV) has a formal authorization to humanely manipulate experimental animals.

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9.2 ANEXO B. “The in vivo influence of microbial transglutaminase-induced cross-linking on the nutritional quality of milk and wheat proteins” – Journal of the Science of Food and Agriculture, submetido em maio de 2008.

The *in vivo* influence of microbial transglutaminase-induced cross-linking on the nutritional quality of milk and wheat proteins

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Abstract

BACKGROUND: The improvement of functional properties of milk and wheat proteins modified by microbial transglutaminase (TGase) has been the subject of many studies in food technology. However, the nutritional quality of these proteins, as a consequence of the cross-linking reaction mediated by TGase, has not been elucidated. Thus, the present study was designed to evaluate the *in vivo* nutritional quality of milk and wheat proteins after cross-linking by microbial TGase.

RESULTS: White Wistar rats were divided into six groups receiving diets that contained casein, cross-linked milk protein, milk protein, cross-linked wheat protein, wheat protein, or a non-proteic diet. Results showed that cross-linked milk and wheat proteins can support growth and the animals had a positive nitrogen balance. True digestibility was similar between casein and non-cross-linked milk protein diets. It was also observed that milk and wheat proteins were not affected by cross-linking concerning several quality parameters: protein efficiency ratio, food efficiency ratio, food transformation index, apparent nitrogen digestibility, true digestibility, biological value, net protein utilization, net protein ratio, and protein retention efficiency.

CONCLUSION: Based on the obtained results, it can be suggested that the use of microbial TGase does not affect the nutritional quality of milk and wheat proteins, while improving their physicochemical properties.

Keywords: Protein quality; Milk proteins; Wheat proteins; Protein Cross-linking; Transglutaminase

INTRODUCTION

Foods are multi-components matrices of complex structures. The macromolecular structures of foods influence their mechanical and physical properties, chemical and microbiological stability, sensory properties and nutrition value. The nutritional and sensory properties of food play a major role in product quality because they are recognized by consumers and are the main factors behind food choices,[1] and many of these nutritional and sensory properties are determined by proteins.[2] The nutritional quality of a protein is mainly due to its essential amino acid composition and digestibility.[3] Functional properties such as solubility, water-holding capacity, viscosity, gelation, coagulation, emulsification and foaming, will all influence the food characteristics.[4]

Modifications of food proteins can be achieved by chemical and enzymatic methods that have been shown to be powerful tools for improving the functional properties of the final product.[5] The use of enzymes to modify the functional properties of food proteins is an area that has attracted considerable interest because consumers perceive enzymes to be more *natural* than chemicals.[6] Protein functionality can be modified by intra- or intermolecular cross-linking.[7] The cross-linking of food proteins by enzymatic reactions produces substantial changes in their structures and can modify many properties of the food such as texture, viscosity, solubility, water-holding capacity, thermal stability, emulsification, and gelation.[8] Transglutaminase (TGase) is an enzyme that has received much attention for its ability to catalyze the cross-linking reaction in proteins.[9]

TGases (protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) are a family of enzymes that catalyses acyl-transfer reactions between the γ-carboxyamide groups of peptide-bound glutamine residues and a variety of primary amines.[10] When the ε-amino group of a peptide bound lysine residue acts as substrate, the two peptide chains are covalently linked through an ε-(γ-glutamyl)lysine bond. Thus these enzymes are capable of introducing covalent intra- or intermolecular cross-linking of the proteins. TGases have been found in animal and plant tissues, and produced by microorganisms.[11] Microbial TGase has widespread and growing applications in the food processing industry.[9] The enzyme has been used for improving the functional properties of several proteins including meat, soybean, milk, and wheat proteins.[9,10,11] Among dairy proteins, casein has shown to be a good substrate for TGase.[12] It has been shown that the microstructure of yogurt is improved by treatment of milk with TGase,[13] and the cross-linking of milk proteins by

TGase appears to be an acceptable alternative to the addition of extra protein or stabilizer in the production of non-fat yogurt.[14] The treatment of milk with TGase improves its heat stability, probably by preventing dissociation of *k*-casein from the micelles.[15] Wheat gluten was also shown to be cross-linked by TGase and the action of the enzyme reinforces the protein network structure causing the change of viscoelastic properties of the dough.[16] TGase applications increased volume and improved structure of breads and dough stability,[17] and also improved baking qualities of weak wheat flours.[18] Although these studies show the improvement of functional properties of milk and wheat proteins by microbial TGase cross-linking, information about changes in nutritional properties is missing. The introduction of covalent cross-links between proteins in food produces substantial changes in their structures and therefore are expected to have some effects on the nutritional quality of the final product.[5] The formation of covalent cross-linking of amino acids in the same or in another protein molecule may decrease digestibility and biological availability of essential amino acids that are involved in these reactions.[19] Therefore, the present study was designed in order to investigate some possible nutritional effects of cross-linking by microbial TGase of milk and wheat proteins in a biological system.

MATERIALS AND METHODS

Materials

Commercial milk powder was obtained from Eleva SA (Rio Grande do Sul, Brazil), while commercial wheat flour was bought from Bunge Alimentos SA (Santa Catarina, Brazil). Microbial TGase was kindly provided by Ajinomoto Inc. (Tokyo, Japan), with a declared enzymatic activity of 100 U g^{-1}. All reagents were of analytical grade.

Texture evaluation

A study was performed to determine the effect of TGase reaction time on texture of samples. Milk powder and wheat flour samples were incubated with 1 % (w/w) of TGase at 37 °C for an incubation time of 0, 1, 2, 3 and 4 h. Control samples were incubated without enzyme. The texture was determined using a Texture Analyzer TA-XT2 (Stable Micro Systems Ltd., New York, USA). Breaking force (g) was determined at room temperature using a cylindrical stainless steel probe with a pressing surface diameter of 35

mm. The instrument was adjusted to the following conditions: probe area, 9.62 cm^2; measurement time, 14 s; penetration speed, 0.5 mm s^{-1}; penetration distance, 7 mm into surface. Gel strength was carried out in triplicate and expressed as kg m^{-2} of probe area.

Sample preparation

The cross-linking of both milk and wheat proteins was prepared by mixing either milk powder or wheat flour with 1 % (w/w) of TGase. The mixtures were incubated at 37 °C for 2 and 3 h, respectively. These samples were designated cross-linked milk protein (CMP) and cross-linked wheat protein (CWP), respectively. The milk powder and wheat flour were incubated at 37 °C for 2 and 3 h, without TGase, and these samples were designated, respectively, milk protein (MP) and wheat protein (WP). All four samples were dried in an air circulation oven at 45 °C and powdered in a food micro-homogenizer to pass through a 60-mesh sieve. These flours were stored at 4 ^oC for the subsequent chemical analysis and for preparation of the respective diets.

Diets composition

Table 1 shows the compositions of the four test protein diets (CMP, MP, CWP, and WP), one standard protein diet (casein) and one non-protein diet, used as controls to estimate the endogenous nitrogen excretion of the rats. Standard and test diets were adjusted to 10 % protein content, whereas the non-protein diet was devoid of protein. In addition to the protein sources, the diets contained vitamin and mineral mix (AIN-93G) according to Reeves *et al*..[21] For the preparation of the diets, the ingredients were homogenized and passed through a 60-mesh sieve to ensure uniform distribution of minerals and vitamins. All the diets were analyzed for their moisture, protein, lipid, and ash contents by the AOAC methods.[20]

Table 1. Composition of the experimental diets

Ingredients (g kg^{-1})	Basal diet	Casein diet	Test diets			
			CMP	MP	CWP	WP
Casein	-	123.5	37.1	37.1	37.1	37.1
CMP flour	-	-	290.0	-	-	-
MP flour	-	-	-	305.3	-	-
CWP flour	-	-	-	-	658.5	-
WP flour	-	-	-	-	-	675.7
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Soybean oil	70.0	70.0	34.4	28.1	59.1	66.7
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Salt mixture	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin mixture	10.0	10.0	10.0	10.0	10.0	10.0
L-cystine	-	3.0	-	-	-	-
Choline bitartrate	-	2.5	-	-	-	-
Corn starch	735.0	606.0	443.5	434.5	50.3	25.5

Animal tests

All animal experiments were approved by a submission to the Committee of Bioethics of the University (see note at the end of this work). Young, 21–25 days old white male Wistar rats weighting 54.5 ± 5.8 g were obtained from the Central Animal House, Pelotas Federal University, Rio Grande do Sul, Brazil. The rats were randomly divided into six groups, each consisting of six individuals. The animals were separately housed in stainless steel metabolic cages designed for separate collection of faeces and urine. Cages were located in a room with a 12 h light/dark cycle, at a temperature of 21 ± 2 °C, fitted with an appropriate ventilation system. Food and water were given *ad libitum*.

Effect of diets on animal growth

For growth studies, the rats were individually housed in stainless steel metabolic cages. One group of rats was fed on a casein diet (standard diet), while other four groups were fed on test diets (CMP, MP, CWP, and WP). The rats were weighed at the start of experiment and then again on every other day. Food and water were given *ad libitum*. Diets were given

daily and the unconsumed feed was collected and weighed. The rats fed on different experimental and control diets were weighed for four weeks and the gain in weight during this period was recorded. The consumption of protein was calculated as nitrogen consumed, based on the content of nitrogen in the diets. Protein efficiency ratio (PER), food efficiency ratio (FER), and food transformation index (FTI) were calculated by the following formulas, as described by FAO/WHO:[22]

$$PER = \frac{[Gain\ in\ body\ weight\ (g)]}{[\mathrm{Pr}otein\ consumed\ (g)]} \qquad (1)$$

$$FER = \frac{[Gain\ in\ body\ weight\ (g)]}{[Food\ consumed\ (g)]} \qquad (2)$$

$$FTI = \frac{[Food\ consumed\ (g)]}{[Gain\ in\ body\ weight\ (g)]} \qquad (3)$$

The corrected protein efficiency ratio (C-PER) was calculated according to Chapman *et al.*,[23] where 2.5 is the standard value for casein:

$$C - PER = \frac{PER \times 2.5}{[Deter\min ed\ \ PER\ \ for\ \ reference\ casein]} \qquad (4)$$

At the end of four weeks the final body weights of animals were recorded. After sacrifice, the liver, both kidneys, and the spleen were carefully resected and immediately weighed.

Nitrogen balance experiments

The studies of nitrogen balance were carried out according to Miller and Bender[24]. Rats were individually housed in stainless steel metabolic cages. One group of rats was fed on a non-protein diet (basal diet) and another on a casein diet (control diet). Four groups of rats were fed on the test diets (CMP, MP, CWP, and WP). The experiment was conducted for fourteen days, which included an initial conditioning period of four days. During the last ten days, urine and faeces of each rat were separately collected. Food and water were given *ad libitum* and the change in body weight was recorded. The faeces were oven-dried at 100 °C for 24 h. The dried samples were grounded to 20 meshes. The concentration of nitrogen in the urine and faecal powder was estimated by the microKjeldhal method.[20] The non-

protein diet group was used to measure the metabolic faecal nitrogen and the endogenous urinary nitrogen. Data obtained from this experiment were used to calculate nitrogen absorbed (NA), nitrogen retention (NR), apparent nitrogen digestibility (AND), true digestibility (TD), biological value (BV), net protein utilization (NPU) and net protein retention (NPR), as described by FAO/WHO;[22] and protein retention efficiency (PRE), as described by Bender and Doell,[25] by employing the following formulas:

$$NA = NI - NF_1 \tag{5}$$

$$NR = NI - (NF_1 + NU_1) \tag{6}$$

$$AND = \frac{[NI - NF_1]}{NI} \times 100 \tag{7}$$

$$TD = \frac{NI - (NF_1 - NF_2)}{NI} \times 100 \tag{8}$$

$$BV = \frac{NI - (NF_1 - NF_2) - (NU_1 - NU_2)}{NI - (NF_1 - NF_2)} \times 100 \tag{9}$$

$$NPU = \frac{BV \times TD}{100} \tag{10}$$

$$NPR = \frac{[Weight\ gain\ of\ test\ group + Weight\ loss\ of\ protein - free\ group]}{[Weight\ of\ test\ protein\ consumed]} \tag{11}$$

$$PRE = NPR \times 16 \tag{12}$$

where, NI is nitrogen intake of animals fed test diet; NF_1 the excreted nitrogen in faeces of animals fed test diet; NF_2 the excreted nitrogen in faeces of animals fed non-protein diet (basal diet); NU_1 the excreted nitrogen in urine of animals fed test diet; NU_2 the excreted nitrogen in urine of animals fed non-protein diet.

Statistical analysis

Experimental data were analyzed by one-way ANOVA and Tukey's highly significant difference test. Differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Texture evaluation

The effects of TGase reaction time on the texture of samples of milk and wheat proteins, cross-linked and non-cross-linked, are shown in Fig. 1. The maximal gel strength of milk and wheat proteins was obtained at 2 and 3 h, respectively. Results show that gel strength for the milk powder (Fig. 1A) is similar ($P > 0.05$) for incubation times of 2, 3 or 4 h, and for wheat flour samples the gel strength (Fig. 1B) is similar ($P > 0.05$) only after 3 and 4 h of incubation with the enzyme. The susceptibility of a protein to TGase-induced cross-linking depends on the macromolecular structure of the protein. Previous studies have shown that wheat gluten proteins can be cross-linked by TGase, despite their low lysine content because of their high glutamine content.[16] Among the milk proteins, the casein fraction has been shown to be a good substrate for TGase.[12]

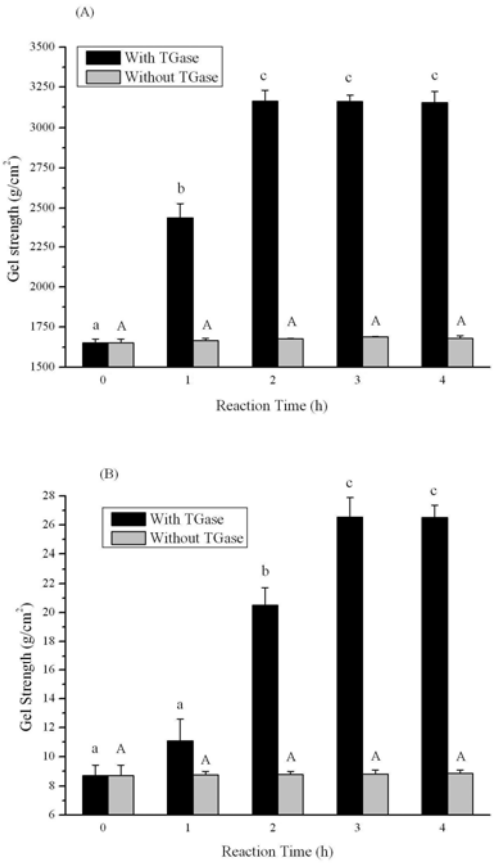

Fig. 1. Texture of non-crosslinked and cross-linked samples of milk powder and wheat flour modified by TGase (measured by gel strength). (A) milk protein; (B) wheat protein. Equal letters indicate that samples do not significantly differ ($P > 0.05$) (capital caps: samples without enzymatic treatment; lower: enzymatically treated samples).

Proximate chemical composition

Table 2 shows the chemical composition of the flour of cross-linked milk protein (CMP), milk protein (MP), cross-linked wheat protein (CWP), and wheat protein (WP). The protein content was 1.2 % higher in CMP flour than in MP flour and only 0.27 % higher in CWP flour than in WP flour. Some authors have shown that the protein content increased after the cross-linking with TGase. Ahn *et al.*[26] observed that after the treatment with TGase, the protein contents of wheat, barley, and soy flours increased 0.6, 0.8, and 1.6 %, respectively. Similarly, Rosell *et al.*[27] found that the wet gluten content of wheat flours was slightly increased with TGase treatment due to a polymerization of proteins.

Table 2. Chemical composition (g kg^{-1}) of the non-crosslinked and cross-linked flours

Samples	Moisture	Protein	Lipids	Ash
CMP	137.3 ± 0.4	241.3 ± 0.2	122.8 ± 1.3	53.3 ± 0.3
MP	106.4 ± 0.2	229.3 ± 1.2	137.2 ± 0.9	55.6 ± 0.2
CWP	109.8 ± 0.1	106.3 ± 1.1	16.5 ± 0.6	4.9 ± 0.5
WP	115.5 ± 0.3	103.6 ± 0.7	4.9 ± 0.3	5.9 ± 0.9

Effect of diets on animal growth

Data on the growth studies of the rats treated with milk and wheat proteins cross-linked by microbial TGase and non-cross-linked are shown in Table 3.

Table 3. Growth assay values of rats fed experimental diets

Parameters	Casein diet	Test diets			
		CMP	MP	CWP	WP
Food intake (g)	379.67 ± 70.22[a]	397.77 ± 28.91[a]	373.80 ± 51.29[a]	370.07 ± 32.02[a]	376.62 ± 12.99[a]
Body weight gain (g)	145.35 ± 26.60[a]	123.81 ± 12.43[a]	115.41 ± 20.43[ac]	91.06 ± 6.54[bc]	91.19 ± 7.77[bc]
PER	3.78 ± 0.22[a]	3.32 ± 0.18[b]	3.31 ± 0.22[b]	2.34 ± 0.30[c]	2.38 ± 0.16[c]
C-PER	2.50 ± 0.00[a]	2.20 ± 0.12[b]	2.19 ± 0.15[b]	1.55 ± 0.20[c]	1.58 ± 0.10[c]
FER	0.38 ± 0.02[a]	0.31 ± 0.02[b]	0.31 ± 0.02[b]	0.25 ± 0.03[c]	0.24 ± 0.02[c]
FTI	2.61 ± 0.16[a]	3.22 ± 0.17[b]	3.26 ± 0.21[b]	4.08 ± 0.47[c]	4.15 ± 0.27[c]

Means with different superscripts in the same horizontal row are significantly different ($P < 0.05$).

Fig. 2 shows the cumulative body weight gain of the rats fed on control and test diets during the 28 days of treatment, with a linear increase of the body weight been observed for all groups. Rats fed on control diet (casein diet) grew faster than rats fed with other diets, while the growth rate of rats fed on wheat protein (WP) was the lowest. However, rats fed either cross-linked milk protein (CMP) or milk protein (MP) grew at a rate that was not significantly different ($P < 0.05$) from casein diet (Table 3). Rats fed either cross-linked wheat protein (CWP) or wheat protein (WP) had growth rates not significantly different (Table 3). As shown in Fig. 2, the body weight gain of rats fed on cross-linked protein diets was slightly higher than the respective non-cross-linked diet.

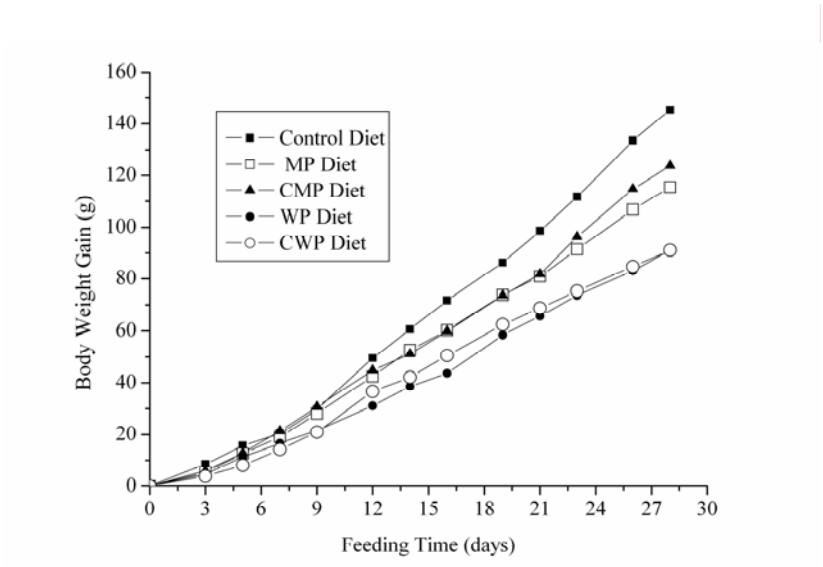

Fig. 2. Body weight gain of rats fed experimental diets

Food intakes after 28 days on the CMP and casein diets were the highest, 397.77 and 379.67 g per rat, respectively. However, these values did not significantly differ ($P > 0.05$) from the other three diets where food intake ranged from 370.07 to 376.62 g per rat. The results of food intake for the rats fed on CMP and CWP diets, 397.77 and 370.07 g per rat, respectively, were similar to the values obtained by Seguro *et al*.[28] for the rats fed with diets containing cross-linked casein.

Body weight gain on the control group (casein diet) was the highest (145.35 g per rat) after 28 days, but the difference was not significantly ($P > 0.05$) different from CMP

and MP diets (123.81 and 115.41 g per rat). The body weight gain was significantly lower ($P < 0.05$) in the groups fed on wheat protein and cross-linked wheat protein. The cross-linking reaction by TGase of the milk and wheat proteins showed no effect on the food intake and body weight gain of the animals after 28 days. Our results suggest that cross-linked milk and wheat proteins support the growth of young rats similarly to the non-cross-linked proteins. Moreover, the cross-linked milk protein supports the growth of the test animals similarly to the standard casein diet.

The casein diet produced a PER of 3.78, which was significantly higher ($P < 0.05$), than cross-linked and non-cross-linked milk protein diets (3.32 and 3.31, respectively). The PER of these three formulations were significantly higher ($P < 0.05$) than the PER of the cross-linked and non-cross-linked wheat proteins (2.34 and 2.38, respectively), probably due to limiting amino acids in the wheat gluten, a lysine-poor protein. Protein quality, weight gain and PER are inter-related. The better the protein quality, the higher the weight gain and the higher would be the PER.[29] The C-PER values of four test diets 1.55 (CWP), 1.58 (WP), 2.19 (MP), and 2.20 (CMP) were significantly lower ($P < 0.05$) than the casein diet (2.50) The differences between the PER and C-PER of the cross-linked and non-cross-linked proteins were not significantly different ($P > 0.05$), so the formation of ε-(γ-glutamyl)lysine cross-links in milk and wheat proteins did not appear to have any influence on body weight gain. Seguro *et al*.[28] studied the bioavailability of the ε-(γ-glutamyl)lysine moiety in cross-linked casein in rats and obtained the C-PER value of 2.47 for the diet containing cross-linked casein.

The FER (food efficiency ratio) was the highest for the group on the casein diet (0.38). This value differed significantly ($P < 0.05$) from the milk protein diets (0.31), and from the cross-linked and non-cross-linked wheat protein diets (0.25 and 0.24, respectively). The food transformation index (FTI) was higher for rats fed on casein diet. This group required less feeding to increase one gram (2.61) compared to the rats fed on CMP (3.22), MP (3.26), CWP (4.08), and WP (4.15) diets. Statistical analysis showed a similar trend to the food efficiency ratio. The polymerization of milk and wheat proteins by TGase treatment did not affect the FER and FTI of the animals.

Table 4 shows the relative weight of the organs of rats fed on casein and test diets. No differences ($P > 0.05$) were observed for the liver, kidneys, and spleen weights of rats fed on the control or test diets. The slightly lower liver weight for rats fed on CWP diet

was regarded as a normal variability in an animal study. No visual macroscopic abnormalities were observed in the studied organs.

Table 4. Ratio of organ weight per final body weight of rats fed experimental diets*

Organs	Casein diet	Test diets			
		CMP	MP	CWP	WP
Liver	3.97 ± 0.42	3.95 ± 0.40	3.84 ± 0.25	3.55 ± 0.27	3.70 ± 0.44
Right kidney	0.44 ± 0.05	0.42 ± 0.03	0.45 ± 0.05	0.44 ± 0.04	0.42 ± 0.02
Left kidney	0.42 ± 0.04	0.42 ± 0.04	0.43 ± 0.04	0.43 ± 0.02	0.41 ± 0.02
Spleen	0.29 ± 0.03	0.26 ± 0.05	0.28 ± 0.05	0.26 ± 0.02	0.27 ± 0.02

Differences among diets were not significant at $P < 0.05$.

* Ratio = (organ weight/final body weight) x 100.

Protein cross-linking can have a profound effect on the structure and function of proteins in foods, so it could affect their nutritional qualities.[30] However, our results for body weight gain, PER, FER, and FTI, especially for the milk proteins, have shown the good nutritional quality of the cross-linked proteins. According to Sgarbieri,[29] when experimental animals gain weight and other indexes as FER and PER are good, it is an excellent indicator that the fed diets are of high quality.

Nitrogen balance experiments

Data on the nitrogen balance studies of milk and wheat proteins cross-linked by microbial TGase are shown in Table 5. The nitrogen consumed, nitrogen absorbed and nitrogen retained were similar for casein and the test group. The nitrogen retained was positive in all groups, showing that the amount nitrogen intake was higher than the faecal and urinary excretion of it. The cross-linking of milk and wheat proteins increased the content of both nitrogen intake absorbed and nitrogen intake retained by the animals. Nitrogen excreted in faeces was lower ($P < 0.05$) for animals on control and MP diets. However, the nitrogen excreted in urine was higher ($P < 0.05$) for the animals on both wheat proteins diets, probably due to limiting amino acids in the wheat gluten, causing deamination of most of protein content for energy production. Although the methodology used for measuring the balance of nitrogen metabolism is based upon classical techniques,[24] perhaps the more

accurate measurement of ileal nitrogen digestibility could be more elucidating as if the amount of excreted nitrogen was directly related to the flow of undigested protein entering the large intestine.[31]

Table 5. Nitrogen balance evaluation values of rats fed experimental diets

Parameters	Casein diet	Test diets			
		CMP	MP	CWP	WP
Daily food intake (g)	13.17 ± 2.34^{a}	13.63 ± 1.32^{a}	13.27 ± 2.15^{a}	14.61 ± 0.95^{a}	13.19 ± 0.72^{a}
Daily body weight gain (g)	5.12 ± 1.09^{a}	4.28 ± 0.84^{ac}	4.31 ± 1.13^{ac}	3.62 ± 0.23^{bc}	3.12 ± 0.37^{bc}
Body weight loss* (g)	9.73 ± 1.19	9.73 ± 1.19	9.73 ± 1.19	9.73 ± 1.19	9.73 ± 1.19
Nitrogen consumed (g)	3.00 ± 0.53^{ab}	2.86 ± 0.28^{ab}	2.77 ± 0.45^{b}	3.48 ± 0.23^{a}	2.99 ± 0.16^{ab}
Nitrogen absorbed (g)	2.88 ± 0.53^{a}	2.70 ± 0.26^{a}	2.63 ± 0.45^{a}	3.26 ± 0.23^{a}	2.80 ± 0.16^{a}
Nitrogen retained (g)	2.72 ± 0.51^{a}	2.54 ± 0.21^{a}	2.46 ± 0.36^{a}	2.76 ± 0.23^{a}	2.36 ± 0.15^{a}
Nitrogen faecal (g)	0.12 ± 0.01^{bc}	0.16 ± 0.02^{ac}	0.14 ± 0.01^{bc}	0.22 ± 0.01^{a}	0.20 ± 0.01^{a}
Nitrogen urinary (g)	0.16 ± 0.06^{b}	0.16 ± 0.11^{b}	0.17 ± 0.12^{b}	0.50 ± 0.01^{a}	0.44 ± 0.08^{a}
AND (%)	95.98 ± 0.79^{a}	94.52 ± 0.57^{bc}	94.93 ± 0.89^{ac}	93.76 ± 0.53^{bcd}	93.18 ± 0.46^{d}
TD (%)	96.81 ± 0.64^{a}	95.36 ± 0.58^{bc}	95.82 ± 0.73^{ac}	94.45 ± 0.49^{bd}	93.98 ± 0.43^{d}
BV (%)	94.79 ± 1.96^{a}	94.59 ± 3.55^{a}	94.19 ± 3.83^{a}	85.01 ± 1.55^{b}	84.70 ± 2.68^{b}
NPU (%)	91.76 ± 1.74^{a}	90.20 ± 3.38^{a}	90.23 ± 3.27^{a}	80.29 ± 1.49^{b}	79.59 ± 2.41^{b}
NPR	4.35 ± 0.27^{a}	3.88 ± 0.39^{a}	4.02 ± 0.40^{a}	2.78 ± 0.12^{b}	2.85 ± 0.19^{b}
PRE	69.60 ± 4.27^{a}	62.12 ± 6.29^{a}	64.25 ± 6.37^{a}	44.54 ± 1.96^{b}	45.52 ± 3.05^{b}

Means with different superscripts in the same horizontal row are significantly different ($P < 0.05$).

*Group the rats fed on the protein-free diet.

Results indicate that the true digestibility (TD) was significantly lower ($P < 0.05$) for both cross-linked milk (95.36 %) and wheat proteins (94.45 %) than the TD value obtained for rats on casein diet (96.81 %). TD and apparent nitrogen digestibility (AND) followed a similar trend. The AND and TD were similar ($P > 0.05$) between casein and non-cross-linked milk protein diets, but due to the cross-linking process, both parameters were significantly higher ($P < 0.05$) for the rats fed on the casein diet than for the rats fed on the CMP diet. The polymerization process of milk and wheat proteins by TGase have no influence on TD and AND of these proteins, since these nutritional parameters were no statistically different ($P > 0.05$), between CMP and MP or between CWP and WP. Research on the *in vitro* degradation of ε-(γ-glutamyl)lysine show that after ingestion of cross-linked proteins, normal mammalian gastrointestinal digestive enzymes cleave them into amino acids but leave the ε-(γ-glutamyl)lysine dipeptide intact. The resistant ε-(γ-glutamyl)lysine dipeptide may be absorbed through the intestinal brush-border and transported to the kidney.[32]

The biological value (BV) was higher (94.79 %) for rats on the control diet but similar ($P > 0.05$) to the values obtained for rats on the CMP diet (94.59 %) and MP diet (94.19 %). The BV values obtained for rats on wheat protein diets (84.70 % to WP and 85.01 % to CWP) were similar ($P > 0.05$), but lower ($P < 0.05$) than the others. According to Whitney and Rolfes,[33] a protein with a BV of 70 % or more can support human growth and tissue maintenance as long as energy intake is adequate. Since the BV of the milk and wheat cross-linked protein diets were respectively 94.59 and 85.01 %, these proteins could support growth and tissue maintenance. TGase action involves cross-linking of free primary amino groups of lysine with glutamine residues, but the results indicate that the biological value of the milk and wheat proteins is not reduced by this process. The protein nutritive value of a food reflects its ability to meet nitrogen and amino acid requirements assuring proper animal growth and maintenance. This ability is a function of several factors, including protein content, digestibility, and amino acid composition.[34] According to Seguro *et al*.,[28] the cross-linking by TGase is thought to protect nutritionally valuable lysine residues in food from various deteriorative reactions. Furthermore, the use of TGase potentially allows production of food proteins of higher nutritional quality, through cross-linking of different proteins containing complementary amino acids.[11]

The net protein utilization (NPU) value was higher (91.76 %) for rats on the control diet but similar ($P > 0.05$) to the value obtained for rats fed on the cross-linked milk protein (90.20 %) and milk protein (90.23 %) diets. The NPU values obtained for rats on diets of CWP and WP, 80.29 % and 79.59 %, respectively, were similar ($P > 0.05$), but lower ($P < 0.05$) than the standard protein diet. The NPU has been suggested to be more of a practical magnitude than BV in protein quality evaluation. This is because digestibility is an important and integrated part of the nutritive value of a dietary protein source. NPU is a measure of both digestibility and BV of the amino acid mixture absorbed from food.[33] Thus the results indicate that both milk and wheat proteins nutritional qualities were not affected by the TGase -induced cross-linking.

Daily food intakes of animals on the control and test diets were not significantly ($P > 0.05$) different. However, animals on the casein, CMP, and MP diets gained body weight rapidly and had significantly ($P < 0.05$) higher NPR than the animals on cross-linked and non-cross-linked wheat protein diets**.** The differences between the NPR values of the proteins cross-linked and non-cross-linked were non-significant ($P > 0.05$), so the cross-linking process by microbial TGase in milk and wheat proteins had not influenced the ability of proteins to support both maintenance and growth.

The protein retention efficiency (PRE) indicates the distinct superiority of the casein diet over the other diets, since this value to casein diet was the highest 69.60, and for test diets, this ranged from 44.54 to 64.25. Casein is a pure protein source with well balanced amino acid profile and hence the choice as a standard protein diet (control diet) against other diets can be compared. The results suggest that the PRE of milk and wheat proteins is not affected by the cross-linking process.

Based on the *in vivo* biological values examined, the milk and wheat protein quality cross-linked by TGase was no affected. Therefore, the results obtained in this study contrast with those reported by Tang *et al.*.[35] These researchers, studying the effect of *in vitro* digestibility of soy proteins cross-linked by microbial TGase, suggested that this enzyme may negatively affect the nutritional properties of food proteins. According to them, the nutritive value of a protein can be evaluated by various *in vitro* and *in vivo* methods, but due to their simplicity and speed, the *in vitro* digestibility methods have been more widely used than *in vivo* methods. However, the biological evaluation of protein provides useful information regarding their overall quality and it is the best tool for judging

the protein nutritional quality, since many factors can affect the quality of a specific protein *in vivo*.[36]

CONCLUSIONS

This study shows that milk and wheat proteins cross-linked by microbial TGase support the growth of young rats. The polymerization process of these proteins by this enzyme did not negatively affect some nutritional parameters such as protein efficiency ratio, food efficiency ratio, food transformation index, apparent nitrogen digestibility, true digestibility, biological value, net protein utilization, net protein ratio, and protein retention efficiency, indicating that the cross-linking process by TGase had no influence on the nutritional quality of milk and wheat proteins. These results are important because protein cross-linking is interesting for industrial applications in order to improve food texture, but these modifications should come without causing the loss of nutritional quality.

ACKNOWLEDGEMENTS AND STATEMENT OF ETHICAL PRINCIPLES

The authors wish to thank CNPq and CAPES for their financial support and scholarships. The animal experimentation was authorized by Resolution 01/2007 of the Research Committee of the Food and Science Institute of Technology of The Federal University of Rio Grande do Sul State. One of the authors (JGV) has a formal authorization to humanely manipulate experimental animals. Our group supports the ethically and morally justified use of animals in experimentation, but we also support the search for proper models to completely replace animal experimentation in the near future.

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9.3 ANEXO C. Comprovantes de publicação dos artigos apresentados em Resultados II e IV, respectivamente

Comprovante de publicação do artigo apresentado em Resultados II

To: mazayub@ufrgs.br
Sent: Wednesday, July 30, 2008 10:19 PM
Subject: Decision on your manuscript #ABAB-727R1

Dear Author:

I am pleased to inform you that your manuscript, "Environmental Effects on Transglutaminase Production and Cell Sporulation in Submerged Cultivation of Bacillus circulans" has been accepted for publication in Applied Biochemistry and Biotechnology.

For queries regarding your accepted paper, please click the following link, complete the query form and click "Submit".

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Thank you.

Congratulations and best regards,

Carlos Ricardo Soccol, PhD
Applied Biochemistry and Biotechnology

Comprovante de publicação do artigo apresentado em Resultados IV

To: mazayub@ufrgs.br
Cc: alaskin@hughes.net ; mllopez@ual.es
Sent: Tuesday, July 29, 2008 10:35 PM
Subject: JIMB: Your manuscript entitled Solid state bioreactor production of transglutaminase by Amazonian Bacillus circulans BL32 strain

Ref.: Ms. No. JIMB-D-08-00097R2
Solid state bioreactor production of transglutaminase by Amazonian Bacillus circulans BL32 strain
Journal of Industrial Microbiology & Biotechnology

Dear Professor Záchia Ayub,

I am pleased to tell you that your work has now been accepted for publication in Journal of Industrial Microbiology & Biotechnology.

It was accepted on 28-07-2008.

Thank you for submitting your work to this journal.

With kind regards

Allen I. Laskin, Ph.D.
Editor-in-Chief
Journal of Industrial Microbiology & Biotechnology

10. CURRICULUM VITAE RESUMIDO

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2. FORMAÇÃO: Graduação em Quimica Industrial (Universidade Federal do Rio Grande do Sul - UFRGS, 1993 – 1997), Graduação em Química – Licenciatura (Universidade Federal do Rio Grande do Sul - UFRGS, 1998 – 2002), Mestrado em Microbiologia Agrícola e do Ambiente (Universidade Federal do Rio Grande do Sul – UFRGS, 2000 – 2002).

3. ESTÁGIOS: Estágio curricular obrigatório na empresa Samrig S A Moinhos Rio Grandense – SAMRIG (Esteio, março a novembro de 1991); Estágio curricular obrigatório na empresa PPH Companhia Industrial de Polipropileno – PPH (Triunfo, fevereiro a

dezembro de 1992); Bolsista CNPQ/UFRGS no Centro de Biotecnologia da UFRGS (Porto Alegre, de março de 1995 a março de 1997, sob orientação do professor Dr. Marco Antônio Záchia Ayub); Estágio curricular obrigatório no Laboratório de Controle de Qualidade da empresa Moinhos de Trigo Indígena S.A. – MOTRISA (Canoas, maio de 1997 a fevereiro de 1998); Estágio curricular obrigatório na Escola Municipal de Ensino Fundamental Flôres da Cunha – EMEFFC (Esteio, setembro a dezembro de 2000); Estágio curricular obrigatório no Colégio de Aplicação da UFRGS – APLICAÇÃO (Porto Alegre, março de 2001 a janeiro de 2002).

4. EXPERIÊNCIA PROFISSIONAL OU DIDÁTICA

- Chefe do Laboratório de Controle de Qualidade da empresa Moinhos de Trigo Indígena S.A. – MOTRISA, no período de fevereiro de 1998 a fevereiro de 2000.
- Professor substituto do Departamento de Química Inorgânica da UFRGS (40 horas), atuando nas disciplinas de Química Geral Experimental, Química Analítica Qualitativa, Química Analítica Quantitativa e Química Geral para os cursos de Química Industrial, Farmácia, Engenharia Química, Engenharia de Alimentos, Farmácia e Biologia, no período de março de 2002 a fevereiro de 2004.
- Professora da Escola de Educação Profissional Sojuin atuando na disciplina de Química Geral para o curso de Auxiliar de Análises Químicas, no período de outubro de 2002 a junho de 2005.
- Responsável Técnico pela empresa de massas alimentícias frescas Mercoflour Ltda., no período de março de 2003 a abril de 2005.
- Professor do curso de Especialização em Ciência e Tecnologia de Alimentos do ICTA/UFRGS, atuando na disciplina de Química e Bioquímica de Alimentos.
- Professor assistente do Centro Universitário – UNIVATES, atuando nas disciplinas Química Analítica Qualitativa, Bromatologia, Tecnologia de Produtos Cárneos, Tecnologia das Fermentações, Tecnologia de Produtos Farináceos e Oleaginosos, Química Industrial, Organizações e Normas, Segurança do Trabalho, Tecnologia de Sabões e Detergentes, Estágio Supervisionado IV e Tecnologia de Bebidas e Conservas para os cursos de Química Industrial, Farmácia, Nutrição e Biomedicina. Desde agosto de 2003.

- Professor do curso de Especialização em Tecnologia de Alimentos da UNIVATES, atuando nas disciplinas de Microscopia de Alimentos, Tecnologia de Carnes e Tecnologia de Doces/Farináceos. Desde março de 2006.

- Professor substituto do Departamento de Química da Escola Técnica da UFRGS (40 horas), atuando nas disciplinas de Análise Qualitativa Orgânica e Inorgânica, Técnicas Básicas de Laboratório, Físico-Química, Tecnologia Química, Química Analítica Quantitativa, Química Orgânica Aplicada e Química Geral para os cursos de Técnico em Biotecnologia e Técnico Analista de Processos – Química. Desde março de 2008.

5. ARTIGOS COMPLETOS PUBLICADOS

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6. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

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SOUZA CFV, ROSA TD, FREIMÜLLER S, AYUB MAZ. Changes in microflora during ripening of Serrano Cheese. In: XXI Congresso Brasileiro de Microbiologia, 2001, Foz do Iguaçu - PR.

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