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HIDRÓLISE DA LACTOSE E SÍNTESE DE GALACTOOLIGOSSACARÍDEOS UTILIZANDO *β*-GALACTOSIDASE IMOBILIZADA EM SUPORTES À BASE DE QUITOSANA

Tese apresentada ao Programa de Pós-Graduação em Engenharia de Alimentos do Centro Tecnológico da Universidade Federal de Santa Catarina, como requisito final à obtenção do título de Doutor em Engenharia de Alimentos.

Orientador: Prof. Dr. Jorge L. Ninow
Coorientador: Prof. Dr. Plinho F. Hertz

MANUELA POLETTO KLEIN

Engenheira de Alimentos (2008)

MSc. Ciência e Tecnologia de Alimentos (2010)

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HIDRÓLISE DA LACTOSE E SÍNTESE DE GALACTOOLIGOSSACARÍDEOS UTILIZANDO *β*-GALACTOSIDASE IMOBILIZADA EM SUPORTES À BASE DE QUITOSANA

Por

MANUELA POLETTO KLEIN

Tese de doutorado apresentada ao Programa de Pós-Graduação em Engenharia de Alimentos do Departamento de Engenharia Química e de Alimentos, Centro Tecnológico de Universidade Federal de Santa Catarina, como requisito parcial para a obtenção do Título de Doutor em Engenharia de Alimentos pela Comissão Julgadora composta pelos membros:

_____
Prof. Dr. Jorge Luiz Ninow
Orientador

_____
Prof. Dr. Plinho Francisco Heris
coorientador

_____
Prof. Dr. João Borges Laurindo
Coordenador

Banca Examinadora:

_____
Prof. Dr. Jorge Luiz Ninow

_____
Prof. Dr. Adriano Brandelli

_____
Profª. Drª. Maria Manuela Camilo Feltes

_____
Profª. Drª. Maria da Graça Nascimento

_____
Profª. Drª. Débora de Oliveira

_____
Drª. Silvana Licodiedoff

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RESUMO

A *β*-galactosidase (E.C 3.2.1.23) é uma das enzimas mais empregadas na indústria de alimentos sendo principalmente utilizada na hidrólise da lactose para obtenção de produtos com baixo teor de lactose e na síntese de galactooligossacarídeos (GOS). A imobilização desta e outras enzimas é uma forma eficaz de permitir o reuso do biocatalisador e aumentar sua estabilidade térmica. Esta imobilização pode ser feita em suportes à base de quitosana, pois, além de serem seguros, possuem grupos funcionais que permitem a imobilização direta ou a modificação com outros grupos funcionais de interesse. Desta forma, o objetivo deste trabalho foi estudar a hidrólise da lactose e a síntese de GOS utilizando a *β*-galactosidase imobilizada em suportes à base de quitosana. Primeiramente, macro e nanopartículas de quitosana foram obtidas, caracterizadas e utilizadas para a imobilização da *β*-galactosidase de *Kluyveromyces lactis.* Avaliou-se a carga de enzima imobilizada em cada um dos suportes, bem como a estabilidade térmica e operacional do biocatalisador imobilizado. Subsequentemente, as macropartículas de quitosana foram utilizadas em um reator de leito fixo a fim de avaliar a hidrólise da lactose e a síntese de GOS em sistema contínuo. A imobilização da *β*-galactosidase de *Aspergillus oryzae* em macropartículas de quitosana foi realizada utilizando a genipina como agente de entrecruzamento para substituir o glutaraldeído, dada sua toxicidade. Tanto o suporte como a enzima imobilizada obtidos foram caracterizados e aplicados na hidrólise da lactose e na síntese de GOS. Por fim, investigou-se a estabilidade térmica da *β*-galactosidase de *Aspergillus oryzae* imobilizada em macropartículas de quitosana em presença de lactose e GOS, a fim de simular as condições operacionais de obtenção deste prebiótico. Apesar das macro e nanopartículas de quitosana terem apresentado algumas diferenças entre si (área superficial, capacidade de carga, estabilidade térmica e retenção de atividade da enzima), ambos biocatalisadores puderam ser reutilizados por 50 bateladas de hidrólise. A hidrólise da lactose permaneceu estável ao longo de mais de 15 dias de operação, resultando em 90 % de conversão de lactose, a 37 ºC. Nesta mesma temperatura, a produtividade máxima de GOS foi de 484,5 g L^{-1} h^{-1}. A estabilidade térmica da enzima foi melhorada em presença de lactose, glicose e galactose, o que sugere que sistemas de operação contínua podem contribuir na estabilização térmica da enzima. A imobilização da enzima de *A. oryzae* em macropartículas de quitosana entrecruzadas com genipina também foi bem sucedida, rendendo biocatalisadores

operacionalmente estáveis, com hidrólise da lactose efetiva e estável durante 25 bateladas de reuso da enzima imobilizada. O parâmetro que mais teve influência na síntese de GOS foi a concentração de lactose, sendo que a concentração máxima de GOS obtida foi de 146 g L^{-1}, utilizando uma solução de lactose 500 g L^{-1}. A melhoria na estabilidade térmica da *β*-galactosidase de *A. oryzae* imobilizada em macropartículas de quitosana foi bastante acentuada em presença de GOS, o que sugere que os processos de síntese deste composto podem ser otimizados em relação à temperatura, já que altas temperaturas facilitam o processo de dissolução da lactose e evitam a contaminação microbiana.

PALAVRAS-CHAVE: Hidrólise da Lactose; Galactooligossacarídeos; *β*-Galactosidase; Imobilização; Quitosana; Genipina; Estabilidade Térmica.

ABSTRACT

The *β*-galactosidase (E.C 3.2.1.23) is one of the most used enzymes in the food industry, being mainly applied in lactose hydrolysis and galactooligosaccharides (GOS) synthesis. The immobilization of *β*-galactosidase and others enzymes is an effective way to allow the reuse and to improve the thermal stability of the biocatalyst. Immobilization can be carried out on chitosan based supports, since they are safe and presents functional groups to direct enzyme immobilization or for modification with others functional groups. Then, the objective of this work was to study the lactose hydrolysis and GOS synthesis by using the enzyme *β*-galactosidase immobilized on chitosan based supports. Firstly, chitosan macro and nanoparticles were obtained, characterized and used for immobilization of the *β*-galactosidase from *Kluyveromyces lactis.* The enzyme load, the thermal stability and the operational stability were evaluated for each support. Subsequently, chitosan macroparticles were applied in a packed-bed reactor in order to evaluate the lactose hydrolysis and GOS synthesis in a continuous system. The immobilization of *Aspergillus oryzae β*-galactosidase on chitosan macroparticles was carried out using genipin as crosslinking agent in order to replace glutaraldehyde, given its toxicity. The support and the immobilized enzyme obtained were characterized and applied in the lactose hydrolysis and in the GOS synthesis. Lastly, the thermal stability of *A. oryzae β*-galactosidase immobilized on chitosan macroparticles was investigated in the presence of lactose and GOS, in order to simulate operational conditions for obtaining this prebiotic compound. Although chitosan macro and nanoparticles having shown some differences (surface area, load capacity, thermal stability and activity retention), both biocatalysts were reused for 50 batches of hydrolysis. The lactose hydrolysis remained stable during more than 15 days of continuous operation, yielding 90 % of lactose conversion, at 37 ºC. In the same temperature, maximum GOS productivity was 484.5 g L^{-1} h^{-1}. The enzyme thermal stability was improved in the presence of lactose, glucose and galactose, this suggests that systems of continuous operation could contribute for the thermal stability of the enzyme. The immobilization of *A. oryzae β*-galactosidase immobilized on chitosan macroparticles cross-linked with genipin was also satisfactory, with stable and effective lactose hydrolysis during 25 batches of reuse. The parameter that most influenced GOS synthesis was the initial lactose concentration, being 146 g L^{-1} the maximum GOS concentration achieved when 500 g L^{-1} lactose buffered solution was used. The

improvement of *A. oryzae* *β*-galactosidase thermal stability was substantial in the presence of GOS, suggesting that the synthesis of this compound can be optimized related to the temperature, once higher temperatures facilitates the process of lactose dissolution and avoid microbial contamination.

KEYWORDS: Lactose Hydrolysis; Galactooligosaccharides; *β*-Galactosidase; Immobilization; Chitosan; Genipin; Thermal Stability.

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1. INTRODUÇÃO

As β-galactosidases pertencem ao grupo de enzimas que têm despertado crescente interesse por parte da indústria de alimentos, devido a sua capacidade de hidrolisar a lactose em seus monossacarídeos (glicose e galactose). A hidrólise da lactose permite o consumo de leite e outros produtos lácteos por pessoas intolerantes à este dissacarídeo, sem os efeitos adversos causados pela má absorção de lactose no intestino. A hidrólise da lactose apresenta outras vantagens tecnológicas como a prevenção da cristalização da lactose em produtos como sorvete, doce de leite e leite condensado, melhorando assim suas características sensoriais, como suavidade e cremosidade. Além disso, durante a hidrólise da lactose, pode haver a formação simultânea de galactooligossacarídeos, um importante ingrediente prebiótico.

A aplicação de prebióticos em alimentos, como os galactooligossacarídeos (GOS), tem aumentado consideravelmente nos últimos anos devido às suas importantes propriedades funcionais, como por exemplo: melhoria da microbiota intestinal, alívio de transtornos gastrointestinais, prevenção de tumores, melhoria na absorção de minerais como o cálcio, e, além disso, a possibilidade de utilização como adoçantes naturais não-cariogênicos e de baixa caloria.

Atualmente, os GOS podem ser obtidos comercialmente utilizando distintas β-galactosidases. Entre as β-galactosidases de origem fúngica, a de *Aspergillus oryzae* é descrita como tendo boa atividade de transgalactosilação. Entre as de origem bacteriana, destaca-se a β-galactosidase de *Bacillus circulans.* Além da fonte de enzima, outros parâmetros devem ser otimizados para alcançar um bom rendimento reacional, como por exemplo a concentração de lactose no meio, a temperatura empregada na reação enzimática, a concentração de enzima e o tempo de reação.

A utilização da β-galactosidase imobilizada tem se tornado cada vez mais usual, uma vez que esta tecnologia permite a reutilização do biocatalisador, aumenta sua estabilidade operacional e térmica, evita a contaminação do produto final pela enzima além de permitir a utilização de diferentes configurações de reatores com enzima imobilizada para uso em processos contínuos.

Entre os diversos materiais disponíveis para o desenvolvimento de suportes para a imobilização de enzimas, a quitosana se destaca por apresentar importantes características como: biocompatibilidade, alta afinidade por proteínas, propriedades antimicrobianas, fácil preparação

em diferentes formatos físicos, disponibilidade de grupos funcionais para adsorção de enzimas ou para reação com agentes de entrecruzamento, entre outros.

A caracterização do suporte e da enzima imobilizada é de fundamental importância do ponto de vista de aplicação industrial, uma vez que as propriedades mecânicas e físicas, a estabilidade operacional, térmica e ao armazenamento do conjunto suporte-enzima obtido irão definir as condições de operação, bem como os tipos de reatores a serem utilizados, determinando assim, o sucesso dos processos industriais.

Dentro deste contexto, este trabalho tem como **objetivo principal** estudar os processos de hidrólise da lactose e de síntese de galactooligossacarídeos utilizando a enzima β-galactosidase imobilizada em distintos suportes à base de quitosana.

Como **objetivos específicos** têm-se:

- Produção e caracterização de suportes à base de quitosana com diferentes propriedades físicas e químicas;
- Imobilização da β-galactosidase nos suportes obtidos e caracterização do derivado imobilizado obtido;
- Aplicação da enzima imobilizada na hidrólise da lactose e na síntese de GOS em batelada e em processo contínuo;
- Estudo dos principais fatores que influenciam tais processos;
- Estudo dos mecanismos, além da própria imobilização, que auxiliam na preservação da atividade enzimática dos derivados imobilizados.

Este trabalho encontra-se organizado da seguinte forma: no Capítulo 1 está apresentada a revisão bibliográfica abordando os principais pontos do tema proposto. Os Capítulos 2, 3, 4, 5 e 6 apresentam, em forma de artigo científico, os materiais e métodos utilizados nos experimentos, juntamente com os resultados obtidos. No Capítulo 7 são apresentadas as considerações finais com as principais conclusões obtidas e as perspectivas para trabalhos futuros.

CAPÍTULO 1: REVISÃO BIBLIOGRÁFICA

1.1. Hidrólise da Lactose

A hidrólise da lactose é um dos mais importantes processos biotecnológicos na indústria de alimentos, apresentando benefícios e vantagens em diversas áreas como saúde, tecnologia de alimentos e meio ambiente. É realizada por catálise enzimática pela enzima *β*-galactosidase, permitindo a utilização de condições moderadas de temperatura e pH (Figura 1) (Gekas e Lopezleiva, 1985).

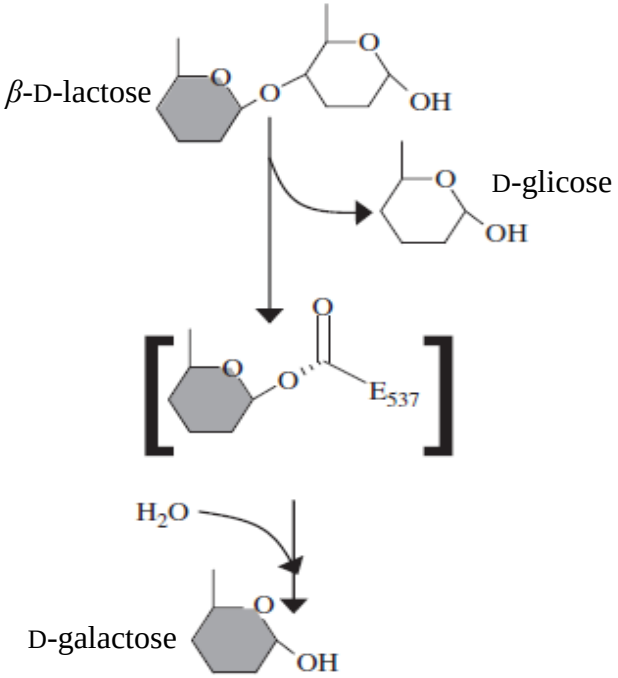


Figura 1: Representação esquemática da reação de hidrólise da ligação glicosídica *β*(1→4) da lactose. Fonte: Ganzle e colaboradores (2008).

O mecanismo de hidrólise da lactose pela *β*-galactosidase envolve, primeiramente, uma etapa chamada de *galactosilação*, que é o ataque nucleofílico por parte da glutamina presente no sítio ativo da enzima (Glu537 na *β*-galactosidase de *Escherichia coli*), formando uma

ligação covalente com a galactose, com liberação simultânea de glicose. Esta etapa é iniciada pela doação de prótons por outra glutamina presente no sítio ativo da enzima (Glu461 na *β*-galactosidase de *Escherichia coli*). Na segunda etapa, ocorre a transferência de um próton da água (ou de outro aceptor) para o resíduo de glutamina G461. Esta etapa é chamada *desgalactosilação* uma vez que a ligação covalente entre a galactose e Glu537 é rompida. Se água está envolvida na etapa de desgalactosilação, galactose é formada. Quando outros aceptores estão envolvidos, a reação é chamada de *transgalactosilação* (Juers et al., 2012).

A lactose é naturalmente encontrada em alta concentração somente no leite e em produtos lácteos. O leite humano contém aproximadamente 7% de lactose (Ganzle *et al.*, 2008) enquanto o leite de vaca contém de 4,5 a 5% de lactose, o que perfaz mais de um terço do seu teor de sólidos totais, aproximadamente 20% do teor de sólidos do sorvete e em torno de 72% do teor de sólidos totais no soro do leite (Grosova *et al.*, 2008). Este açúcar não pode ser absorvido prontamente pelo organismo e precisa ser hidrolisado em seus monossacarídeos pela ação da *β*-galactosidase presente no intestino delgado (Rossi e Lentze, 1984).

Normalmente, em mamíferos, a atividade de lactase diminui após o período de lactação com taxas distintas e em tempos variáveis como consequência normal da auto-regulação da atividade de *β*-galactosidase (Lomer *et al.*, 2008). Aproximadamente 75% da população mundial adulta possuem alguma deficiência de *β*-galactosidase na mucosa do intestino delgado, resultando em diferentes níveis de intolerância à lactose. A má absorção de lactose no intestino delgado resulta em desconforto abdominal (Swagerty *et al.*, 2002), má absorção de cálcio e aumenta o risco de osteopenia (Laaksonen *et al.*, 2009). Estratégias para reduzir os sintomas da intolerância à lactose incluem a substituição de leite por leite com baixo teor de lactose ou iogurte e o uso de probióticos, já que a microbiota intestinal possui um importante papel na redução dos sintomas da intolerância à lactose (Zhong *et al.*, 2004; Shaukat *et al.*, 2010).

Em produtos como sorvete, doce de leite e leite condensado pode ocorrer a excessiva cristalização da lactose resultando em produtos com textura arenosa. A hidrólise prévia da lactose do leite utilizado no processamento de tais produtos reduz a concentração de lactose a níveis aceitáveis e aumenta a concentração de glicose e galactose, que são açúcares mais solúveis quando comparados à lactose e, portanto menos propensos à cristalização (Klein *et al.*, 2010). Desta forma, a hidrólise

enzimática da lactose melhora as características tecnológicas e sensoriais de produtos lácteos, aumentando sua digestibilidade, cremosidade e suavidade (Grosova *et al.*, 2008).

Em relação à questão ambiental, a hidrólise da lactose presente no soro do leite é interessante uma vez que oferece um destino mais adequado para este subproduto. A partir da hidrólise da lactose do soro podem ser obtidos xaropes doces que são amplamente utilizados em confeitaria e na indústria de bebidas (Grosova *et al.*, 2008). Além disso, a hidrólise prévia da lactose do soro de leite aumenta significativamente o número de microrganismos comercialmente interessantes capazes de utilizar este substrato, aumentando também a faixa de produtos que podem ser obtidos, como exemplo, biomoléculas (lactatos, acetatos, etanol, butanodiol, etc.), biopolímeros e biomassa (Cote *et al.*, 2004; Guimaraes *et al.*, 2010).

1.2. Prebióticos

O termo *prebiótico* foi definido pela primeira vez, em 1995, por Gibson e Roberfroid como sendo "um ingrediente alimentício não digerível que afeta a saúde do hospedeiro de forma benéfica pois estimula seletivamente o crescimento e/ou atividade de bactérias benéficas, como as bifidobactérias e os lactobacilos. (Gibson e Roberfroid, 1995). A Figura 2 representa as condições que um ingrediente alimentício deve apresentar para ser considerado prebiótico.

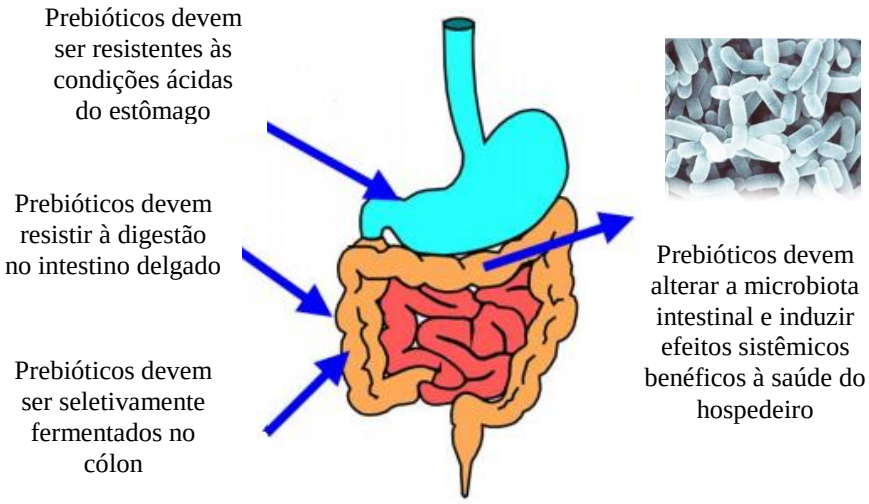


Figura 2: Propriedades que um ingrediente alimentício deve apresentar para ser considerado prebiótico. Adaptado de Gibson e Roberfroid (1995) e Tuohy e colaboradores (2005).

Devido aos avanços científicos dos últimos anos em relação aos prebióticos e seus efeitos benéficos sobre a saúde humana, o conceito de prebiótico foi revisado e atualizado em relação a primeira definição. Assim: "Prebióticos são ingredientes alimentícios seletivamente fermentados que induzem mudanças específicas tanto na composição como na atividade da microbiota intestinal conferindo benefícios ao bem-estar e saúde do hospedeiro" (Gibson *et al.*, 2004).

Entre os ingredientes alimentícios, os carboidratos não digeríveis (oligo- e polissacarídeos), alguns peptídeos e proteínas, e certos lipídeos (éteres e ésteres) são candidatos à prebióticos. Devido à sua estrutura química, estes compostos não são absorvidos na parte superior do trato gastrointestinal nem hidrolisados pelas enzimas digestivas humanas. Assim, tais ingredientes chegam ao cólon e servem como substratos para as bactérias endógenas, fornecendo indiretamente ao hospedeiro, energia, substratos metabólicos e micronutrientes essenciais (Gibson e Roberfroid, 1995).

Carboidratos não digeríveis incluem compostos como amido resistente, polissacarídeos de parede celular, hemicelulose, pectinas,

gomas e oligossacarídeos (Delzenne e Roberfroid, 1994). Para a maioria destes compostos, o processo de fermentação no cólon não é específica, uma vez que estimulam o crescimento e/ou a atividade metabólica de distintas espécies bacterianas, incluindo espécies potencialmente prejudiciais (Drasar *et al.*, 1976; Maczulak *et al.*, 1993; Wang e Gibson, 1993). Assim, tais compostos não podem ser classificados como prebióticos, já que não possuem seletividade para bactérias benéficas, como os lactobacilos e bifidobactérias (Gibson e Roberfroid, 1995).

Segundo Gibson e colaboradores (2004), os únicos carboidratos não digeríveis que satisfazem os critérios para serem considerados prebióticos são os frutooligossacarídeos, os galactooligossacarídeos e a lactulose, embora outros carboidratos da dieta também sejam promissores candidatos. Devido ao grande interesse na obtenção de produtos com propriedades prebióticas, pesquisas estão sendo feitas para avaliar o potencial prebiótico de novos oligossacarídeos como a lactosacarose, obtida a partir de uma mistura de sacarose e lactose por levansacarases, β-galactosidases e β-frutosidases (Diez-Municio *et al.*, 2012; Seki e Saito, 2012); glicooligossacarídeos, sintetizados empregando sacarose como doadora e distintos aceptores (gentiobiose, maltose, etc.) mediante glicosiltransferases como alternansacarases ou dextransacarases (Cote, 2009; Cote *et al.*, 2009); xilooligossacarídeos (Moura *et al.*, 2007), isomaltooligossacarídeos (Rudeekulthamrong *et al.*, 2013), entre outros.

Diversos estudos, tanto *in vitro* como *in vivo*, incluindo algumas intervenções em humanos, têm demonstrado as propriedades benéficas da ingestão de prebióticos (Rycroft *et al.*, 2001; Rastall, 2010; Roberfroid *et al.*, 2010), dentre elas se encontram:

1. Prevenção da produção de compostos genotóxicos e promotores de tumores por bactérias não benéficas, através do aumento de bifidobactérias e lactobacilos;
2. Diminuição dos sintomas causados por doenças inflamatórias do cólon como a doença de *Crohn* e a colite ulcerosa, mediada pela microbiota intestinal;
3. Diminuição da probabilidade de colonização de bactérias patogênicas, como o *Clostridium difficile*, causador de diarreia, pela produção de compostos antimicrobianos como as bacteriocinas e os ácidos graxos de cadeia curta, que diminuem o pH do cólon;
4. Melhoria na absorção de minerais como o cálcio, provavelmente pela diminuição do pH pela produção de ácidos graxos de cadeia curta por parte da microbiota intestinal, o que aumenta a biodisponibilidade de

cálcio ou pela abertura, mediada por alguns prebióticos, de canais epiteliais por onde o cálcio é absorvido;
5. Redução da incidência de enfermidades atópicas tais como dermatites ou urticária alérgica, provavelmente pelo aumento de bifidobactérias no intestino grosso.

Embora o efeito dos prebióticos sobre a microbiota intestinal seja bem conhecido, sobretudo na estimulação do crescimento de bifidobactérias e lactobacilos, existem discrepâncias entre alguns trabalhos e opiniões de especialistas sobre muitos destes efeitos benéficos. Consequentemente, mais estudos devem ser feitos, sobretudo *in vivo*, que permitam confirmar tais efeitos e ainda outros como os relacionados com obesidade e o tratamento da diabetes tipo 2 (Roberfroid *et al.*, 2010).

1.2.1. Galactooligossacarídeos

Galactooligossacarídeos (GOS) são oligossacarídeos sintetizados a partir de soluções concentradas de lactose utilizando a enzima *β*-galactosidase (Crittenden e Playne, 1996). Possuem estrutura Gal_n–Glc, onde *n* indica o grau de polimerização, sendo, geralmente, de 1-5 (Mussatto e Mancilha, 2007). A estrutura dos GOS pode diferir na composição dos açúcares, na regioquímica das ligações glicosídicas e no grau de polimerização, dependendo da origem da enzima utilizada (Figura 3). Tais diferenças estruturais acarretam em propriedades químicas distintas em relação à estimulação do crescimento de bactérias probióticas (Rycroft *et al.*, 2001).

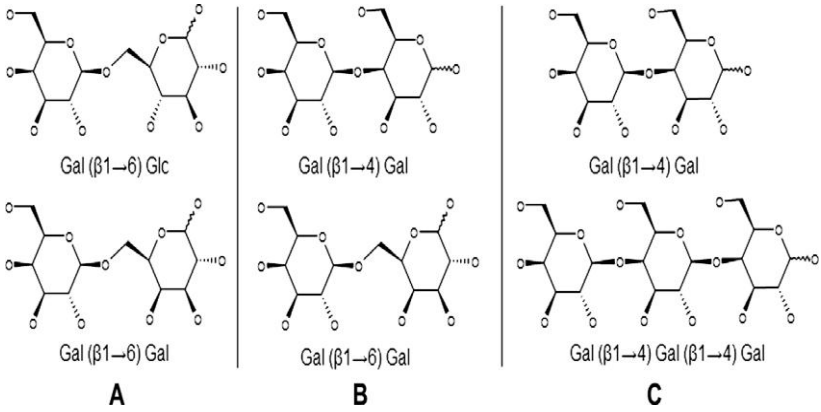


Figura 3. Exemplos de estruturas dos diferentes GOS em relação (A) à composição, (B) à regioquímica e (C) ao grau de polimerização (Gosling *et al.*, 2010). Gal: galactose e Glc: glicose.

Os GOS podem ser obtidos por reações de transgalactosilação catalisadas por *β*-galactosidases de diferentes origens (leveduras, fungos e bactérias) durante a hidrólise da lactose. Mesmo que muitos mecanismos tenham sido propostos para a ação desta enzima (Prenosil *et al.*, 1987), demonstrou-se que muitas *β*-galactosidases possuem dois resíduos de ácido glutâmico, Glu^{482} e Glu^{551}, que atuam, respectivamente, como doador de prótons e como nucleófilo, de modo simultâneo na reação enzimática (Figura 4).

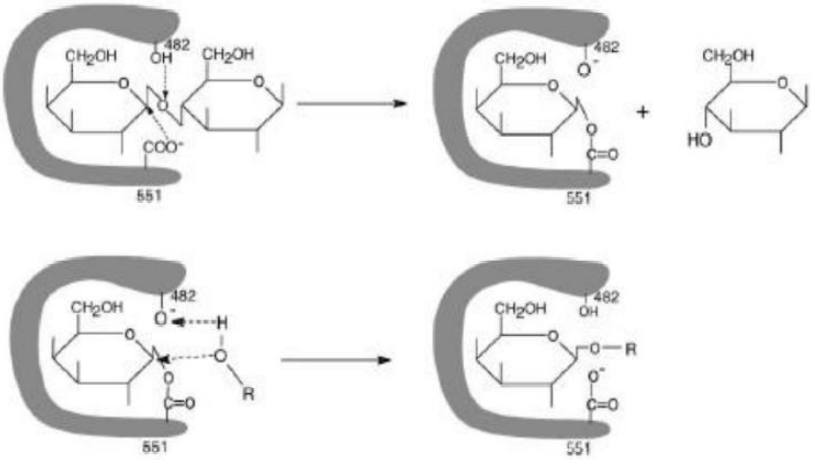


Figura 4. Mecanismo de ação da enzima β-galactosidase na obtenção de GOS a partir da lactose (Zhou e Chen, 2001). Primeiramente ocorre a ligação da lactose ao resíduo de glutamina (Glu551 na figura) presente no sítio ativo da enzima e a liberação simultânea de glicose. Este mecanismo se inicia pela doação de um próton por outro resíduo de glutamina presente no sítio ativo (Glu482 na figura). Posteriormente, a água (ou sacarídeo) age como doadora de prótons e galactose (ou galactooligossacarídeo) é liberada.

A produção de GOS durante a hidrólise da lactose envolve três etapas principais: primeiramente, há a formação do complexo enzima-galactose, seguida pela hidrólise da ligação glicosídica β 1→4 da lactose e a liberação simultânea da glicose. Então, a galactose é transferida a aceptores nucleofílicos contendo um grupo hidroxil (água ou sacarídeos). Em soluções com baixa concentração de lactose, este aceptor é a água, resultando na formação de galactose. Em soluções concentradas de lactose (e outros di- e trissacarídeos), o sacarídeo atua como aceptor e se liga ao complexo enzima-galactose resultando na formação de galactooligossacarídeos de maior peso molecular, sendo esta a última etapa (Mahoney, 1998; Rustom *et al.*, 1998).

A escolha da enzima utilizada na síntese de GOS tem importante efeito no rendimento, grau de polimerização e nos tipos de galactooligossacarídeos formados (Boon *et al.*, 2000; Rabiu *et al.*, 2001). O produto comercial Oligomate 55 é preparado utilizando a enzima β-galactosidase de *Aspergillus oryzae* e *Streptococcus thermophilus* (Crittenden e Playne, 1996) e contém 36% de tri-, tetra-,

penta-, e hexa-galactooligossacarídeos, 16% de dissacarídeos (galactosil-glicose e galactosil-galactose), 38% de monossacarídeos e 10% de lactose, sendo que os GOS formados possuem principalmente ligações do tipo β 1→6 (Rastall, 2010). Já o produto comercial Vivinal GOS, o qual é produzido a partir da enzima de *Bacillus circulans* contém 59% em GOS e 41% de glicose, galactose e lactose, sendo que os GOS formados possuem principalmente ligações do tipo β 1→4 (Rastall, 2010).

Além disso, a concentração de GOS obtida pode variar amplamente dependendo das condições reacionais utilizadas. De modo geral, quanto maior a concentração de lactose no meio maior será o rendimento em GOS, uma vez que, posteriormente à hidrólise, o aceptor final do grupo β-galactosil passa a ser a própria molécula de lactose ao invés da água (Gosling *et al.*, 2011). A síntese de GOS também pode ser beneficiada em altas temperaturas uma vez que a lactose possui solubilidade relativamente baixa a 25ºC (220 g L^{-1}). Em altas temperaturas é possível utilizar concentrações de lactose elevadas (600 g L^{-1}) e evitar sua cristalização (Gosling *et al.*, 2010). A concentração de enzima e tempo devem ser controlados uma vez que a reação é cineticamente controlada e ocorre em competição com a hidrólise da lactose e dos produtos formados (Rastall, 2010). De modo geral, quanto maior a concentração de enzima menor será o tempo necessário para obter o maior rendimento em GOS.

1.3. β-Galactosidases

A enzima β-galactosidase [lactase; β-D-galactoside galactohidrolase; EC 3.2.1.23] pertence ao grupo das enzimas conversoras de sacarídeos da família das hidrolases e é responsável pela hidrólise do resíduo terminal β-galactopiranosil da lactose (Galβ1→4Glc) dando origem a uma mistura equimolecular de glicose e galactose (Grosova *et al.*, 2008).

As β-galactosidases podem ser encontradas na natureza, distribuídas entre vegetais, particularmente amêndoas, pêssego, damasco, maçã, em órgãos de animais como intestino, cérebro, testículos, placenta e também são produzidas por grande quantidade de microrganismos tais como fungos filamentosos, bactérias e leveduras, sendo as leveduras e fungos as fontes preferidas para aplicações comerciais (Santiago, 2004). A legislação brasileira especifica, por meio da Resolução RDC nº 205/2006, que a enzima lactase, utilizada na indústria de alimentos, deve ser de origem microbiana, proveniente dos

seguintes microrganismos: *Aspergillus niger, Aspergillus oryzae, Candida pseudotropicalis, Kluyveromyces lactis, Kluyveromyces fragilis, Kluyveromyces marxianus, Saccharomyces sp*. (Brasil, 2006). Tais espécies são classificadas como *Generally Recognized As Safe* (GRAS) pela *Food and Drug Administration* (FDA), sendo esse um importante critério para aplicações alimentícias.

As propriedades das diferentes *β*-galactosidases dependem de sua origem. Em geral, lactases de fungos filamentosos possuem pH ótimo de atuação numa faixa ácida (2,5-4,5) enquanto o pH ótimo de atuação de lactases provenientes de leveduras e bactérias está numa região mais neutra (6,0-7,0 e 6,5-7,5, respectivamente). Estas diferentes condições de pH ótimo permitem selecionar a lactase mais apropriada para uma aplicação específica. Assim, lactases de fungos filamentosos são mais adequadas para hidrólise de soro ácido, enquanto lactases de leveduras e bactérias são mais apropriadas para hidrólise do soro doce e do leite (Gekas e Lopezleiva, 1985).

Duas *β*-galactosidases, de fontes diferentes, foram utilizadas neste estudo. Uma delas, Maxilact LX 5000, é uma preparação comercial líquida derivada da levedura *Kluyveromyces lactis*, de cor castanho pálido a castanho escuro, possui glicerol como estabilizante e atividade hidrolítica declarada de $\geq$ 5000 U g^{-1}. Esta enzima é uma glicoproteína contendo 45 % (m/m) carboidrato. Apresenta-se como um tetrâmero formado pela associação de dímeros, sendo que cada monômero consiste de 1024 resíduos de aminoácidos com massa molecular de 119 kDa (Pereira-Rodríguez *et al.*, 2012). Sua temperatura ótima está entre 35 - 40 ºC (Cavaille e Combes, 1995). Galactose e glicose são inibidores competitivos e não-competitivos, respectivamente, sendo a inibição por glicose (Ki = 794 mM) muito menor que a inibição por galactose (Ki = 42 mM). Sua atividade enzimática é inibida por zinco e cobre, sendo que os íons K^{+} e Mg^{+} são importantes para a estabilidade da enzima e o íon Mn^{+2} tem efeito estimulatório sobre sua atividade (Cavaille e Combes, 1995; Pereira-Rodríguez *et al.*, 2012). Sabe-se que no sítio ativo desta enzima, os grupos envolvidos na catálise enzimática são dois resíduos de ácido glutâmico (Glu482 e Glu551), localizados no centro de cada monômero (Pereira-Rodríguez *et al.*, 2012). É ainda caracterizada por possuir alta atividade hidrolítica (Gekas e Lopezleiva, 1985); porém apresenta algumas desvantagens no que diz respeito à sua atividade de transgalactosilação e estabilidade térmica para a produção de galactooligossacarídeos (Park e Oh, 2010).

Por outro lado, a *β*-galactosidase do fungo filamentoso *Aspergillus oryzae*, também utilizada neste estudo, apresenta alta atividade de transgalactosilação e é mais termoestável quando comparada à *β*-galactosidase de *K. lactis*. Esta enzima sintetiza trissacarídeos (GOS-3) como produto principal, com pequenas quantidades de tetrassacarídeos (GOS-4) e outros oligossacarídeos de maior grau de polimerização (Albayrak e Yang, 2002). Apresenta-se como um monômero de massa molecular de aproximadamente 90 kDa, não requer íons ativadores e sua atividade de hidrólise é inibida pelos íons cobre, prata e mercúrio, além de ser inibida competitivamente pela galactose. Possui pH ótimo entre 4,5-5 e temperatura ótima entre 50-55 ºC (Tanaka *et al.*, 1975; Park *et al.*, 1979). A *β*-galactosidase de *A. oryzae*, utilizada neste estudo, é uma enzima parcialmente purificada proveniente da Sigma-Aldrich®. Possui dextrina como veículo e atividade hidrolítica declarada de ≥ 8 U mg^{-1} de sólido.

1.4. Imobilização de Enzimas

Enzimas podem ser consideradas catalisadores sustentáveis, uma vez que são biocompatíveis, biodegradáveis e procedentes de fontes renováveis. Processos enzimáticos são conduzidos em condições moderadas de pH, temperatura e pressão atmosférica, em meio aquoso e com altas taxas reacionais e seletividades. Além disso, os processos enzimáticos proporcionam rotas sintéticas mais econômicas, que geram menos resíduos e são mais eficientes energeticamente do que a síntese orgânica convencional (Sheldon e Van Pelt, 2013).

Apesar de todas estas vantagens, a aplicação industrial de enzimas é dificultada pela baixa estabilidade operacional e dificuldade de recuperação e reutilização do biocatalisador. Muitos métodos têm sido propostos para superar essas limitações, sendo a imobilização de enzimas o de maior sucesso (Rejikumar e Devi, 2001; Grosova *et al.*, 2008). Mimetizando o modo natural de ocorrência em células vivas, onde a maioria das enzimas está ligada às membranas celulares, a imobilização pode estabilizar a estrutura da enzima e, consequentemente, sua atividade (Krajewska, 2004).

Além da facilidade no manuseio de enzimas imobilizadas, a imobilização permite sua separação do meio reacional, minimizando ou eliminando a contaminação do produto final pela enzima. Além disso, a enzima imobilizada não penetra facilmente na pele, e por isso, exibe baixa ou nenhuma alergenicidade (Sheldon e Van Pelt, 2013). A imobilização também permite o eficiente reuso da enzima, reduzindo

custos e permitindo o emprego de operação contínua em biorreatores. Outro benefício é, em muitos casos, o ganho em estabilidade, tanto sob condições de armazenamento ou operacionais, em relação a desnaturação por calor, por solventes orgânicos ou por autólise (Krajewska, 2004; Grosova *et al.*, 2008). A melhoria nas características catalíticas da enzima, associada às repetidas reutilizações se reflete em ganhos em produtividade (kg de produto por kg de enzima) o que, por sua vez, determina o custo da enzima por kg de produto (Sheldon e Van Pelt, 2013).

Resumidamente, os métodos de imobilização de enzimas podem ser divididos em três categorias: ligação a um suporte, inclusão (ou encapsulação) e entrecruzamento (Figura 5).

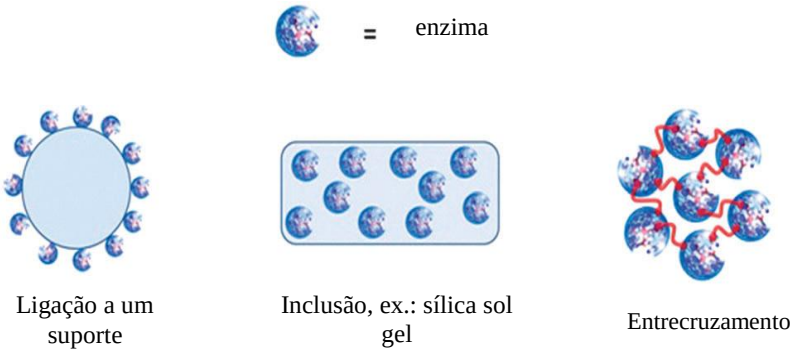


Figura 5: Diferentes métodos de imobilização de enzimas (Sheldon e Van Pelt, 2013).

A ligação da enzima a um suporte pode ser de natureza física (interações van der Waals, pontes de hidrogênio ou hidrofóbicas), iônica ou covalente (Cao, 2005). Apesar da simplicidade da ligação por interações físicas, este método é limitado pela tendência de dessorção da enzima do suporte e por ser sensível às condições do ambiente, como temperatura e concentração de íons (Grosova *et al.*, 2008). Ligações iônicas são geralmente mais fortes e ligações covalentes previnem que a enzima se solte do suporte. Por outro lado, a imobilização por ligações covalentes tem a desvantagem de que, caso a enzima perca sua atividade, ambos, enzima e suporte, não poderão ser reutilizados em um novo processo de imobilização (Sheldon e Van Pelt, 2013).

Dependendo do tipo de suporte utilizado, uma grande diferença pode ser observada na localização da enzima. Em suportes nanométricos, a enzima se liga à superfície acessível, enquanto que em suportes porosos, a maior parte de enzima está localizada na superfície interna (Figura 6). Entretanto, a aglomeração de suportes nanométricos é um fenômeno comumente observado, principalmente quando se trata de suportes paramagnéticos ou ferromagnéticos. Tais aglomerados podem ainda ser vantajosos em relação aos suportes porosos devido a menor dificuldade de difusão do substrato até o sítio ativo da enzima (Liese e Hilterhaus, 2013).

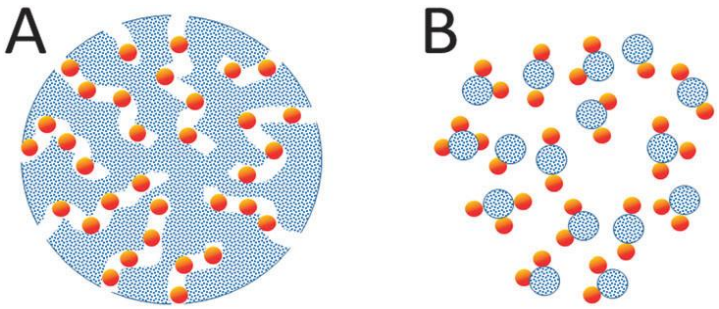


Figura 6: Comparação de um suporte poroso (A) e nanopartículas (B) para imobilização de enzimas (Liese e Hilterhaus, 2013).

A encapsulação pode ser definida como a inclusão de uma enzima em redes poliméricas, normalmente matrizes poliméricas orgânicas ou inorgânicas, como poliacrilamida e sílica sol-gel, respectivamente, ou membranas como fibras ocas ou microcápsulas (Sheldon e Van Pelt, 2013). A restrição física é, geralmente, muito fraca, entretanto, previne completamente a perda da enzima. Um procedimento comum é preparar a solução contendo a enzima e o material de inclusão e na sequência utilizar uma técnica (secagem, polimerização) para formar o material polimérico, contendo a enzima, na forma desejada. Em alguns casos, uma etapa de entrecruzamento adicional é necessária para garantir a imobilização e a estabilização da enzima (Grosova *et al.*, 2008).

O entrecruzamento de agregados ou cristais enzimáticos, empregando um reagente bifuncional como o glutaraldeído, é utilizado para preparar macropartículas livres de suporte. O uso de um suporte leva, inevitavelmente, a uma "diluição da atividade", devido a introdução de uma porção não-catalítica. Este problema não é resolvido

ao utilizar uma maior carga de enzima devido à dificuldade de acesso do substrato ao sítio ativo de algumas moléculas de enzima quando estas formam multicamadas ou quando estão situadas mais ao interior dos poros do suporte. A situação mais adequada, do ponto de vista de atividade específica, é a formação de uma monocamada na superfície do suporte (Sheldon e Van Pelt, 2013). Consequentemente, é interessante a utilização de enzimas imobilizadas sem suporte, como os cristais de enzima entrecruzados (Roy e Abraham, 2004) e os agregados enzimáticos entrecruzados (Sheldon, 2011). Estes métodos oferecem vantagens como alta concentração de atividade enzimática no biocatalisador, alta estabilidade e baixo custo já que não é necessária a utilização de um suporte (Sheldon e Van Pelt, 2013).

1.4.1. Imobilização de Enzimas em Suportes à Base de Quitosana

As características de enzimas imobilizadas são governadas pelas propriedades tanto da enzima como do suporte de imobilização. As interações entre os dois proporciona propriedades químicas, bioquímicas, mecânicas e cinéticas específicas (Cantone *et al.*, 2013). Suportes normalmente utilizados para imobilização de enzimas são resinas sintéticas, biopolímeros como polissacarídeos, ou sólidos inorgânicos como sílica mesoporosa ou zeólitas (Grosova *et al.*, 2008).

Além da atividade catalítica e estabilidade operacional, térmica e ao armazenamento, a estabilidade mecânica é um importante parâmetro que pode limitar a utilização a longo prazo da enzima imobilizada. Dependendo do material do suporte, o efeito do estresse mecânico pode causar desintegração do catalisador, dificultando os processos de recuperação e purificação dos produtos (Liese e Hilterhaus, 2013). As características do suporte (diâmetro de partícula, susceptibilidade ao intumescimento, estabilidade mecânica e comportamento durante a compressão) são de fundamental importância na performance de sistemas imobilizados e irão determinar as condições dos processos, bem como o tipo de reator a ser utilizado. Em particular, o volume de poro, diâmetro de poro e tamanho de partícula determinam a área superficial disponível para imobilização e afetam diretamente a capacidade de carga do suporte (Cantone *et al.*, 2013).

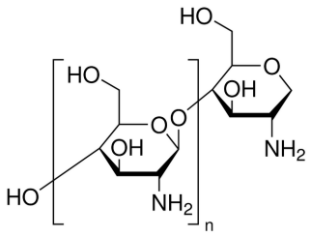
Biopolímeros naturais representam uma alternativa atrativa como suporte para imobilização de enzimas do ponto de vista econômico. Carboidratos insolúveis como celulose, amido, dextranas, agarose e quitosana, bem como proteínas como albumina e gelatina têm sido amplamente utilizados como suportes para imobilização de enzimas

(Cao, 2005). Deste grupo, os polissacarídeos são de especial interesse, uma vez que são seguros do ponto de vista biológico e bastante hidrofílicos, o que proporciona um ambiente adequado para muitas enzimas (Cantone *et al.*, 2013).

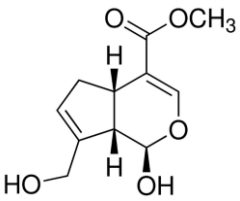
Como suporte para imobilização de enzimas, a quitosana oferece uma série de características desejáveis, incluindo: alta afinidade por proteínas, disponibilidade de grupos funcionais para reação direta com enzimas ou para modificação química, hidrofilicidade, biocompatibilidade, resistência à degradação química, propriedades antimicrobianas e facilidade de preparação em uma variedade de formas físicas (Hsieh *et al.*, 2000; Juang *et al.*, 2001; Krajewska, 2004).

Comercialmente, a quitosana [(1→4)-2-amino-2-deoxi-*β*-D-glucana] resulta da *N*-desacetilação da quitina (com uma solução de NaOH 40–45%, seguido de etapas de purificação), a qual, por sua vez, é obtida a relativo baixo custo do exoesqueleto de crustáceos (principalmente caranguejo, camarão, lagostas e krill), resíduos da indústria pesqueira (Shahidi *et al.*, 1999; Krajewska, 2004).

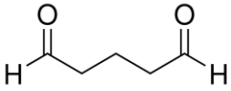
A quitosana é insolúvel em água, mas a presença de grupamentos amino faz com que ela seja solúvel em soluções ácidas (pH menor que 6,5) e pode ser precipitada em pH alcalino, assim partículas de quitosana podem ser facilmente obtidas gotejando uma solução ácida de quitosana em uma solução alcalina de coagulação (Krajewska, 2004). Além disso, anteriormente à imobilização, as partículas de quitosana podem ser ativadas por vários agentes como glutaraldeído (Muzzarelli, 1980), glicidol ou epicloridrina (Rodrigues *et al.*, 2008) e genipina (Muzzarelli, 2009), a fim de gerar grupos específicos para a imobilização da enzima. Em particular, a genipina, um composto isolado do jenipapo e de flores de gardênia, é uma alternativa interessante para substituir o glutaraldeído como agente de entrecruzamento da quitosana, já que a genipina é 5000-10000 vezes menos citotóxica que o glutaraldeído (Sung *et al.*, 1999; Muzzarelli, 2009). O tratamento da quitosana com agentes de entrecruzamento aumenta sua estabilidade mecânica, sua capacidade de adsorção e previne sua dissolução em soluções ácidas (Juang *et al.*, 2001). A Figura 7 mostra a estrutura química da quitosana, bem como dos agentes de entrecruzamento, glutaraldeído e genipina.



Quitosana



Genipina



Glutaraldeído

Figura 7: Estrutura química da quitosana e dos agentes de entrecruzamento, glutaraldeído e genipina, utilizados neste trabalho.

1.5. Reatores Enzimáticos

Um reator enzimático pode ser definido como um dispositivo no interior do qual as reações enzimáticas são realizadas, pela catálise de enzimas, para gerar produtos sob determinadas condições. Para sua aplicação, consideram-se duas fases: a fase líquida, que corresponde aos substratos e aos produtos dissolvidos, e a fase sólida, composta pelo biocatalisador imobilizado (Illanes, 2008).

Reatores enzimáticos são muito utilizados em processos industriais que envolvem catálise enzimática. Estes reatores podem ser utilizados com enzimas livres ou imobilizadas. Enzimas imobilizadas são preferidas nestes processos por serem, em geral, mais estáveis e de fácil reutilização, além de permitirem uma variedade maior de configurações de reatores (Illanes, 2008).

Várias configurações de reatores têm sido propostas para utilização de enzimas imobilizadas em operações contínuas, nos quais tais biocatalisadores são embalados em colunas e utilizados em um sistema de fluxo contínuo. Desta forma, são utilizados repetidamente durante o tempo que permanecerem ativos, minimizando custos, tempo de produção e tornando sua utilização economicamente viável. Possuem vantagens em termos de produtividade e controle de processo, contudo, ciclos de lavagem podem ser necessários para contornar problemas como entupimento (Girelli e Mattei, 2005).

A seleção de um reator enzimático para determinada aplicação é geralmente baseada no custo, espaço, na transferência de massa e na capacidade de reutilização do biocatalisador imobilizado. Os principais tipos de reatores são: reator em batelada, reator contínuo de leito fixo e reator contínuo de leito fluidizado (Xue e Woodley, 2012).

Reatores em batelada do tipo tanque agitado, devido à sua configuração relativamente simples, flexibilidade e facilidade de operação, é o tipo mais comum de reator, podendo ser aplicado para reações catalisadas tanto por enzimas livres ou imobilizadas (Tufvesson *et al.*, 2010; Xue e Woodley, 2012). Possuem a desvantagem de que, em larga escala, apresentam baixa produtividade. Além disso, a enzima ou célula imobilizada é exposta à estresse mecânico devido à agitação, o que pode levar à perda física do preparado enzimático e, assim, à contaminação do produto e ao acentuado decréscimo da atividade catalítica (Watanabe *et al.*, 2005; Hilterhaus *et al.*, 2008; Tufvesson *et al.*, 2010).

A utilização de reatores de leito fixo pode ser uma boa alternativa para reações utilizando enzimas imobilizadas (Hills, 2003). A principal vantagem de reatores de leito fixo em comparação a reatores do tipo tanque agitado é a alta produtividade volumétrica alcançada e a facilidade de operação em modo contínuo. Além disso, neste tipo de reator, possíveis forças de cisalhamento são eliminadas e a separação da enzima do meio reacional contendo os produtos também é simplificada (Xu, 2003). Por outro lado, limitações na transferência de massa, formação de caminhos preferenciais e queda de pressão (dependendo do suporte de imobilização utilizado) são fatores relacionados às desvantagens da utilização de reatores de leito fixo (Tufvesson *et al.*, 2010).

Reatores contínuos de leito fluidizado são reatores nos quais o meio reacional é mantido fluidizado por alimentação contínua a partir da base do reator e, geralmente, a fluidização é feita pela recirculação do meio. Estes reatores têm sido aplicados para facilitar o contato e a transferência de massa durante a reação, sendo aplicados em reações com mais de uma fase (líquido–gás, líquido–líquido e sólido–líquido) em uma variedade de processos químicos. A queda de pressão neste tipo de reator é menos acentuada em relação à reatores de leito fixo e o fluxo é mais uniforme, minimizando a formação de caminhos preferenciais. Da mesma forma, o estresse mecânico é menor quando comparado à reatores do tipo tanque agitado (Gomez *et al.*, 2007; Xue e Woodley, 2012).

1.6. Estabilização de Enzimas

Enzimas são biocatalisadores geralmente sensíveis a condições extremas de operação e, naturalmente, não evoluíram, do ponto de vista termodinâmico e cinético, para suportar condições industriais de temperatura, pressão e alta concentração de substratos e produtos. Entretanto, quando estabilizadas, mantêm sua conformação ideal e são bastante eficientes para catalisar suas respectivas reações (Burioni *et al.*, 2004; Robic, 2010).

A estabilidade termodinâmica é um importante parâmetro a ser avaliado para aplicação industrial de enzimas e está relacionado aos processos conformacionais de *folding* e *unfolding* (Li *et al.*, 2005). A instabilidade termodinâmica pode ser parcialmente atribuída a falta de rigidez dentro da estrutura terciária da enzima. Esta falta de rigidez pode ser causada por frações de enrolamentos aleatórios e α-hélices pouco estáveis e mais flexíveis, devido às poucas interações iônicas, em comparação às folhas *β*, mais estáveis (Li *et al.*, 2005). A estabilidade termodinâmica da proteína é indicada por sua temperatura de fusão, T_f , ou por sua energia livre de Gibbs, 8G (kJ mol^{-1}). Para um processo de desnaturação (*unfolding*) de duas etapas (N↔U), na temperatura de fusão, a constante de equilíbrio, *K*, é igual a unidade (*K*=[*U*]/[*N*]). E $8G_U = 0$ na T_f, uma vez que $\Delta G = RT\ln(k)$ (Bommarius e Paye, 2013).

A estabilidade cinética está relacionada à resistência à degradação e manutenção da eficiência catalítica da enzima numa dada condição. É geralmente reportada como tempo de meia-vida, $t_{1/2}$ (h ou d), ou como uma constante de desativação, k_d (h^{-1} ou d^{-1}). Para enzimas com taxas de inativação de primeira ordem, k_d está relacionado com o tempo de meia-vida (Equação 1) (Bommarius e Paye, 2013).

$$t_{1/2} = \frac{-\ln(0{,}5)}{k_d} \qquad (1)$$

Para aumentar a estabilidade termodinâmica e cinética em relação às condições do meio reacional, métodos incluindo imobilização, engenharia de proteínas e engenharia do meio reacional têm sido considerados (De Barros *et al.*, 2010) (Figura 8).

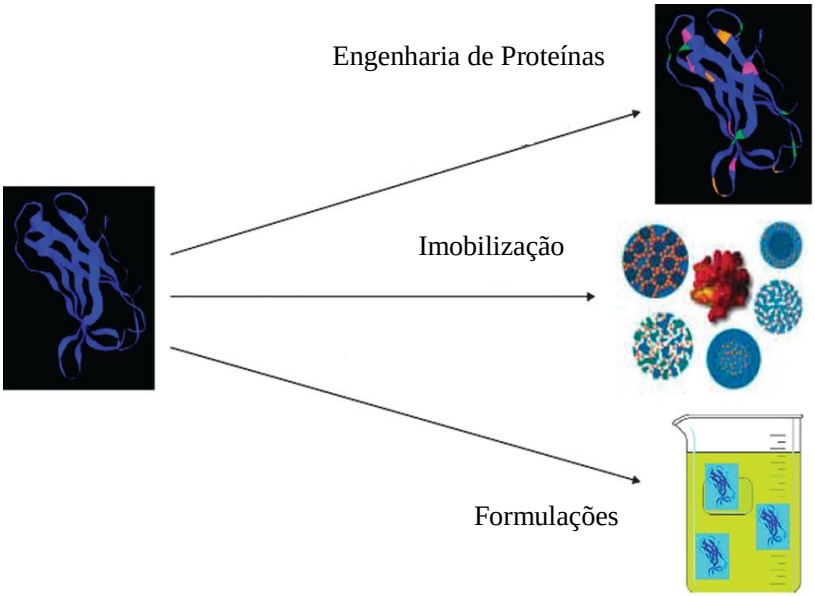


Figura 8: Três modos de estabilização de proteínas: (i) engenharia de proteínas (ii) imobilização e (iii) formulação (engenharia do meio).

A estabilidade de proteínas em solução pode ser aumentada pela adição de determinadas substâncias de relativo baixo peso molecular e em alta concentração (1 M ou mais). Estes aditivos incluem sais, polióis, açúcares e polímeros como polietilenoglicol (Fagain, 1995) e podem ser divididos em solutos osmolíticos ou estabilizantes iônicos (Schein, 1990). Osmólitos geralmente não possuem carga, afetam a viscosidade do meio e a tensão superficial. Nesta categoria estão os polióis, polissacarídeos e aminoácidos. Já estabilizantes iônicos parecem "blindar" as cargas superficiais da proteína (Schein, 1990). O mecanismo de estabilização por solutos ou co-solventes resulta da hidratação preferencial da proteína: o aditivo é excluído da vizinhança da molécula proteica, a qual é cercada somente por água. A proteína desnaturada é menos compacta que sua forma nativa, levando a intensificação da exclusão do co-solvente, o que é termodinamicamente desfavorável. Assim, a estrutura mais compactada da proteína nativa é favorecida, resultando na sua estabilização (Bhat e Timasheff, 1992; Timasheff, 1992).

Osmólitos de maior massa ou volume molecular parecem ter uma maior capacidade de induzir a estabilidade em proteínas, uma vez

que o efeito de exclusão estérica do aditivo aumenta com seu volume. Além disso, osmólitos maiores têm tendência de auto-agregação, o que aumenta ainda mais sua exclusão preferencial da superfície da proteína, contribuindo para o efeito estabilizante (Liu *et al.*, 2010). Assim, é provável que, galactooligossacarídeos, produto e substrato natural de *β*-galactosidases, sejam mais eficientes para promover a estabilidade térmica da enzima em comparação com o também substrato natural, lactose, e seus produtos, glicose e galactose.

1.6.1. Modelagem Cinética de Inativação Térmica

Modelos matemáticos consistem em equações que fornecem um resultado com base em conjunto de dados de entrada. É uma forma concisa de expressar comportamento físico em termos matemáticos. Segundo Haefner (2005), a modelagem matemática pode ter três objetivos: compreensão, predição e controle. A modelagem cinética de inativação térmica de enzimas é uma ferramenta que pode contribuir para a compreensão dos mecanismos físicos e químicos do sistema, tendo como resultado parâmetros fundamentais como energia de ativação, entalpia e entropia. Quanto à predição, o objetivo da modelagem matemática de inativação térmica é avaliar o efeito de diferentes tratamentos térmicos sobre a atividade residual, podendo então estimar a perda de atividade durante o tratamento, sem a necessidade de executar ensaios numerosos. Por outro lado, a finalidade da modelagem no controle do sistema implica na definição das condições de processamento para alcançar determinado objetivo (Van Boekel, 2008).

Modelagens de inativação têm sido descritas para microrganismos, componentes de alimentos e enzimas (Naim *et al.*, 2008; Shalini *et al.*, 2008; Mercali *et al.*, 2013). Considerando-se que a inativação prossegue normalmente através de uma rede de reações, incluindo a desnaturação, agregação, coagulação e decomposição química, os modelos cinéticos são baseados em mecanismos diferentes: de primeira ordem, reações consecutivas e paralelas (Tabela 1).

Tabela 1: Principais equações cinéticas utilizadas para análise da inativação térmica de enzimas.

Modelo		Equação
1ª Ordem	(2)	$\frac{A}{Ao} = \exp(-kt)$
Distintas Isoenzimas	(3)	$\frac{A}{Ao} = A_L \exp(-k_L t) + A_R \exp(-k_R t)$
2 Frações	(4)	$\frac{A}{Ao} = a \times \exp(-k_L t) + (1-a) \times \exp(-k_R t)$
Conversão Fracionada	(5)	$\frac{A}{Ao} = A_r + (1-A_r) \times \exp(-kt)$
Multi-Componente	(6)	$\frac{A}{Ao} = \frac{[\exp(-k_1 t) + r \exp(-k_2 t)]}{1+r}$
*n*th-Order	(7)	$\frac{A}{Ao} = \left\{A_o^{1-n} + (n-1) \times kt\right\}^{1/(1-n)}$
Weibull	(8)	$\frac{A}{Ao} = \exp(-bt^n)$

Nestas equações, A/A_0 representa a atividade residual da enzima no tempo t (min), e k (min^{-1}) é a constante de inativação térmica em uma dada temperatura.

A cinética de primeira ordem (Equação 2) tem sido usada para descrever a inativação térmica de muitas enzimas e outros compostos (Ludikhuyze *et al.*, 1999; Zhu *et al.*, 2013). Os parâmetros temperatura-dependentes dessa modelagem são dados pela equação de Arrhenius (Equação 9), que descreve a relação entre a constante de inativação térmica (k) e a temperatura.

$$\ln(k) = \ln(C) - \frac{E_a}{RT} \quad (9)$$

onde C é a constante de Arrhenius, E_a é a energia de ativação, R a constante universal dos gases e T é a temperatura absoluta.

Modelos paralelos, apresentados nas Equações (3), (4) e (5) levam em consideração que a enzima é uma mistura de, pelo menos, duas formas ativas (isoformas, isoenzimas) com diferentes sensibilidades, representadas pela fração "lábil" (A_L) e pela fração "resistente" (A_R), cada uma delas seguindo uma reação de 1ª ordem, com suas respectivas constantes (k_L e k_R, para as porções lábil e resistente, respectivamente) (Aymard e Belarbi, 2000). Na equação (4), o coeficiente α representa a atividade da porção lábil da enzima em relação à atividade total (Chen e Wu, 1998). A equação (5), conversão fracionada, se refere a um processo de inativação de 1ª ordem e leva em consideração a atividade da enzima diferente de zero após o aquecimento prolongado, devido à presença de uma fração da enzima extremamente resistente (Rizvi e Tong, 1997).

O modelo multicomponente de 1ª ordem (equação 6) é expresso pela soma da cinética de seus componentes, cada um seguindo uma cinética de inativação de 1ª ordem durante o aquecimento (Fujikawa e Itoh, 1996). Para reações de ordem-*n* ($n \neq 1$), onde *n* é a ordem da reação, a atividade enzimática residual é dada pela equação (7) (Shalini *et al.*, 2008).

Por fim, o padrão de distribuição de Weibull (1951) se baseia no pressuposto de que, nas condições analisadas, a taxa momentânea da sensibilidade térmica ao calor é um fator da intensidade do aquecimento transiente e da atividade residual, e não da taxa na qual a atividade residual foi atingida (Peleg e Penchina, 2000). A equação (8) representa a forma cumulativa da distribuição de Weibull, onde o parâmetro *b* é característico de cada reação e equivale a taxa de inativação térmica.

1.7. Considerações sobre o estado da arte

A análise da literatura científica disponível sobre o assunto da presente Tese de Doutorado evidencia a existência de um grande número de trabalhos que tratam da hidrólise da lactose, mecanismo, enzimas envolvidas e importância do processo na obtenção de produtos destinados a pessoas intolerantes à lactose.

Verifica-se também que vários tipos de carboidratos não digeríveis têm sido estudados e caracterizados por suas propriedades benéficas relacionadas à manutenção da microbiota dita “probiótica” e até mesmo por suas ações diretas na saúde dos consumidores. Assim, um futuro promissor está reservado para a área alimentícia e nutracêutica na obtenção e uso de compostos prebióticos, como os

galactooligossacarídeos (GOS), que podem ser obtidos a partir de subprodutos da indústria alimentícia, como o soro de leite.

Nos trabalhos analisados, a síntese de GOS é feita empregando altas concentrações de lactose, utilizando β-galactosidases de diferentes origens. A enzima utilizada na síntese de GOS tem importante efeito no rendimento, grau de polimerização e nos tipos de GOS formados. Na maioria dos casos, os estudos são realizados com a β-galactosidase em sua forma livre, no entanto, a aplicação industrial de enzimas livres pode ser limitada devido a baixa estabilidade operacional e dificuldade de recuperação e reutilização do biocatalisador. Em vista disso, vários trabalhos trazem contribuições significativas sobre a imobilização de β-galactosidases, destacando as diversas vantagens do uso de enzimas imobilizadas em relação ao uso de enzimas livres, em particular, no que se refere ao aumento de estabilidade térmica e operacional, e a possibilidade de uso em diferentes configurações de reatores.

Entretanto, ainda que o número de trabalhos seja bastante grande, tendo em vista a importância do tema tratado, algumas lacunas ainda persistem e estudos que visem imobilizar a β-galactosidase em suportes de baixo custo, gerando biocatalisadores estáveis, fáceis de usar, biocompatíveis e seguros para utilização em indústrias de alimentos, são de fundamental importância para sua satisfatória aplicação na síntese de GOS, bem como na hidrólise da lactose. Além disso, o estudo da estabilidade térmica da enzima em condições operacionais pode fornecer informações fundamentais, de modo a melhorar os processos de síntese de GOS e de hidrólise da lactose, contribuindo assim para aumentar o conhecimento existente na área específica e, por consequência, preencher, ao menos em parte, as lacunas existentes.

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CAPÍTULO 2: EFFECT OF THE SUPPORT SIZE ON THE PROPERTIES OF *β*-GALACTOSIDASE IMMOBILIZED ON CHITOSAN: ADVANTAGES AND DISADVANTAGES OF MACRO AND NANOPARTICLES

Neste trabalho, as macro e nanopartículas de quitosana foram produzidas e caracterizadas em função do seu tamanho, área superficial, porosidade, estabilidade térmica e carga de enzima aplicada. Da mesma forma, a retenção de atividade, a estabilidade térmica e a estabilidade operacional na hidrólise da lactose foram avaliadas para ambos biocatalisadores imobilizados obtidos. Os resultados estão apresentados no artigo a seguir, publicado na revista *Biomacromolecules*, 2012, v. 13, p. 2456-2464. DOI: dx.doi.org/10.1021/bm3006984.

Effect of the support size on the properties of *β*-Galactosidase immobilized on chitosan: advantages and disadvantages of macro and nanoparticles

ABSTRACT: The effect of the support size on the properties of enzyme immobilization was investigated by using chitosan macroparticles and nanoparticles. They were prepared by precipitation and ionotropic gelation, respectively, and were characterized by Fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), transmission electron microscopy (TEM), light scattering analysis (LSA) and N_2 adsorption-desorption isotherms. *β*-Galactosidase was used as a model enzyme. It was found that the different sizes and porosities of the particles modify the enzymatic load, activity and thermal stability of the immobilized biocatalysts. The highest activity was shown by the enzyme immobilized on nanoparticles when 204.2 mg protein (g dry support)$^{-1}$ were attached. On the other hand, the same biocatalysts presented lower thermal stability than macroparticles. *β*-Galactosidase immobilized on chitosan macro and nanoparticles exhibited excellent operational stability at 37 ºC, since it was still able to hydrolyze 83.2 and 75.93% of lactose, respectively, after 50 cycles of reuse.

Keywords: *β*-Galactosidase, Enzyme Immobilization, Chitosan, Macroparticles, Nanoparticles, Support Characterization.

INTRODUCTION

Enzyme immobilization is a usual pre-requisite as a solution to obtain reusable biocatalysts and thus decrease the price of these relatively expensive compounds. Moreover, immobilization also improves the control over the reaction, avoids product contamination by the enzyme (especially relevant in food chemistry) and permits the use of different reactor configurations (Garcia-Galan et al., 2011) .

Among the various materials available for the development of new supports for enzyme immobilization, chitosan can be considered appropriate due to many characteristics, including: high affinity to proteins, availability of reactive functional groups for direct reactions with enzymes and for chemical modifications, hydrophilicity, good biocompatibility, reduced non-specific adsorption, improved resistance to chemical degradation, anti-bacterial properties and ease of preparation in a variety of physical forms (Adriano et al., 2008; Hsieh et al., 2000; Juang et al., 2001; Krajewska, 2004). Moreover, chitosan appears economically attractive since chitin is the second most abundant natural polymer, after cellulose (Bailey et al., 1999). Commercially, chitosan results from the *N*-deacetylation of chitin, which is obtained at a relatively low cost from the shells of shellfish (mainly crabs, shrimps, lobsters and krill) and wastes from the seafood processing industry (Bailey et al., 1999; Krajewska, 2004; Shahidi et al., 1999). Immobilization of enzymes on chitosan supports is readily performed by using a bifunctional reagent, such as glutaraldehyde, once its functional groups (–CHO) simultaneously react with the binding sites of chitosan ($–NH_2$) and the amino terminal group from the enzyme (Chiou & Wu, 2004). Furthermore, the treatment of chitosan with glutaraldehyde improves its mechanical strength, its adsorption performance and prevents them from dissolution in acidic solutions (pH lower than 2) (Juang et al., 2001).

The enzyme *β*-galactosidase (EC 3.2.1.23) has several applications in the food industry (Grosova et al., 2008; Husain, 2010). It catalyses the hydrolysis of lactose that is present in milk allowing people, with lactose intolerance, to consume milk and other dairy products without suffering adverse effects caused by lactose malabsorption. Immobilized *β*-galactosidase is used both for hydrolysis of lactose and, under special conditions, for galactooligosaccharide synthesis (Grosova et al., 2008). Using immobilized *β*-galactosidase instead of its free equivalent form is an effective way to perform the

enzyme reuse and to improve its thermal and operational stability (Grosova et al., 2008; Husain, 2010).

Covalent immobilization of *β*-galactosidase on chitosan supports has been studied by many authors; it usually occurs on particles of a great diameter, namely, 2 - 5 mm (Chang & Juang, 2005; Dwevedi & Kayastha, 2009; Kumari & Kayastha, 2011; Singh et al., 2011). There are few works describing the use of small particles of chitosan in the immobilization of enzymes (Biro et al., 2008; Ilgu et al., 2011; Tang et al., 2006). Many studies were mostly related to the preparation of chitosan nanoparticles and their applications as drugs carriers (Berthold et al., 1996; De Campos et al., 2001; Janes et al., 2001). The use of smaller-diameter supports for enzyme immobilization has the advantage of offering a high specific surface area for enzyme attachment. Nanoparticles also offers high surface/volume ratio for immobilization which can result in higher concentration of the biocatalyst when compared with others immobilization protocols (Ansari & Husain, 2012). Furthermore, because of the smaller size of the support particles, the diffusion hindrance can be reduced (Zheng et al., 2003). Many protocols about the immobilization of enzymes on nanoparticles have been developed until now (Ansari & Husain, 2011; Ansari & Husain, 2012; Husain et al., 2011; Pan et al., 2009; Schilke et al., 2010). Therefore, it is important to consider the advantages and disadvantages of using nanoparticles for enzyme immobilization in comparison with the traditional methods already existing.

Based on these aspects, the main purpose of this work was to study the effect of the support size on the properties of *β*-galactosidase from *Kluyveromyces lactis* covalently immobilized on chitosan macroparticles and nanoparticles, prepared by precipitation and ionotropic gelation, respectively. Then, the effect of the immobilization approach and the support size on the immobilization yield and activity retention, enzymatic load, thermal stability and operational stability was verified in order to compare the prepared biocatalysts. Besides, the physicochemical characteristics and structures of the particles obtained were analyzed by FTIR, DSC, LSA, TEM and N_2 adsorption-desorption isotherms.

EXPERIMENTAL SECTION

Materials

β-galactosidase from *K. lactis* (Maxilact LX 5000) was obtained from Globalfood (Brazil). Chitosan (from shrimp shells, ≥ 75% deacetylated), *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) and lactose were

obtained from Sigma-Aldrich (Brazil). A glucose determination kit was purchased from Labtest Diagnóstica SA (Brazil) and glutaraldehyde 25% is from Nuclear (Brazil). All other chemicals were of analytical or HPLC grade.

Methods

Preparation and Activation of Chitosan Particles

Chitosan macroparticles were prepared by the precipitation method as follows: chitosan (2% w/v) was dissolved in 0.35 M acetic acid, sonicated during 20 min to remove the air bubbles and added dropwise into the coagulation solution (sodium hydroxide 1 M and ethanol 26% v/v) under slow agitation. The macroparticles obtained were washed with distilled water until neutrality was reached. The activation was performed by incubating the macroparticles with glutaraldehyde (5% v/v) prepared in 0.1 M phosphate-potassium buffer (pH 7.0) during 3 h under slow rotation. The activated support was exhaustively washed with the same buffer to remove the excess glutaraldehyde.

Chitosan nanoparticles were prepared by ionotropic gelation using sodium sulfate as the gelation agent, according to Berthold and co-workers (1996), with some modifications: 0.5 mL of sodium sulfate aqueous solution (1.4 M) were added dropwise into 9.5 mL of chitosan (0.25% w/v) dissolved in 0.35 M acetic acid containing Tween 80 (1% v/v) under sonication. This suspension was magnetically stirred for 2 h (500 rpm) and the particles formed were collected by centrifugation (3,500 ×g, 15 min, 4 °C). The particles obtained were washed with distilled water and activated by suspending them in 10 mL of glutaraldehyde (1.25% v/v) in 0.1 M phosphate-potassium buffer (pH 7.0) under agitation during 30 min. The glutaraldehyde excess was removed with successive washings using the same buffer.

Chitosan Particles Characterization

Changes on the molecular structure of chitosan macro and nanoparticles were determined before and after glutaraldehyde activation by Fourier Transform Infrared (FT-IR) spectroscopy with a Varian 640-IR spectrometer. Samples previously lyophilized were crushed and thoroughly mixed with powdered KBr, then pressed to form a transparent pellet (1% w/w). The spectra were obtained at room temperature with 40 accumulative scans and 4 cm^{-1} of resolution.

The Differential Scanning Calorimetry (DSC) was carried out on a DSC Q20 Instruments. The samples were encapsulated in

aluminum DSC pans and the measurements were taken between 25 ºC and 400 ºC at a heating rate of 5 ºC.min^{-1} under a nitrogen flow of 40 mL.min^{-1}. The Thermogravimetric Analysis (TGA) was performed using a Shimadzu Thermal analyzer Model TA50, at a heating rate of 10 ºC.min^{-1}, from room temperature up to 600 ºC under argon atmosphere.

Morphological images of chitosan nanoparticles were obtained by Transmission Electron Microscopy (TEM). Nanoparticles were suspended in distilled water and dispersed in an ultrasonic bath for 5 min. A drop of sample was dropped on a copper grid and negatively stained with uranyl acetate solution (2% w/v). The excess liquid was absorbed at the periphery of the grid by using filter paper. The remaining sample was air-dried at room temperature. All preparations were observed using a JEOL JEM 1200ExII transmission electron microscope (JEOL, Tokyo) operating at 120 kV.

Moreover, the mean size and polydispersity index (PDI) of the nanoparticles in suspension were evaluated by light scattering performed on a Brookhaven Instruments standard setup (BI-200 M goniometer, BI-9000AT digital correlator) with a He–Ne laser (k = 632.8 nm) as the light source, as previously described by Malheiros and co-workers (2010) DLS measurements were made before nanoparticles activation to avoid its aggregation and after sample filtration (0.45 μm membrane filter) in order to remove any particle that may cause interference in the analysis.

Textural characteristics of the samples were evaluated by N_2 adsorption-desorption isotherms at 77 K, using a Tristar II Krypton 3020 Micromeritics equipment. The lyophilized samples were degassed at 40 °C, under vacuum, for 10 h. The specific surface areas were determined by using the BET method and the pore size distribution were estimated using BJH method (Gregg, 1982) .

β-Galactosidase Immobilization

Chitosan particles were incubated overnight with *β*-galactosidase solution prepared with 0.1 M of phosphate-potassium buffer (pH 7.0) containing $MgCl_2$ 1.5 mM (activity buffer) during 15 h at room temperature, under gentle stirring. After immobilization, successive washings with the buffer removed the excess enzyme, until no more activity was detected in the washing fractions. Between these washes, immobilized enzyme was washed with NaCl (1 M) and ethylene glycol (30% v/v) to eliminate ionic and hydrophobic interactions between enzyme and support. Different amounts of *β*-galactosidase were

offered to the chitosan particles in order to investigate the best enzyme activity.

The immobilized activity was calculated taking the difference between the applied and the recovered activities in the pooled supernatant and washing fractions. The immobilization yield (IY) and the activity retention (AR) was calculated according to the following equations:

$$IY(\%) = \frac{\text{Immobilized Activity}}{\text{Applied Activity}} x100 \quad (1)$$

$$AR(\%) = \frac{\text{Activity measured on the support}}{\text{Immobilized Activity}} x100 \quad (2)$$

Activity Assays of β-Galactosidase

Free and immobilized *β*-galactosidase activities were measured using ONPG as substrate. The measurements were performed in 0.5 mL activity buffer containing ONPG (10 mM) at 37 ºC during 2 min. The reaction was stopped by addition of 1.5 mL borate buffer pH 9.8. The released *o*-nitrophenol (ONP) was determined spectrophotometrically at 415 nm. One unit of *β*-galactosidase activity (U) was defined as the amount of enzyme which catalyses the conversion of 1 μmol of ONPG to *o*-nitrophenol per minute under the conditions previously stated. Protein content of the enzyme solutions was determined by the Lowry assay. The immobilized protein was estimated as the difference between the amount of protein applied to the support and the amount recovered in the pooled supernatant and washing fractions.

As chitosan particles were in different physical format depending on the preparation method used, to get comparable activity values, a known amount of wet macro and nanoparticles were lyophilized and the activities were expressed as U (g dry support) $^{-1}$.

Thermal Stability of Free and Immobilized β-Galactosidase

The thermal stability of both free and immobilized enzymes was evaluated incubating the biocatalysts in sealed tubes with activity buffer in a thermostatically controlled water bath at 50 ºC. Different conditions were tested: the presence and absence of substrate (lactose - 5%) and the enzyme concentration. After the exposure time, samples

were removed from the water bath and placed in ice bath to stop thermal inactivation instantaneously. The remaining enzyme activity was determined as described above. Assays were performed in duplicate.

Operational Stability of Immobilized β-Galactosidase in the Lactose Hydrolysis

Lactose hydrolysis in batch was performed with *β*-galactosidase immobilized on macro and nanoparticles of chitosan incubated in Erlenmeyer flasks containing 5% (w/v) of a buffered (pH 7) lactose solution. Samples were withdrawn periodically and analyzed enzymatically for glucose formation. After its first use, the immobilized enzyme was incubated repeatedly in the same conditions of its first use in order to evaluate its operational stability in the successive hydrolysis batches.

RESULTS AND DISCUSSION

Characterization of Chitosan Particles

FTIR Analysis

Spectra of chitosan macro and nanoparticles are presented in Figure 1. The broad band between 3100 and 3700 cm^{-1} is attributed to the O–H stretching vibration, mainly from water, which overlaps the amines stretching vibrations (N–H) in the same region. The bands between 2800 and 3000 cm^{-1} are attributed to the C–H stretching vibration (Colthup, 1975). The spectrum of chitosan particles before activation show a characteristic peak around 1640 cm^{-1} correlated to the N-H bending vibrations. The peak at 1380 cm^{-1} were attributed to –C–O–H stretching of primary alcoholic group in chitosan. Chitosan characteristic spectral peaks before glutaraldehyde activation were in agreement with the ones reported in the literature (Liu et al., 2012; Zhang et al., 2009). Comparing chitosan macro and nanoparticles before activation, similarities in their spectra were observed related to the characteristic peaks.

After activation, the strong peak at around 1660 cm^{-1} can be attributed to an imine bond (N=C) produced after the reaction of aldehyde with the amine groups of chitosan (Monteiro & Airoldi, 1999). The weak shoulder at 1710 cm^{-1} can be assigned to the carbonyl stretching mode of remaining aldehyde groups of glutaraldehyde (Collins et al., 2011). The other peaks are related to the chitosan structure as previously discussed. Collins and co-workers (2011) in their studies about FTIR-ATR characterization of free and chitosan

immobilized *Rhizomucor meihei* lipase reported similar findings when comparing characteristic spectral bands of chitosan after glutaraldehyde activation.

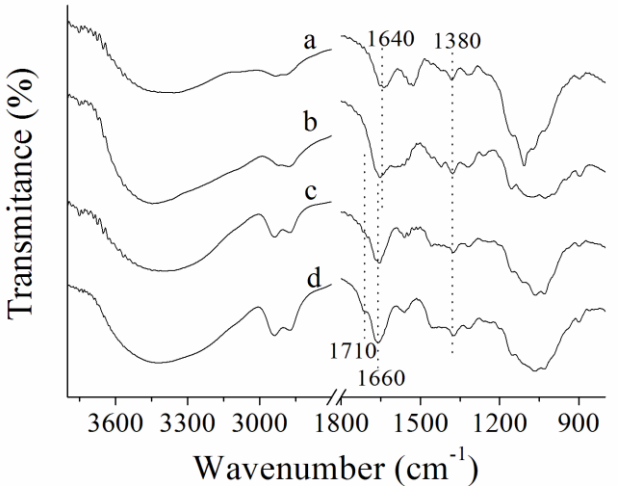

Figure 1. FTIR spectra of (a) non-activated macroparticles, (b) non-activated nanoparticles, (c) activated macroparticles and (d) activated nanoparticles.

Thermal Analyses

The DSC results for chitosan particles are shown in Figure 2. The thermal processes occurred between 25 °C and 350 °C. The endothermic peaks with maxima between 80 °C and 95 °C, correspond to the energy required to desorb water (Yang & Su, 2011) and exothermic peaks at higher temperatures are attributed to the thermal decomposition of chitosan (Tripathi et al., 2009; Yang & Su, 2011).

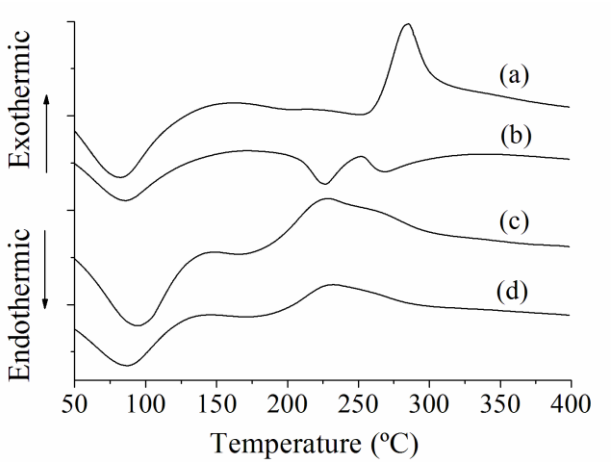

Figure 2. DSC thermogram of (a) non-activated macroparticles, (b) non-activated nanoparticles, (c) activated macroparticles and (d) activated nanoparticles.

After activation, endothermic peaks for nanoparticles remained almost constant, whereas they underwent a slight shift to higher temperatures for macroparticles. This can indicate that, for macroparticles, the water interaction with the activated network is stronger than with the non-activated chitosan. According to Rueda and co-workers (1999), in chitosan, water is bound to the hydroxyl groups more strongly than to the amine groups. Once the treatment of chitosan particles with glutaraldehyde replaces the amino groups by aldehyde groups, it is expected that the interaction of water-chitosan molecules will be stronger in the activated particles and higher temperatures would be necessary to remove the water molecules. The difference observed between macro and nanoparticles can denote that particles sizes and/or the different methodology applied for particles preparation can influence the strength of the water–chitosan interaction.

At higher temperatures, it is possible to observe exothermic peaks due to decomposition of chitosan. However, for the non-activated nanoparticles (curve 2b), it was also possible to observe an unexpected endothermic peak near 220 ºC, which can be due to desorption of some residues of the nanoparticles synthesis, considering that the synthesis methods of macro and nanoparticles were different; and/or to desorption of volatile chitosan oligomeric species generated in the synthesis of nanometric materials.

From the DSC curves it is also possible to observe that macro and nanoparticles are thermally stable at least, up to 170 ºC, and the curves (2c and 2d) of activated particles, which will be after used for β-galactosidase immobilization, were very similar, indicating that the size of the particles probably does not affect their thermal stability.

It was observed that the activated chitosan particles begin to decompose at lower temperatures, when compared with their non-activated forms. This can be attributed to the interactions of the internal chitosan chains promoted by glutaraldehyde, which interferes with pre-existing attractive hydrogen bonds, weakening the chitosan structure (Neto et al., 2005).

The TGA analysis of the particles is presented in Figure 3. It is possible to observe that all samples showed weight loss of near 45% in the same region of DSC exothermic peaks. The particles were thermally stable, at least, up to 180 ºC, in accordance to the DSC results. Similar thermal stability was reported in other studies on chitosan materials (Pereira-Rodríguez et al., 2012; Tripathi et al., 2010; Zhang et al., 2012). Regarding the non-activated nanoparticle curve (curve 3b), a slight difference in the curve slope can be observed, which is in accordance to the DSC analysis interpreted on the occurrence of two events: the endothermic species desorption and the exothermic decomposition.

TGA curves of activated samples were equivalent, and this behavior was also observed in the DSC analysis. It indicates that the thermal properties of macro and nanoparticles became very similar after the glutaraldehyde treatment.

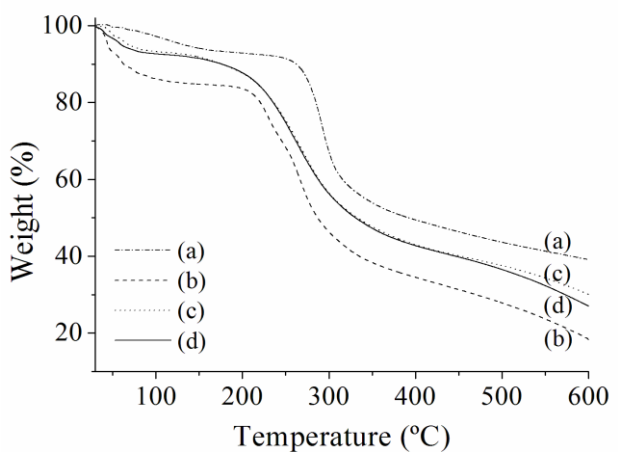

Figure 3**.** TGA curves of (a) non-activated macroparticles, (b) non-activated nanoparticles, (c) activated macroparticles and (d) activated nanoparticles.

Additionally, the DSC and TGA curves indicate that the obtained supports would be thermally stable at the temperature range used in most enzymatic reactions (up to 100 ºC), informing future researchers about the maximum temperature to which these materials could be exposed without any damage.

Morphology of Chitosan Macro and Nanoparticles

Results obtained from the light scattering analysis (LSA) show that filtered chitosan nanoparticles presented sizes of approximately 410 nm, with a low polydispersity of 0.266, which is an indicative of the homogeneity of the sample. Similar results were found in the work of Biró and co-workers (2008) in which the use of sodium sulfate as gelation agent yielded nanoparticles with a medium size of 517 nm before activation with glutaraldehyde. LSA was done before nanoparticles activation because the effect of cross-linking promoted by the glutaraldehyde caused stronger aggregation of the particles, which would make this analysis unfeasible. It is noteworthy that glutaraldehyde not only activates but also cross-links the chitosan. The two terminal aldehyde groups of glutaraldehyde can react with amino groups of D-glucosamine units of different chains, resulting in cross-linking. The Schiff's base linking aldehyde with amino group also

provides to the chitosan particles operational stability and resistance against lower pH (Singh et al., 2011).

Figure 4 (a) and (b) shows the non-activated and activated chitosan macroparticles, respectively. The pictures were made using a Nikon Coolpix P100, 10.3 megapixel camera. The prepared macroparticles have a regular round shape with a mean diameter of 2 mm. After glutaraldehyde activation, the particles turned slightly yellow due to the Schiff's base formation. The pictures (c) and (d) show the colloidal suspension of non-activated and activated nanoparticles, respectively. After glutaraldehyde activation, it is possible to see phase separation, probably due to the formation of heavier aggregates from the cross-linking reaction between different chains of chitosan.

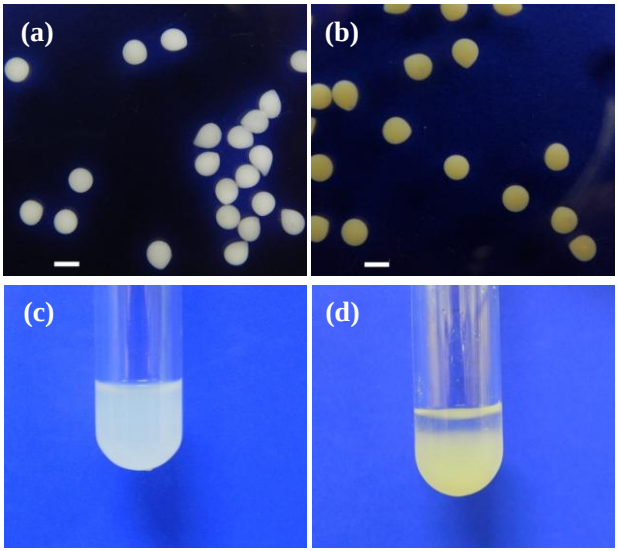

Figure 4. Pictures of (a) non-activated macroparticles, (b) activated macroparticles, (c) non-activated nanoparticles and (d) activated nanoparticles, taking with Nikon Coolpix P100, 10.3 megapixel camera. Bar, 2 mm.

The Transmission Electron Microscopy images (TEM) of the nanoparticles revealed that the nanoparticles have an irregular morphology and variation in size. It is expected that nanoparticles agglomerate more than their non-activated form, due to the cross-linking caused by glutaraldehyde during the activation treatment. However, at a microscopic level, the agglomeration was also observed before glutaraldehyde activation as was shown in Figure 5. The tendency to

agglomeration of nanoparticles is well known and is reported to be due to their high surface area and energy. Several strategies have been developed in order to achieve outstanding colloidal stability of nanoparticles (Zhang et al., 2011).

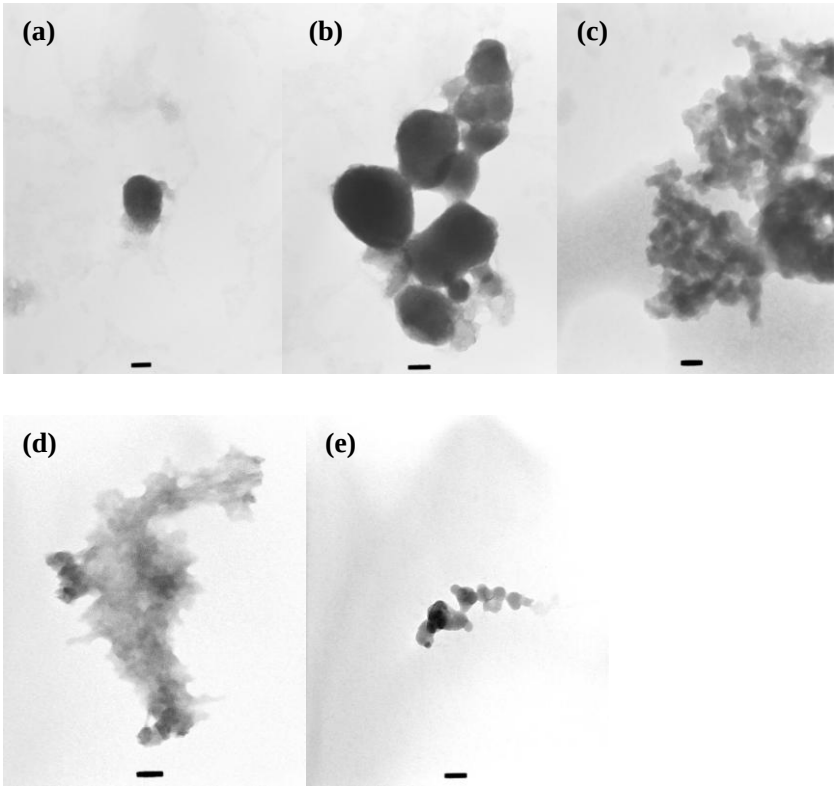

Figure 5**.** Transmission Electron Microscopy images obtained for: nanoparticles before glutaraldehyde activation (a), (b) and (c), and nanoparticles after glutaraldehyde activation (d) and (e). Bar, 50 nm.

Textural Characteristics of Macro and Nanoparticles

The N_2 adsorption-desorption isotherms of the macroparticles and nanoparticles after glutaraldehyde activation, with and without *β*-galactosidase, are shown in Figure 6, together with the BJH pore size

distribution curves. The BET surface area and pore volume are presented in Table S1 of the Supporting Information.

Before the enzyme immobilization, the isotherm curves (a) seemed to show type IV profile of mesoporous materials for macro and nanoparticle samples. However, the amount of adsorbed N_2 was very low, mainly for the nanoparticles. The samples presented a small amount of mesopores with diameters in the range between 2 and 20 nm. The BET specific surface area for the macroparticles, before the enzyme immobilization, was 87 $m^2.g^{-1}$. This value was higher than that obtained for nanoparticles, 29 $m^2.g^{-1}$, as macroparticles present a higher mesoporosity than nanoparticles (see the y scale of BJH curves (a) showed in Inset Figure 6). Additionally, as observed in TEM images, nanoparticles agglomeration is possible, reducing their surface area.

After *β*-galactosidase immobilization, porosity decrease is evident in all diameters range (see Inset Figure 6). The surface area reduces from 87 to 46 $m^2.g^{-1}$and from 29 to 8 $m^2.g^{-1}$, for macroparticles and nanoparticles, respectively. This surface area reduction accompanied a drastic reduction in N_2 adsorption. The lower degree of surface occupation observed for the macroparticles can be due to the inaccessibility of some pores for enzyme immobilization.

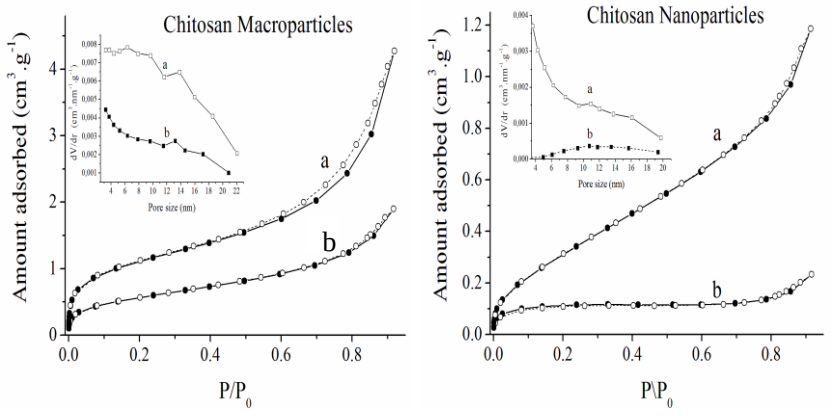

Figure 6. N_2 adsorption desorption isotherms of chitosan macro and nanoparticles: (a) without *β*-gal and (b) with *β*-gal. Inset Figure: BJH pore size distribution.

Enzyme Immobilization and Loading Capacity of the Chitosan Particles

The β-galactosidase activities of nano and macroparticles related to the weight of dry supports, as well as the immobilization yields and activity retention after immobilization for different amounts of enzyme are summarized in Figure 7 below and Tables S2 and S3 in Supporting Information. For both, chitosan macro and nanoparticles, the immobilization yield was reduced when the enzymatic load was increased, since the support saturation was being achieved. Similarly, the activity retention was decreased. This effect can be attributed to some factors: (a) the active site blockage by the immobilization effect itself; (b) the presence of neighboring molecules of enzyme that reduce the access of the substrate to active sites (de Fuentes et al., 2001) (c) random attachment of proteins on the particles surface which may undergo changes in the enzyme conformation to a less active one and (d) the support overload, with a consequent formation of a multilayer of enzymes molecules at the particles surface (Pereira et al., 2001).

Less specific activity was reached after the β-galactosidase immobilization on macroparticles than on nanoparticles (Figure 7). The maximum activity achieved by the macroparticles was 210 U (g dry support) $^{-1}$ while for the nanoparticles this value was 18330 U (g dry support) $^{-1}$, much higher than previous reports (Elnashar & Yassin, 2009a; Elnashar & Yassin, 2009b). In this condition approximately 46.2 and 204.2 mg of protein were immobilized per gram of dry support, respectively.

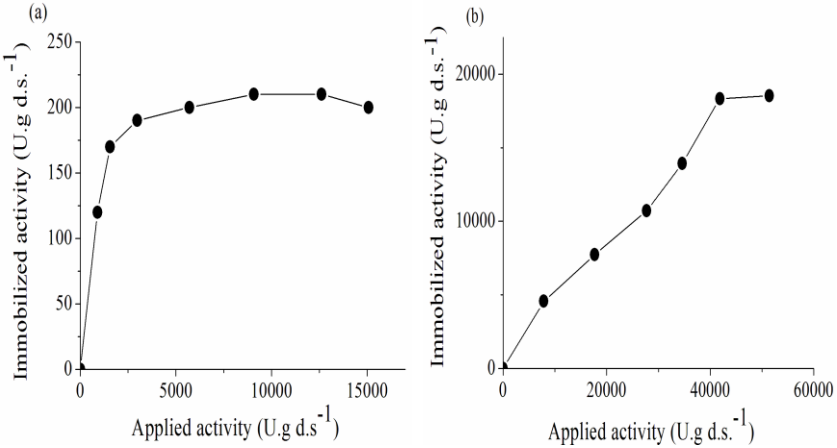

Figure 7. Loading capacity of (a) chitosan macroparticles and (b) chitosan nanoparticles.

d.s. - dry support

Regarding the activity retention after immobilization, macroparticles presented the lowest values. The less activity retention may evidence that not all surface area of macroparticles are available for enzyme attachment. The enzymes molecules probably cannot enter all macroparticles pores, getting immobilized mostly in their outer surfaces. Furthermore, the molecules of enzyme immobilized at the support surface could hinder the access of the substrate to the active sites of the enzymes that could be attached into the inner surface of the macroparticles. In this way, it is likely that not all the molecules are responsible for the activity expressed, which also explains these lowest values.

The results found by Di Serio and co-workers (2003) is according to our findings. The immobilization of *β*-galactosidase from *Kluyveromices marxianus (Saccharomyces) lactis* on different oxides porous supports presented a low retention of activity (not exceeding 5%). The authors mentioned that the result can be a consequence of different effects: the enzyme molecules aggregation, a greater number of plugged macropores, uneven enzyme distribution and, eventually, a low effectiveness factor. Furthermore, the activity of the immobilized enzyme was dependent on the chemical nature and physical structure of the supports. As the particle size of the supports was increased, the retention of activity after immobilization decreased strongly, which could be attributed to the distribution of enzyme active sites inside the macropores in a non-uniform way. Biró and co-workers (2008) also found that the immobilization of *β*-galactosidase on chitosan macroparticles reached considerably less activity than when the enzyme was immobilized on chitosan nanoparticles. Husain and co-workers reported that *β*-galactosidase was adsorbed in a greater extent in zinc oxide nanoparticles (ZnO-NP, 480 U) than in native zinc oxide (ZnO, 360 U) when using 150 mg of each support (Husain et al., 2011).

In order to accurately investigate the immobilization yield and the activity retention, the immobilization process was monitored over time for macro and nanoparticles. The amount of protein offered per gram of dry macro and nanoparticles was 46.2 and 204.2 mg, respectively. The results, expressed in terms of immobilization yield (IY) and activity retention (AR) (Figure 8) showed that at the beginning of the process the percentages of AR are higher whereas the percentages of IY are lower, particularly for the nanoparticles. In other words, when a small amount of protein is attached to the support, its activity is totally expressed. However, when yield increases and more proteins become bounded, their AR reduces.

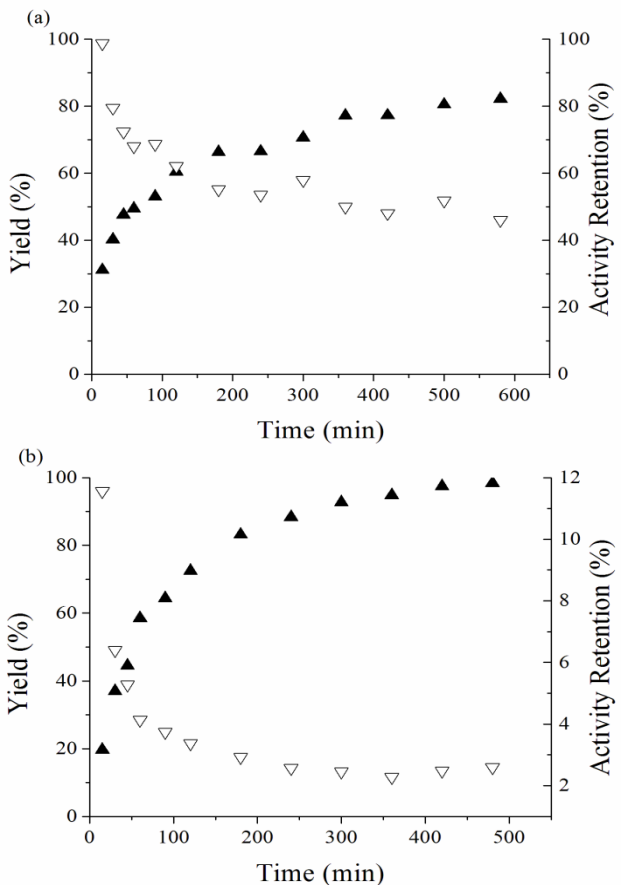

Figure 8. Enzyme loading for (a) nanoparticles and (b) macroparticles. Results are expressed in terms of percentages of yield (▲) and activity retention (▽).

For the nanoparticles (Figure 8a) after the first 15 min of immobilization, the yield and activity retention were 31.1 and 98.7 % and after 580 min of immobilization these values changed to 90 and 46.5 %, respectively. The same pattern was shown by the macroparticles (Figure 8b). In both cases, as time progresses and more protein molecules are immobilized, a lower percentage of activity from the initial applied is recovered. The support overload for the macro and nanoparticles, which hinders the mass transfer between β-galactosidase active sites and the surrounding microenvironment could be the main reason for the results found here, as also already explained above.

Similarly, Pereira and co-workers (2001) in the study of immobilization of *Candida rugosa* lipase on porous chitosan beads by non-specific interaction found that the activity of the immobilized enzyme ranged from 48 to 480 $U.g^{-1}$ support when the loading applied varied from 0.1 g to1 g crude lipase $(g\ chitosan)^{-1}$. The highest retention of activity was obtained when the loading was 120 U lipase $(g\ support)^{-1}$ and a further increase in the enzyme loading had no effect on activity retention. The authors suggested that the formation of an enzyme multilayer might be the major reason for the lower activity retention.

Thermal Stability of Free and Imobilized β-Galactosidase

The inactivation kinetics for free and immobilized *β*-galactosidase is shown in Figures 9, 10 and 11. In these figures, the symbols refer to the average of experimental values. A comparison of thermal stability of free and immobilized biocatalysts, under non-reactive conditions (without lactose) at 50 ºC, is presented in Figure 9. Biocatalyst concentrations used for the free enzyme were 0.25 and 1 $mg.mL^{-1}$ and for the immobilized *β*-galactosidase were 46.2 mg $(g\ dry\ support)^{-1}$ dry support and 204.2 mg $(g\ dry\ support)^{-1}$, the maximum protein load for macro and nanoparticles, respectively. All immobilized enzymes presented higher thermal stability when compared to the free enzyme, proving that the immobilization on chitosan particles had a positive effect on the thermal stability of *β*-galactosidase and it is able to protect the active conformation of the enzyme from damage by heat exchange (Zhang et al., 2010).

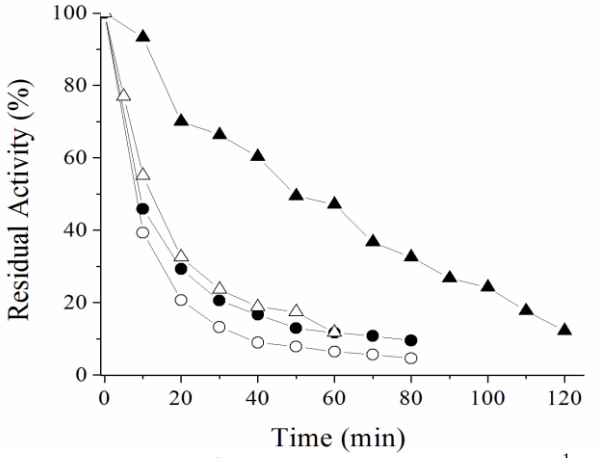

Figure 9. Thermal stability of (●) free *β*-galactosidase (1 mg.mL^{-1}), (○) free *β*-galactosidase (0.25 mg.mL^{-1}), (▲) immobilized *β*-galactosidase on macroparticles (46.2 mg (g dry support)$^{-1}$) and (△) immobilized *β*-galactosidase on nanoparticles (204.2 mg. (g dry support)$^{-1}$).

The thermal stability of *β*-galactosidase immobilized on macroparticles was superior when compared to the free enzyme, retaining around 50% of its initial activity after 50 min of incubation at 50 ºC, against less than 20% for the free enzyme (1 mg.mL^{-1}). For nanoparticles, almost no increase was observed; around 20% of the initial activity was maintained after the same period of incubation. It is noteworthy that the concentration of the free enzyme chosen to evaluate the thermal stability was equivalent to the concentration used for the immobilized enzymes, for both enzyme loads.

Immobilized enzymes with smaller protein concentrations were also analyzed to study the effect of the enzyme loading on the thermal stability of the immobilized biocatalysts. It was found that when increasing the protein concentration from 15.4 to 46.2 mg (g dry support)$^{-1}$, almost no increase was observed in the enzyme thermal stability for the macroparticles, as can be seen in Figure 10. Similar results were found for the nanoparticles, when the support loading increased from 44.4 to 204.2 mg (g dry support)$^{-1}$.

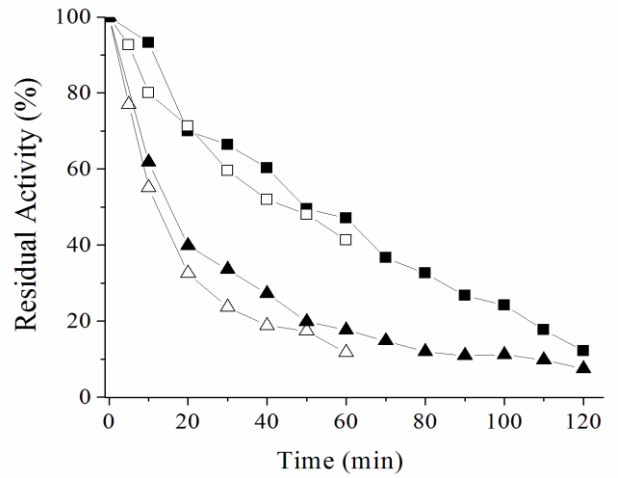

Figure 10. Thermal stability of (■) immobilized *β*-galactosidase on macroparticles (46.15 $mg.mL^{-1}$), (□) immobilized *β*-galactosidase on macroparticles (15.4 $mg.mL^{-1}$), (▲) immobilized *β*-galactosidase on nanoparticles (204.2 mg $(g\ dry\ support)^{-1}$), and (△) immobilized *β*-galactosidase on nanoparticles (44.4 mg $(g\ dry\ support)^{-1}$).

Comparing the immobilized derivatives, the macroparticles show the best results of thermal stability despite having lower protein load per gram of dry support. In this case, it could be said that the macroparticles offer a stronger protecting effect on the enzyme than the nanoparticles. It is possible that the phenomenon of support overload or even the immobilization into the pores makes the enzyme more protected from thermal deactivation. Some studies reported that the molecular confinement, able to restrict the molecular movement, reduces the possibility of enzyme deactivation, increasing its thermal stability (Cao, 2005).

Similar findings were obtained by Huerta and co-workers (2011) where *β*-galactosidase from *A. oryzae* was covalently immobilized on amino-epoxy Sepabeads®, glyoxyl-agarose 6BCL and chitosan granules. Their half-lives at 55 ºC were 20.5 h, 9.3 h and 18.6 h, respectively, while for the free enzyme the value was 7.4 h. The immobilized enzymes half-lives were always higher when compared to that related to the free enzyme. However, as also observed in this present study, there is a dependence of the enzyme thermal stability on the method of immobilization used.

Thermal stability of immobilized biocatalysts under reactive conditions (5% w/v of buffered lactose solutions) is presented in Figure 11. For both immobilized biocatalysts, the presence of lactose on the reaction medium increased its thermal stability. In terms of residual activity, this increasing was from 7.5 to 20.6% for nanoparticles and from 12 to 61.7% for macroparticles after 120 min of incubation at 50 ºC. These findings indicate that *β*-galactosidase immobilized on chitosan has better heat tolerance in the presence of lactose. According to Kovalenko and co-workers (2007), the presence of the substrate generates an enzyme-substrate complex, more tolerant to high temperatures, which should prevent the deactivation of enzyme due to modification of the active site.

From a technological point of view it is important to estimate the thermal stability of *β*-galactosidase in the presence of its substrate for further application in lactose hydrolysis.

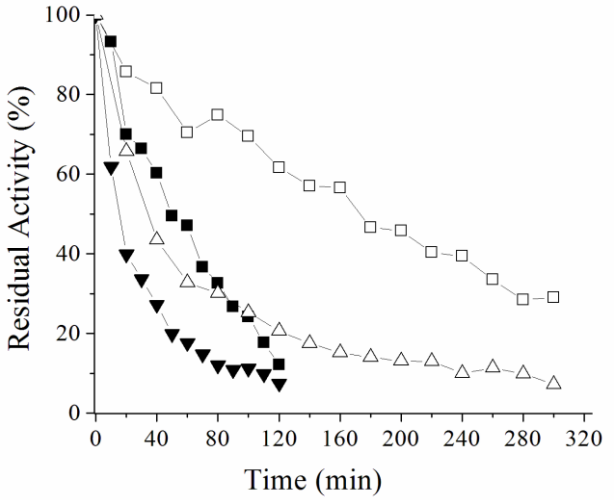

Figure 11**.** Comparison of the thermal stability of *β*-galactosidase immobilized on macroparticles (46.2 mg (g dry support)$^{-1}$) in presence (□) and absence (■) of lactose (5% w/v buffered solutions) and *β*-galactosidase immobilized on nanoparticles (204.2 mg. (g dry support)$^{-1}$) in presence (△) and absence (▼) of lactose.

Operational Stability in Lactose Hydrolysis

The operational stability of the immobilized biocatalysts was evaluated by the hydrolysis of buffered lactose solutions (5% w/v; pH 7) at 37 ºC. Lactose hydrolysis performed with macroparticles (1.5 U.mL^{-1})

in 20 mL of lactose resulted in 92.2% of lactose conversion in 1.33 h for it first use. The reusability of the immobilized enzyme is shown in Figure 12. Repeated batch hydrolysis of buffered lactose solutions by the immobilized enzyme on macroparticles, at 37 ºC, allowed 50 repeated cycles with a small decrease in the rate of lactose hydrolysis, from 91.2% in the first reuse to 83.2% in the last reuse.

In the same way, *β*-galactosidase immobilized on nanoparticles shows an excellent operational stability. Lactose hydrolysis performed with nanoparticles (8.5 $U.mL^{-1}$) in 20 mL of lactose solutions resulted in 96.6% of lactose conversion in 0.67 h for it first use. Repeated batch hydrolysis of buffered lactose solutions by the immobilized enzyme on nanoparticles, at 37 ºC, allowed also 50 repeated cycles with a small decrease in the rate of lactose hydrolysis, from 90.2% in the first reuse to 75.9% in the last reuse. The reusability of the immobilized enzyme on nanoparticles is also shown in Figure 12.

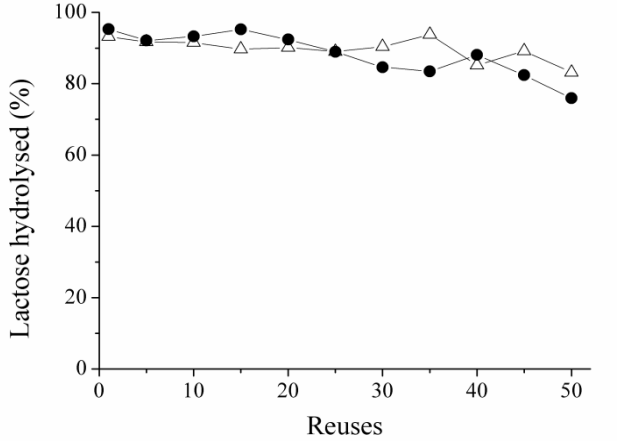

Figure 12. Lactose hydrolysis performed with *β*-galactosidase immobilized on chitosan macroparticles (△) and on nanoparticles (●) at 37 ºC.

From these results, it can be concluded that both biocatalysts show excellent operational stability in the hydrolysis of lactose in the conditions stated above. Comparing these findings with those found in the current literature, it seems that these results are among the best reported. Elnashar and Yassin (2009b) in their studies about the immobilization of *β*-galactosidase on thermally stable carrageenan coated with chitosan (hydrogel), showed that the operational stability was 97% of retention of the enzyme activity after 15 uses. In the same way, *β*-galactosidase from *Aspergillus oryzae* immobilized on chitosan

beads was continuously used at 50 ºC and flow rate of 0.5 mL.min^{-1}, and approximately 40% reduction in the activity of the immobilized *β*-galactosidase/chitosan was observed after repeated use for 20 cycles (400 min) (Rejikumar & Devi, 2001).

CONCLUSIONS

In this work, *β*-Galactosidase from *K. lactis* immobilized on chitosan macro and nanoparticles meets both advantageous requirements in enzyme immobilization: stability and reuse. The biocatalysts thermal stability was improved for macroparticles, especially under reactive conditions (presence of lactose) in comparison with the free enzyme. Besides, both preparations could be reused for 50 repeated batches in the lactose hydrolysis without any outstanding loss of enzyme activity.

The morphological and physicochemical characteristics of the supports obtained were elucidated using different techniques. The nanoparticles showed to have an average diameter of 410 nm and the macroparticles presented a more porous structure than the nanoparticles. The FTIR analysis showed similarities in the spectra of macro and nanoparticles. Thus, it can be concluded that the differences related to immobilization yield and activity retention, load capacity, as well as, thermal stability are due to the influence of the support size and porosity.

From a practical point of view, each biocatalyst prepared presents its proper advantages and disadvantages. While in the hydrolysis of lactose with nanoparticles just a small amount of chitosan can be used due its high activity, a centrifugation step is required for the separation of nanoparticles from the reaction medium. On the other hand, after the hydrolysis of lactose with macroparticles, the separation step can be performed by simple unit operations such as filtration or sedimentation. However, due to the low activity of macroparticles when compared to the nanoparticles, it is necessary to spend more chitosan in the preparation of that biocatalyst. Furthermore, in some process that requires the use of higher temperatures, as the production of galactooligosaccharides, the use of macroparticles could be more adequate once they presented the highest thermal stability.

Supporting Information:
Table S1: Surface area and pore characteristics result for macro and nanoparticles with and without *β*-galactosidase. Table S2: Influence of the enzyme load on the immobilization of *β*-galactosidase on chitosan

macroparticles. Table S3: Influence of the enzyme load on the immobilization of *β*-galactosidase on chitosan nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

Supporting Information for

Effect of the support size on the properties of *β*-Galactosidase immobilized on chitosan: advantages and disadvantages of macro and nanoparticles

Table S1: Surface area and pore characteristics result for macro and nanoparticles with and without *β*-gal*.

Sample	BET surface area ($\pm$ 4 $m^2.g^{-1}$)	Pore volume ($\pm$ 0.005 $cm^3.g^{-1}$)
Nanoparticles with *β*-gal	8	0.006
Nanoparticles without *β*-gal	29	0.04
Macroparticles with *β*-gal	46	0.06
Macroparticles without *β*-gal	87	0.13

**β*-gal - *β*-galactosidase

Table S2: Influence of the enzyme load on the immobilization of β-galactosidase onto chitosan macroparticles.

Applied Activity (U (g d.s.)$^{-1}$*)	Immobilized Activity (U (g d.s.)$^{-1}$)	Immobilization Yield (%)	Activity measured on the support (U (g d.s.)$^{-1}$)	Activity Retention (%)
900	900	100	120	13.4
1560	1560	100	170	10.7
2970	2960	99.6	190	6.4
5710	5630	98.7	200	3.6
9070	8760	96.6	210	2.4
12620	11500	91.1	210	1.8
15070	13500	89.6	200	1.5

*d.s. - dry support

Table S3: Influence of the enzyme load on the immobilization of β-galactosidase onto chitosan nanoparticles.

Applied Activity (U (g d.s.)$^{-1}$*)	Immobilized Activity U (g d.s.)$^{-1}$	Immobilization Yield (%)	Activity measured on the support U (g d.s.)$^{-1}$	Activity Retention (%)
7870	7870	100.00	4570	58.1
17700	17700	100.00	7740	43.7
27730	27440	99.8	10720	39.5
34610	34290	99.4	13940	40.7
41850	37650	89.9	18330	48.7
51390	42440	82.6	18530	43.6

*d.s. - dry support

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CAPÍTULO 3: HIGH STABILITY OF IMMOBILIZED *β*-D-GALACTOSIDASE FOR LACTOSE HYDROLYSIS AND GALACTOOLIGOSACCHARIDES SYNTHESIS

Neste trabalho, as macropartículas de quitosana com *β*-galactosidase imobilizada foram utilizadas em um reator de leito fixo para estudar a hidrólise da lactose e a síntese de GOS em um sistema contínuo. Foram testadas a hidrólise contínua de uma solução de lactose e também de soro de leite, além da síntese de GOS em diferentes fluxos de alimentação do reator. A estabilidade térmica da enzima em presença de lactose, glicose e galactose também foi avaliada. Os resultados estão apresentados no artigo a seguir, publicado na revista *Carbohydrate Polymers*, 2013, v.95, p. 465-470. DOI: 10.1016/j.carbpol.2013.02.044

High stability of immobilized *β*-D-galactosidase for lactose hydrolysis and galactooligosaccharides synthesis

ABSTRACT: *β*-D-Galactosidase from *Kluyveromyces lactis* was immobilized on glutaraldehyde-activated chitosan and used in a packed-bed reactor for the continuous hydrolysis of lactose and the synthesis of galactooligosaccharides (GOS). The biocatalyst was tested for its optima pH and temperature, thermal stability in the presence of substrate and products, and operational stability. Immobilization increased the range of operational pH and temperature, and the enzyme thermal stability was sharply increased in the presence of lactose. Almost complete lactose hydrolysis was achieved for both milk whey and lactose solution at 37 ºC at flow rates up to 2.6 mL min^{-1}. Maximal GOS concentration of 26 g L^{-1} was obtained at a flow rate of 3.1 mL min^{-1}, with a productivity of 186 g L^{-1} h^{-1}. Steady-state operation for 15 days showed the reactor stability concerning lactose hydrolysis.

Keywords: Continuous Enzymatic Packed-Bed Reactor; *β*-D-Galactosidase; Chitosan Macroparticles; Galactooligosaccharides; Lactose Hydrolysis.

INTRODUÇÃO

Lactose (*β*-D-galactopyranosyl-(1→4)-D-glucopyranose), the main sugar of milk, can be hydrolyzed by *β*-D-galactosidase liberating D-glucose and D-galactose, making possible the consumption of milk and other dairy products by lactose-intolerant people (Haider & Husain, 2009). In the presence of highly concentrated lactose, this enzyme can also produce galactooligosaccharides (GOS), by transferring galactosyl residues to lactose molecules. The GOS produced (for example, Gal (*β* 1→4) Gal (*β* 1→4) Glc), usually has the structure Gal_n–Glc, where *n* indicates the degree of polymerisation, which is typically 1–5 (Gosling et al., 2010; Mussatto & Mancilha, 2007). GOS are non-digestible oligosaccharides, which are used as prebiotics food ingredients. The regular consumption of GOS can promote the growth and metabolism of intestinal bifidobacteria, microorganisms that are associated with positive health effects (as the reduction of the level of cholesterol, anticarcinogenic properties, and production of vitamins), when applied in human and other animals diets (Grosova et al., 2008). Therefore, the enzyme *β*-D-galactosidase, in its free or immobilized forms, has an important application in the food industry for lactose hydrolysis and for GOS synthesis.

For the industrial-scale applications of immobilized enzymes, where large amounts of biocatalyst are required, the immobilization protocol must be simple and preferably make use of inexpensive materials as supports (Garcia-Galan et al., 2011). Moreover, concerning food applications, nontoxicity and biocompatibility are also required. In this context, chitosan [(1→4)-2-amino-2-deoxy-*β*-D-glucan], which is a natural polyaminosaccharide derived from chitin [(1→4)-2-acetamido-2-deoxy-*β*-D-glucan], a by-product of the seafood processing industry, is both safe and cheap (Krajewska, 2004; Muzzarelli, 1980). Another unique property of chitosan is its low propensity for microbial contamination, a problem associated with the immobilization of *β*-D-galactosidase for lactose hydrolysis (Benhabiles et al., 2012). Chitosan is soluble in acid solutions, and can be precipitated in alkaline pH, thus chitosan particles can be readily obtained dripping an acid chitosan solution into an alkaline coagulation solution (Krajewska, 2004). Prior to enzyme immobilization, chitosan particles can also be easily activated using various agents such as glutaraldehyde (Muzzarelli, 1980), genipin (Chiou et al., 2007; Muzzarelli, 2009), glycidol or epichlorohydrin (Rodrigues et al., 2008) in order to generate specific groups for enzyme attachment.

Reactors configuration for continuous operations is another important aspect in designing industrial enzymatic processes. Packed-bed reactors (PBRs) are widely and successfully used in many industrial processes. Some of the advantages of using this type of reactor are the reuse of the enzyme without the need of a prior separation; the continuous production of the desired product; the easiness of handling substrates of low solubility by the use of large volumes containing low concentrations of substrate; and the long-term runs and industrial-scale operations. Therefore, PBRs are more cost effective than batch operations (Chang et al., 2007; Halim et al., 2009).

The main objective of this research was to set up a packed-bed reactor filled with chitosan-immobilized *β*-D-galactosidase for the continuous hydrolysis of lactose and the synthesis of galactooligosaccharides. The immobilized enzyme was also characterized for its operational optima pH and temperature, and biocatalyst thermal stability, assayed in the presence of substrates and products. Process performance was evaluated in terms of lactose hydrolysis and GOS synthesis as a function of flow rate of substrate feeding. Finally, continuous lactose hydrolysis was carried out in order to evaluate the operational stability of the PBR under steady-state operation.

MATERIALS AND METHODS

Materials

Whey powder was obtained from a local supplier (Elegê Laticínios S.A., Teutônia, Brazil), *Kluyveromyces lactis* *β*-D-galactosidase was the liquid formulation of Maxilact LX 5000, with a declared activity of ≥ 5000 NLU/g (DSM Food Specialties, Heerlen, Netherlands). Chitosan (from shrimp shells, ≥ 75 % deacetylated), *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG), D-glucose, D-galactose, lactose, and raffinose (*β*-D-fructofuranosyl α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranoside) were obtained from Sigma-Aldrich (St. Louis, USA). A D-glucose determination kit was purchased from Labtest Diagnóstica SA (São Paulo, Brazil). All solvents and other chemicals were of analytical grade.

Methods

Preparation of β-D-galactosidase immobilized on chitosan macroparticles

β-D-galactosidase was covalently immobilized on glutaraldehyde-activated chitosan macroparticles as described in a previous work (Klein et al., 2012). The amount of protein attached on chitosan macroparticles was 46.2 mg g^{-1} of dry support, presenting an activity of 230 U g^{-1} of dry support. The chitosan- *β*-D-galactosidase derivative was stored at 7 ºC in 0.1 M of phosphate-potassium buffer (pH 7.0) containing $MgCl_2$ 1.5 mM (activity buffer).

Activity of free and immobilized β-D-galactosidase

Free *β*-D-galactosidase activity was assayed by incubating the diluted enzyme (50 µL) with 0.5 mL of activity buffer containing ONPG (10 mM) at 37 ºC during 2 min. For immobilized *β*-D-galactosidase, 1.5 mg (dry support) was incubated with 1 mL of activity buffer containing ONPG (10 mM) at 37 ºC during 2 min. The reactions were stopped with the addition of borate buffer pH 9.8. Released *o*-nitrophenol (ONP) was spectrophotometrically determined at 415 nm. One unit of *β*-D-galactosidase activity (U) was defined as the amount of enzyme that hydrolyzes 1 µmol of ONPG to *o*-nitrophenol per minute under the conditions previously stated. Protein content of the enzyme solutions was determined by the Lowry assay.

Optima pH and temperature for free and immobilized β-D-galactosidase

The optimum operational pH of *β*-D-galactosidase activity was studied by monitoring enzyme activity of both free and immobilized preparations in 0.1 M of phosphate-potassium buffer (pH 5.7 to 8.0) containing $MgCl_2$ 1.5 mM, at 37 ºC, while the optimum temperature was determined by measuring the activity between 10 ºC and 70 ºC at pH 7.

Thermal stability of the immobilized β-D-galactosidase

Thermal stability of the immobilized enzyme was evaluated incubating the biocatalyst in sealed tubes in a thermostatically controlled water bath at 55 ºC. Thermal stability was performed under the following conditions: activity buffer, different concentrations of lactose (50 g L^{-1} and 400 g L^{-1}), D-glucose (200 g L^{-1}), D-galactose (200 g L^{-1}), or D-glucose plus D-galactose (200 g L^{-1} of each sugars). Samples were withdrawn at different time intervals and placed in ice bath to stop the thermal inactivation instantaneously. The remaining enzyme activity was determined as described above.

Packed-bed reactor setup

The column type packed-bed reactor (12 × 3 cm) was composed of a water-jacketed glass column, flow-rate controller, and water bath. The reactor was packed with 4 g (dry weight) of chitosan-immobilized *β*-D-galactosidase 2 mm diameter macroparticles, corresponding to approximately 920 U of *β*-D-galactosidase total activity. The substrate solution was fed through the bottom of the column using a peristaltic pump. The topside and the bottom of the column were fitted with a sintered glass disc (4 mm thick). The total volume of the packed-bed reactor was 29 mL.

Lactose hydrolysis

The hydrolysis of lactose was performed by flowing through the reactor either buffered lactose solution or whey, both containing 50 g L^{-1} of sugar. The lactose solution was prepared in activity buffer, while the whey solution was prepared by suspension of whey powder in distilled water and adjusting the pH to 7. Previously to starting the reaction, the packed-bed reactor was washed with activity buffer. Flow rates were tested from 0.26 mL min^{-1} to 3.4 mL min^{-1}. Samples were collected after the steady state was reached and analyzed for D-glucose formation. Previously to the D-glucose quantification, samples of hydrolyzed whey were diluted and filtered through a 0.22 µm acetate cellulose membrane in order to remove proteins that may cause interference in the analysis. The hydrolysis was carried out at two different temperatures, 37 and 7 ºC, in order to simulate real industrial conditions and to avoid possible microbial contaminations.

Continuous synthesis of galactooligosaccharides (GOS)

GOS synthesis was performed by flowing through the reactor buffered lactose solution at high concentration (400 g L^{-1}). Flow rates were tested from 1 to 15 mL min^{-1} for GOS synthesis at 37 ºC. Samples were collected after steady state was reached and the reaction product was analyzed by HPLC.

Analytical procedures

Lactose and products from the transgalactosylation reaction (GOS, D-galactose and D-glucose) were analyzed by HPLC (Shimadzu, Tokyo, Japan) equipped with refractor index and Aminex HPX-87C (300 × 7.8 mm) column. Ultra-pure water was used as eluting solvent at a flow rate of 0.6 mL min^{-1}, at 85 ºC. The concentration of saccharides

was calculated by interpolation from external standards. Standards were used for lactose, D-glucose, and D-galactose. GOS concentration was calculated as raffinose equivalents from an external raffinose standard, as described by Gosling et. al. (2011). The commercial product Vivinal GOS® (Friesland Foods, Netherlands) was used to compare the retention time of the obtained GOS, and its yield (%) was defined as the percentage of GOS produced compared with the weight of initial lactose in the reaction medium.

Operational stability of the packed-bed reactor during lactose hydrolysis

The operational stability of the PBR under steady-state regime was evaluated during 15 continuous days at 37 ºC using a flow rate of 2.6 mL min^{-1} containing 50 g L^{-1} of lactose. Aliquots were periodically collected and analyzed for D-glucose formation.

RESULTS AND DISCUSSION

Properties of the immobilized β-D-galactosidase
Optima pH and temperature

The effect of pH on the relative activity of the free and immobilized *β*-D-galactosidase was evaluated in the range of 5.7 to 8.0 (Fig. 1 A). After immobilization on chitosan macroparticles, the optimum pH of *β*-D-galactosidase was enhanced from 6.5 to a broader range between 6.5 and 8.0. Even at acidic pH, the enzyme activity was remarkably enhanced, with more than 70 % remaining at pH 5.5, compared to less than 25 % for the free form, suggesting the strong stabilization effect on enzyme molecules, provided by the covalent binding to the support. Bayramoglu et. al. (2007), in their studies with *E. coli β*-D-galactosidase immobilized onto magnetic poly(GMA–MMA) beads, found a shift of optimum pH of the enzyme from 7.5 to 7 after immobilization. The authors reported that this effect was probably due to the basic nature of the amino functionalized surface of the magnetic beads. Similarly, *β*-D-galactosidase from *K. lactis* immobilized by adsorption on a mixed-matrix membrane containing zirconium dioxide presented a shift in the optimum pH from 6.5 to 7 (Jochems et al., 2011). The authors suggested that this change was due to the microenvironment in the vicinity of the membrane. The support, indeed, can change the pH value around the enzyme catalytic site by changing

the concentration of the charged species (e.g. hydrogen and hydroxyl ions) in respect to the bulk solution (De Maio et al., 2003).

Changes in activity related to temperature of immobilized *β*-D-galactosidase were investigated in the range of 10 to 70 ºC (Fig. 1 B). The free and immobilized forms presented maximum activities at 45 ºC, while the immobilized enzyme was more active in a wider range of temperatures. Similarly to the changes in pH, the wider range of temperatures with higher activities can be attributed to the effect of immobilization, the protection offered by the chitosan macroparticles to the enzyme. Song et. al. (2010) reported similar results and a broader range of activity was observed for pH and temperature after immobilization of *K. lactis β*-D-galactosidase pretreated with lactose on the surface of silica gel using glutaraldehyde as linker. According to Pereira-Rodríguez et. al. (2012), the tetramer of *β*-D-galactosidase from *K. lactis* is an assembly of dimers, with higher dissociation energy for the dimers than for its tetrameric structure, causing an equilibrium between the dimeric and tetrameric forms of the enzyme when in solution. The binding of the enzyme to the support can prevent the dissociation of subunits of either forms (Fernandez-Lafuente, 2009), consequently causing it structure to be stabilized. Although immobilized enzymes are heterogeneous catalysts, the widened range for pH and temperature can configure another advantage of using immobilized enzymes over the use of their free forms by allowing their applications under different conditions, improving industrial uses.

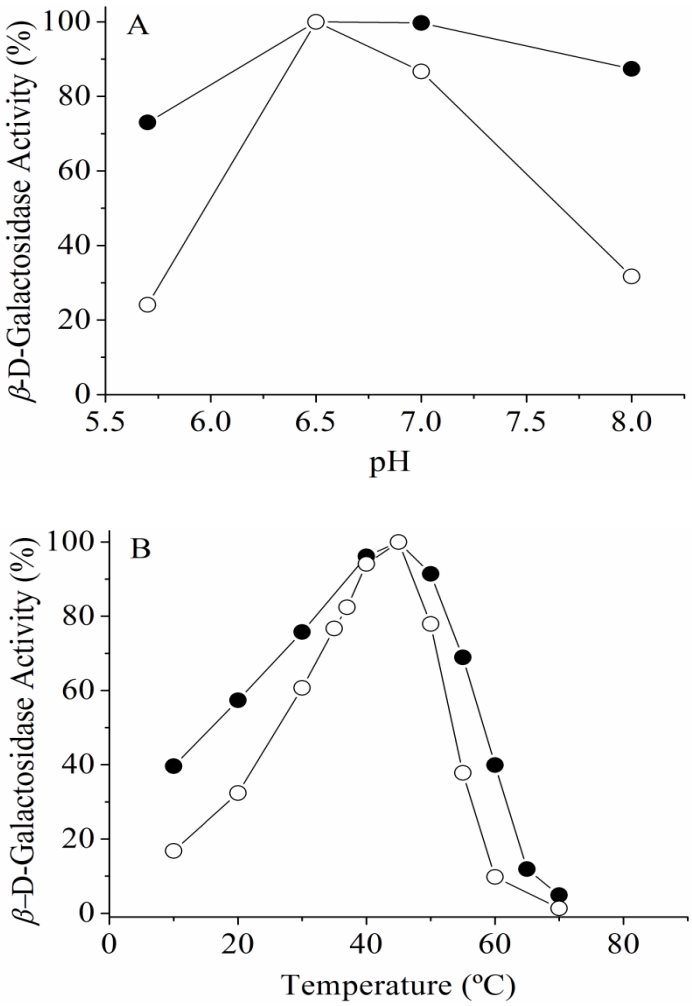

Figure 1. Effect of pH (A) and temperature (B) on the activity of free *β*-D-galactosidase (○) and chitosan-immobilized *β*-D-galactosidase (●).

Thermal stability

Thermal inactivation is an important, limiting factor for prolonged use of enzymes in industrial processes. Inactivation in the presence of substrate and products (reactive conditions) should be evaluated, since this reflects the reaction in an enzyme reactor. Fig. 2 depicts the thermal stability of chitosan-immobilized *β*-D-galactosidase

under different reactive conditions (substrate type and concentration) at 55 ºC.

Activity of the immobilized enzyme under nonreactive conditions (in activity buffer) was approximately 43 % after 5 min of incubation at 55 ºC, which is consistent with the fact that *K. lactis* β-D-galactosidase is relatively thermolabile. According to Jurado et. al. (2004), experiments conducted at 30 ºC and 35 ºC, it was found that this β-D-galactosidase preserved practically all its activity after 3 h of reaction, while at higher temperatures it was virtually deactivated.

In order to assay operational conditions, it was evaluated the thermal stability of the immobilized enzyme in the presence of lactose 50 g L^{-1}, which is the average concentration of lactose present in milk and whey. In the same way, thermal stability test was carried out in presence of whey and lactose 400 g L^{-1} (the concentration used in the GOS synthesis). The results showed that in 50 g L^{-1} of lactose the enzyme retained around 51 % of its activity after 15 min at 55 ºC, while in 400 g L^{-1} the activity was considerably improved (100 % after 15 min and approximately 55.8 % after 8 h of incubation) at the same temperature, suggesting that lactose stabilizes the enzyme. According to Jurado et. al. (2004) and Ladero et. al (2006), lactose (and possibly D-galactose) can stabilize the overall structure of the enzyme by the coupling of the substrate into the active site.

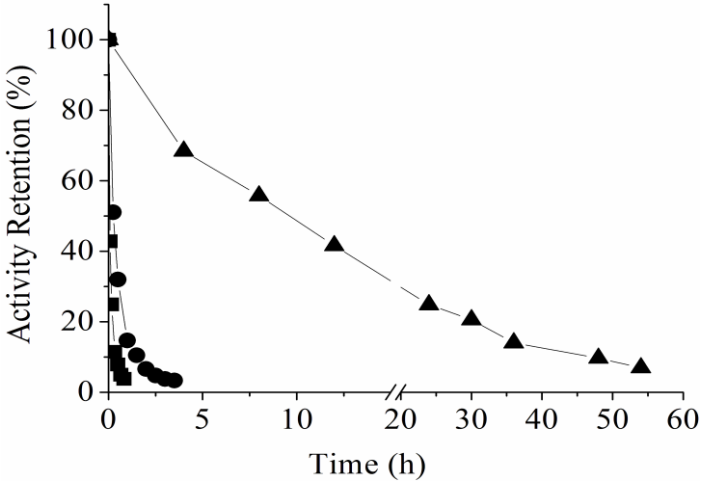

Figure 2. Thermal stability at 55 ºC of chitosan-immobilized β-D-galactosidase in the presence of 400 g L^{-1} lactose solutions (▲), 50 g L^{-1} lactose solutions (●), and activity buffer (■).

The products generated during the reaction can also play some important role in the enzyme stability. Binding reaction products and other ligands to a protein is a simple way to stabilize its conformation, which is a widely used strategy for stabilizing proteins and enzymes during various operations including enzyme purification and enzyme immobilization (Illanes et al., 1998).

Then, in an effort to accurately investigate the effect of substrate and products in the enzyme stability, the thermal stability in the presence of products (D-glucose and D-galactose) was analyzed. As can be seen in the Fig. 3, when only D-glucose (200 g L^{-1}) was present, the chitosan-immobilized *β*-D-galactosidase presented around 50 % of its activity after 11 min of incubation; the stability was not significantly increased compared to the inactivation under nonreactive conditions (4.8 min). For *K. lactis β*-D-galactosidase, D-glucose is known to be a non-competitive inhibitor (K_i^{gli} = 794 mM) (Cavaille & Combes, 1995), which means that lactose and D-glucose will independently bind at different sites of the enzyme.

When inactivation was performed in presence of D-galactose (200 g L^{-1}), the *β*-D-galactosidase residual activity was about 47 % after 30 min of incubation, which was higher than that obtained when D-glucose was used in the same concentration. D-Galactose is a competitive inhibitor for this enzyme (K_i^{gal} = 42 mM) (Cavaille & Combes, 1995) and has a much higher inhibitory power than D-glucose. Since the only difference between D-glucose and D-galactose lies on the binding site to the protein, it can be suggested that the protector effect promoted by D-galactose comes from the binding of this sugar to the active site of the enzyme. It was recently reported the three-dimensional structure of *K. lactis β*-galactosidase and the complex structure of the molecule when D-galactose is at the active site, showing that a tryptophan residue, responsible for the binding of D-glucose in the active site of *β*-galactosidase from *E. coli*, is missing from the active site of *K. lactis β*-galactosidase (Pereira-Rodríguez et al., 2012).

When inactivation was carried out in the presence of D-glucose and D-galactose, both at 200 g L^{-1}, the enzyme stability was improved with 47 % of activity still remaining after 180 min of incubation at 55 ^{o}C, suggesting the stabilizing effect of osmolytes such as sugars, amino acids, and trehalose (Sampedro et al., 2001). Stabilization would be achieved by an increase in the surface tension of the solution, causing the exclusion of the saccharides from the protein domain and reducing backbone movements away from the fully folded protein form

(Bromberg et al., 2008; Butler & Falke, 1996; Lin & Timasheff, 1996; Sampedro et al., 2001). The stabilizing effect also depends on the type of carbohydrate used (Sola-Penna & Meyer-Fernandes, 1998).

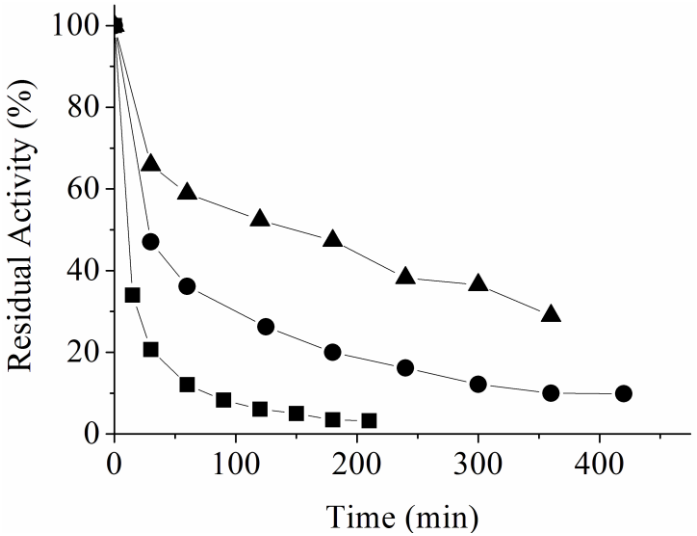

Figure 3. Thermal stability at 55 ºC of chitosan-immobilized *β*-D-galactosidase in the presence of mixture of D-glucose and D-galactose (each 200 g L^{-1}) (▲), 200 g L^{-1} D-galactose solution (●), and 200 g L^{-1} D-glucose solution (■).

The analyzes of the sugars during inactivation in the presence of lactose 400 g L^{-1} showed that lactose was present during all experiment, with the enzyme probably constantly hydrolyzing lactose up to the moment that it was inhibited by the high concentration of produced D-galactose. After 54 h of inactivation at 55 ºC, 63 g L^{-1} of lactose still remained.

Effect of flow rate on lactose hydrolysis in a packed-bed reactor

Since lactose proved to have the major protecting effect on this *β*-D-galactosidase, operations under conditions in which the enzyme is constantly catalyzing lactose hydrolysis and avoiding product inhibition could be interesting to test. The use of PBRs for lactose hydrolysis and GOS synthesis could be advantageous over batch operations. Substrates (lactose solution or whey) were continuously pumped at different flow rates (0.26 to 3.4 mL min^{-1}) through the PBR. Fig. 4 shows the lactose

conversion at 37 ºC and 7 ºC for whey and lactose solution at various flow rates.

At 37 ºC, for both lactose solution (50 g L^{-1}) and whey, more than 90 % of lactose hydrolysis was reached at a flow rate of 2.6 mL min^{-1} (residence time, 11.3 min). Ansari and Husain (2010), reported 95 % of lactose hydrolysis during a 10-days steady-state operation of a PBR filled with *β*-D-galactosidase adsorbed on concanavalin A-cellulose. However, their operation was conducted at slow flow rate of 0.166 mL min^{-1} A pilot scale module (108 cm × 14 cm), packed with *K. lactis β*-D-galactosidase immobilized on cotton fabric, resulted in 30.23 % hydrolysis of lactose from milk, at 37 ºC and residence time of 11.8 min (Li et al., 2007).

Increasing the substrate flow rate to 3.4 mL min^{-1} caused the lactose conversion to drop to 86 and 80 % for lactose solutions (50 g L^{-1}) and whey, respectively. This result can be explained by the short residence time of the substrate inside the reactor.

In order to simulate industrial conditions and reduce the possibility of microbial contamination, lactose hydrolysis was also carried out at 7 ºC. As expect, the degree of lactose hydrolysis was reduced for all flow rates when compared with the reaction at 37 ºC. For lactose solution (50 g L^{-1}), approximately 90 % of lactose was hydrolyzed at a flow rate of 0.26 mL min^{-1}, while for whey 72 % of hydrolysis was achieved under same conditions. It is possible that, at 7 ºC, the difficulty of mass transfer caused by whey proteins are more pronounced than at 37 ºC, which explain the lower lactose conversion observed.

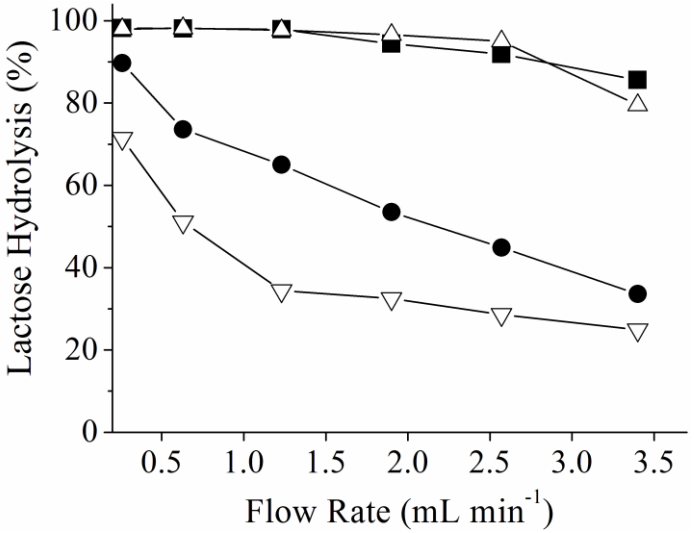

Figure 4. Effect of the flow rate on the lactose hydrolysis by chitosan-immobilized β-D-galactosidase under different conditions: whey at 37 ºC (△), 50 g L^{-1} buffered lactose solution at 37 ºC (■), whey at 7 ºC (▽), and 50 g L^{-1} buffered lactose solution at 7 ºC (●).

Continuous synthesis of galactooligosaccharides

Fig. 5 shows the changes in the concentration of D-glucose, D-galactose, lactose, and GOS as a function of the substrate flow rate, under steady state operation. The maximum GOS concentration of 26 g L^{-1} was achieved using a substrate flow rate of 3.1 mL min^{-1} (residence time of 9.4 min), corresponding to a yield of 6.5 % in GOS and 58 % of lactose conversion. At lower flow rates (1 to 2.6 mL min^{-1}), lower concentrations of GOS were obtained, probably due to the hydrolysis of the synthesized GOS, because the longer residence times (11 to 29 min) allows for its subsequent hydrolysis by the enzyme. Higher than 3.1 mL min^{-1} flow rates (5 to 15 mL min^{-1}) causes the residence time to be insufficient for best synthesis, reducing GOS concentration.

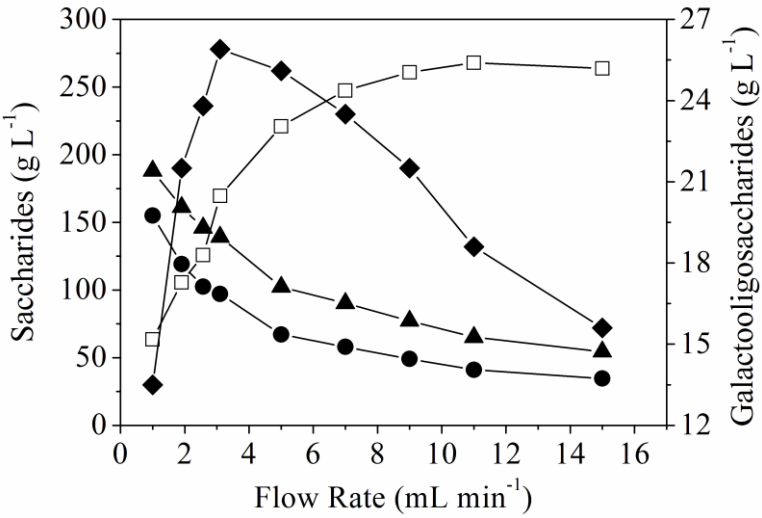

Figure 5. Effect of flow rate on GOS synthesis by chitosan-immobilized *β*-D-galactosidase, using 400 g L^{-1} buffered lactose solution, pH 7 at 37 ºC. D-glucose (▲), D-galactose (●), lactose (□), and galactooligosaccharides (◆).

Comparatively, Nakkharat and Haltrich (2007) reported 16 g L^{-1} of GOS in a packed-bed reactor containing *β*-D-galactosidase from *Talaromyces thermophilus* immobilized on Eupergit C, with 50 % lactose conversion and 200 g L^{-1} of initial lactose concentration. Neri et. al. (2009), worked with *β*-D-galactosidase from *A. oryzae* immobilized on magnetic polysiloxane-polyvinyl alcohol, obtaining 103.4 g L^{-1} of GOS, with lactose conversion of 47 % and 500 g L^{-1} of lactose, in a batch reaction. Although *K. lactis β*-D-galactosidase is known to show low transgalactosylation activity and thermal stability during the production of galactooligosaccharides compared to *A. oryzae* enzyme (Park & Oh, 2010), when applied in a PBR, the immobilized enzyme was able to work continuously, with high productivities of galactooligosaccharides.

The GOS productivity in the PBR related to the operational flow rate is shown in Fig. 6. It increased to a maximum of 484.5 g L^{-1} h^{-1} at 15 mL min^{-1}. Comparatively, Shin et. al. (1998) reported oligosaccharide productivity of 4.4 g L^{-1} h^{-1} with lactose solutions of 100 g L^{-1} in PBR with chitosan-immobilized *Bullera singularis β*-D-galactosidase (970 U g^{-1} resin). The higher GOS productivity so far reported, 6,000 g L^{-1} h^{-1}, was obtained by Albayrak and Yang (2002) using *A. oryzae β*-D-galactosidase immobilized on cotton cloth by

polyethyleneimine and applied in a PBR fed with lactose (400 g L^{-1}) at 2.5 mL min^{-1}.

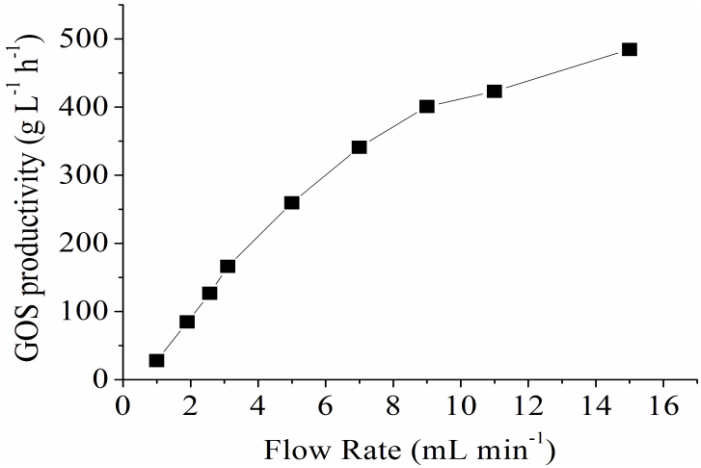

Figure 6. Effect of flow rate on GOS productivity. Experiments were performed using 400 g L^{-1} buffered lactose solution, pH 7 at 37 ºC.

Stability of immobilized β-D-galactosidase in the packed-bed reactor

The operational stability of a system is an important parameter in an industrial process, since it directly affects the costs (Nie et al., 2006). Fig. 7 shows the operational stability of the immobilized enzyme in the PBR. The reactor was operated continuously at 37 ºC at a flow rate of 2.6 mL min^{-1}, mantaining 90 % of lactose hydrolysis for longer than 15 days.

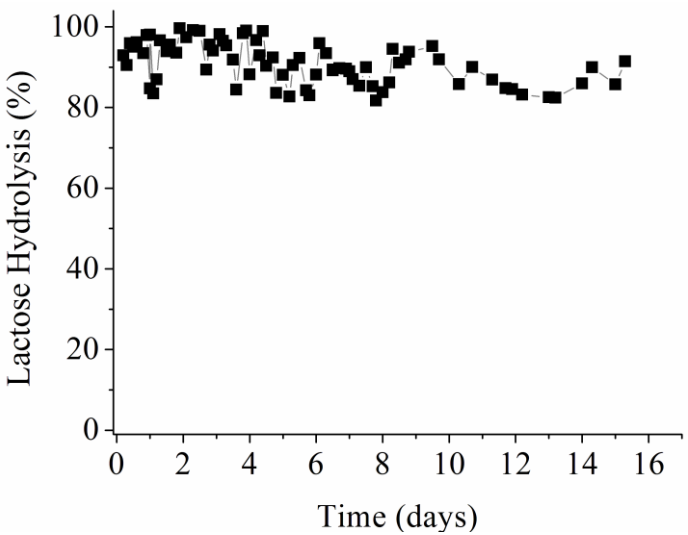

Fig. 7. Operational stability of the PBR filled with chitosan-immobilized *β*-D-galactosidase, operated continuously using 50 g L^{-1} buffered lactose solution, pH 7 at 37 ºC and flow rate of 2.6 mL min^{-1}.

CONCLUSIONS

One of the main objectives in immobilized enzyme technology is to increase the enzyme stability, thus allowing the obtained derivative to be repeatedly used, inclusive at different process conditions, such as in continuous reactors. The use of PBRs for lactose hydrolysis and GOS synthesis may replace batch reactors, with several cost reductions and operation advantages, including reduced reaction inhibition by substrate and products. In the present study, using chitosan macroparticles, a relatively low cost and easily accessible support, improvements in the operational range of pH and temperature of the enzyme were observed as a consequence of the immobilization process. Furthermore, for the first time it was clearly shown that, the combination of continuous flow with a high content of lactose can sharply increase the stability of *K. lactis β*-D-galactosidase. Thus, Maxilact LX 5000, which is generally used for lactose hydrolysis as a free enzyme, could be advantageously employed in its covalent immobilized form to the hydrolysis of lactose and production of GOS in a continuous PBR.

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CAPÍTULO 4: GENIPIN-CROSSLINKED CHITOSAN FOR *β*-GALACTOSIDASE IMMOBILIZATION: SUPPORT CHARACTERIZATION AND APPLICATION ON DAIRY PROCESSES

Neste trabalho foi avaliada a possibilidade de utilizar a genipina, um reagente natural e seguro de entrecruzamento, para imobilizar a enzima *β*-galactosidase de *Aspergillus oryzae* em macropartículas de quitosana e posterior aplicação na hidrólise da lactose e na síntese de GOS. O efeito da imobilização na atividade da enzima, na estabilidade térmica e na estabilidade operacional foi avaliado e comparado com a metodologia tradicional de imobilização com glutaraldeído. Os resultados estão apresentados no manuscrito a seguir, a ser submetido para publicação na revista *Carbohydrate Polymers*.

Genipin-crosslinked chitosan for *β*-galactosidase immobilization: support characterization and application on dairy processes

ABSTRACT: In order to develop safer process for the dairy industry, we prepared a chitosan support with the naturally occurring crosslinking reagent, genipin, for the immobilization of the *Aspergillus oryzae β*-galactosidase. Chitosan particles were obtained by precipitation followed by adsorption of the enzyme and crosslinking with genipin. The particles were characterized by fourier transform infrared (FTIR) spectroscopy and thermogravimetric analysis (TGA). The immobilization of the enzyme by crosslinking with genipin rendered biocatalysts with satisfactory activity retention and thermal stability, comparable with the ones obtained with the traditional methodology of immobilization using glutaraldehyde. Optimization of galactooligosaccharides synthesis, in terms of initial lactose concentration, pH and temperature, were performed and yields of 30 % were achieved. Moreover, excellent operational stability was obtained, since the immobilized enzyme maintained 100 % of its initial activity after 25 batches of lactose hydrolysis.

Keywords: Immobilization, Genipin, Chitosan, *β*-Galactosidase, Lactose Hydrolysis, Galactooligosaccharides.

INTRODUCTION

Thanks to advances in biotechnology it is now possible to produce most enzymes for commercially acceptable prices and to manipulate them such that they exhibit the desired properties (Bornscheuer et al., 2012; Burton et al., 2002; Sheldon & van Pelt, 2013). Various methods including protein engineering, medium engineering of biocatalysts and immobilization can provide suitable enzyme stability, which is often the limiting factor in most bioprocesses (de Barros et al., 2010). Immobilization of enzymes is a relatively simple methodology and offers many benefits, for example: efficient reuse of the enzyme, continuous operation, enhanced stability, under both storage and operational conditions, facile separation from the product, thereby minimizing or eliminating protein contamination of the product, low or no allergenicity, since an immobilized enzyme cannot easily penetrate the skin, among others (Sheldon & van Pelt, 2013).

Beyond kinetic stability, industrial application also requires a biocatalyst with mechanical stability and safety, the latter being essential in food and pharmaceutical industries. As a support for enzyme immobilization, chitosan offers a number of desirable characteristics including nontoxicity, biocompatibility, physiological inertness, biodegradability to harmless products and remarkable affinity to proteins. Commercially, chitosan is obtained from chitin (the major structural component of the exoskeleton of invertebrates and the cell walls of fungi), subjected to *N*-deacetylation by treatment with a 40–45 % NaOH solution, followed by purification procedures. Both the solubility in acidic solutions and aggregation with polyanions impart chitosan with excellent gel-forming properties (Krajewska, 2004). Moreover, mechanical properties of supports obtained from chitosan can be easily improved by crosslinking with glutaraldehyde, genipin and other reagents (CauichRodriguez et al., 1996; Muzzarelli, 2009).

Currently, genipin can be obtained from the fruits of *Genipa americana* and *Gardenia jasminoides* Ellis. After extraction, the geniposide is hydrolyzed into the aglycone genipin with *β*-glucosidase in a microbiological process involving *Penicillium nigricans* (Butler et al., 2003; Muzzarelli, 2009). Genipin has been widely used in herbal medicine (Akao et al., 1994), and the dark blue pigments obtained by its spontaneous reaction with amino acids or proteins have been used in the fabrication of food dyes (Paik et al., 2001). Moreover, it was reported that genipin might be about 5000–10000 times less cytotoxic than glutaraldehyde (Sung et al., 1999).

Is notorious the importance of *β*-galactosidases for dairy industries. This enzyme catalyzes the hydrolysis of lactose into glucose and galactose, allowing the consumption of dairy products by lactose intolerant people. Moreover, in the presence of concentrated lactose, this enzyme can transfer the *β*-galactosyl moiety from lactose hydrolysis to another lactose molecule, thus synthesizing galactooligosaccharides (GOS), an important prebiotic food ingredient, naturally present in human milk (Grosova et al., 2008).

In a recent work (Klein et al., 2012), the *Kluyveromyces lactis* *β*-galactosidase was successfully immobilized on chitosan particles activated by glutaraldehyde, resulting in a biocatalyst with high thermal and operational stability. Given the satisfactory results presented on chitosan as support for *β*-galactosidase immobilization, and the importance of the improvement of bioprocess from a safety point of view, we are prompted to evaluate the possibility of using the naturally occurring crosslinking reagent genipin to immobilize the enzyme *β*-galactosidase from *Aspergillus oryzae* on chitosan particles for further application on dairy industries. The effect of the immobilization approach on the activity retention, thermal stability, operational stability, and on galactooligosaccharides synthesis was verified and compared with the traditional methodology of immobilization with glutaraldehyde. Besides, the physicochemical characteristics of the biocatalyst obtained were determined.

MATERIALS AND METHODS

Materials

Aspergillus oryzae *β*-galactosidase, genipin, chitosan (from shrimp shells, ≥75% deacetylated), *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG), glucose, galactose, lactose, raffinose and stachyose were obtained from Sigma–Aldrich (St. Louis, USA). A glucose determination kit was purchased from Labtest Diagnóstica SA (São Paulo, Brazil). All solvents and other chemicals were of analytical grade.

Methods

Preparation of β-galactosidase immobilized on genipin-crosslinked chitosan particles

Chitosan particles (CS) were prepared by the precipitation method as described in a previous work (Klein et al., 2012). Then, 100

chitosan particles were incubated with *β*-galactosidase solution (2 mL, 20 U mL^{-1}) prepared in 0.02 M of sodium phosphate buffer (pH 7.0), during 8 h at room temperature, under gentle stirring. Crosslinking with genipin was performed according to Wang and co-workers (2011), with some modifications: 500 µL of 0.5 % w/v genipin solution (phosphate buffer pH 7, 0.02 M) was added to the above solution and it was allowed to react during 15 h at room temperature, under gentle stirring. After crosslinking, successive washings with 0.1 M acetate buffer (pH 4.5), NaCl (1 M) and ethylene glycol (30 % v/v) eliminate ionic and hydrophobic interactions between enzyme and support.

Chitosan particles with adsorbed *β*-galactosidase followed by glutaraldehyde crosslinking (CS-GLU) were prepared in order to compare the effect of different crosslinking agents (genipin and glutaraldehyde) on the properties of the immobilized *β*-galactosidase. In this case, the genipin solution was replaced by 100 µL of glutaraldehyde (25 %), and it was allowed to react during 1 h at room temperature. Chitosan particles previously activated with glutaraldehyde (CS-aGLU), followed by *β*-galactosidase immobilization, were also prepared according to a previous report (Klein et al., 2012).

Characterization of genipin-crosslinked chitosan particles

Changes on the molecular structure of chitosan particles were determined before and after genipin crosslinking by Fourier transform infrared (FTIR) spectroscopy with a Varian 640-IR spectrometer. Samples previously lyophilized were crushed and thoroughly mixed with powdered KBr and then pressed to form a transparent pellet (1% w/w). The spectra were obtained at room temperature with 40 accumulative scans and 4 cm^{-1} of resolution. The thermogravimetric analysis (TGA) was performed using a Shimadzu thermal analyzer Model TA50, at a heating rate of 10 °C min^{-1}, from room temperature up to 600 °C under argon atmosphere.

Activity assay of β-galactosidase

β-Galactosidase activity was determined using *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) as substrate. For the free enzyme the measurements were performed in 0.5 mL of acetate buffer containing 15 mM ONPG (pH 4.5) and an adequate amount of free enzyme. After incubation (40 °C for 2 min), the reaction was stopped by adding 1.5 mL of 0.1 M sodium carbonate buffer (pH 10) and the absorbance was measured at 415 nm. The above quantities were doubled for measurements with the immobilized enzyme. One unit (U) of *β*-

galactosidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol per min at the defined assay conditions.

The activity adsorbed was calculated from the difference between the applied and recovered activities in the supernatant before and after adsorption. The adsorption yield (AY) and the activity retention (AR) were calculated according to the following equations:

$$AY(\%) = \frac{\text{Adsorbed Activity}}{\text{Applied Activity}} x100 \quad (1)$$

$$AR(\%) = \frac{\text{Support Activity}}{\text{Adsorbed Activity}} x100 \quad (2)$$

Optima pH and temperature for free and immobilized β-galactosidase

The optimum pH of *β*-galactosidase activity was studied by monitoring enzyme activity of both free and immobilized *β*-galactosidase in different buffers, at 40 ºC: 0.05 M glycine-HCl (pH 2.3-3), 0.1 M Na-acetate (pH 4.0-5.5), 0.1 M Na-phosphate (pH 6.0-7.0) and 0.1 M Tris-HCl (pH 8.0). The optimum temperature was determined by measuring the activity between 20 ºC and 75 ºC at pH 4.5.

Thermal stability of the immobilized β-galactosidase

For thermal stability studies, the immobilized enzyme was incubated in sealed tubes, in thermostatically controlled water bath at 60 ºC. Thermal stability was performed in 0.1 M acetate buffer (pH 4.5) and in the presence of 40 % (w/v) buffered lactose solution (acetate buffer, pH 4.5). At defined time intervals, the immobilized enzyme was withdrawn, chilled immediately and tested for enzyme activity using routine assay.

Operational stability of immobilized β-galactosidase in the lactose hydrolysis

Lactose hydrolysis in batch was performed with *β*-galactosidase immobilized on genipin-crosslinked chitosan macroparticles (2.4 U mL^{-1}) incubated in Erlenmeyer flasks containing 5% (w/v) of buffered lactose solution (acetate buffer, pH 4.5). Samples were withdrawn periodically and analyzed enzymatically for glucose formation. After its first use, the immobilized enzyme was incubated repeatedly in the same

conditions described above to evaluate its operational stability in the successive hydrolysis batches.

Galactooligosaccharides synthesis

Synthesis of galactooligosaccharides from lactose was studied with the immobilized enzyme in different conditions. The reaction kinetics was studied at three different initial lactose concentrations (30, 40 and 50 % w/v), three different pH values (5.25, 6 and 7), and three different temperatures (40, 47.5 and 55 ºC). The lactose solution was prepared by dissolving it in the adequate buffer solution. Samples were taken at appropriate time intervals to obtain the complete reaction profile, filtered using 0.22 µm cellulose acetate membranes, diluted and analyzed for sugar content by high performance liquid chromatography (HPLC).

Analytical procedures

Lactose and products from the transgalactosylation reaction (GOS, galactose and glucose) were analyzed by HPLC (Shimadzu, Tokyo, Japan) equipped with refractor index and Aminex HPX-87C (300 mm x 7.8 mm) column. Ultra-pure water was used as eluting solvent at a flow rate of 0.6 mL min^{-1}, at 85 ºC. The concentration of saccharides was calculated by interpolation from external standards. Standards were used for lactose, glucose, and galactose. GOS concentration was calculated as raffinose and stachyose equivalents from external raffinose and stachyose standards, respectively, as described by Gosling (2011). The yield (%) of GOS synthesis was defined as the percentage of GOS produced compared with the weight of initial lactose in the reaction medium.

RESULTS AND DISCUSSION

Characterization of chitosan particles

Figure 1 shows the chitosan particles without crosslinking (CS, translucent white particles), crosslinked with glutaraldehyde (CS-GLU, yellow particles) and with genipin (CS-GEN, dark blue particles). The obtained particles have a regular round shape with an average diameter of 2 mm. After crosslinking with genipin, the particles turned dark blue, due to oxygen radical-induced polymerization of genipin (Bi et al., 2011). They showed to be resistant to acids pHs solutions, unlike the non-crosslinked chitosan. Moreover, no swelling effects were observed in the CS-GEN particles prepared in this work during more than 4 months of refrigerated storage at pH 4.5. It was reported that the

numerous interchain interactions formed by crosslinking inhibit swelling, since most of the amino groups of chitosan must have reacted with the crosslinker (Berger et al., 2004). Indeed, the lower swelling ability of chitosan gel is attributed to the increased intermolecular or intramolecular linkage of the $–NH_2$ sites in chitosan, which could be achieved by a more complete crosslinking reaction (Mi et al., 2001). Swelling at low pHs values can be observed with the incorporation of an additional polymer that should perturb covalent crosslinking between chitosan chains, hence, decreasing crosslinking density and making available more protonable amino groups (Berger et al., 2004). Bigi (2002) reported that the degree of swelling of gelatin films treated with genipin, at pH 7.4 during 24 h, decreases on increasing genipin concentration. In the same way, Chiono and co-workers (2008) obtained genipin crosslinked chitosan/gelatin blends for biomedical applications using different amounts of genipin as crosslinker. It was found that stiffness and crosslinking degree increased with increasing genipin concentration. In turn, genipin crosslinking reduced wettability, dissolution and swelling degree of gelatin and blend samples.

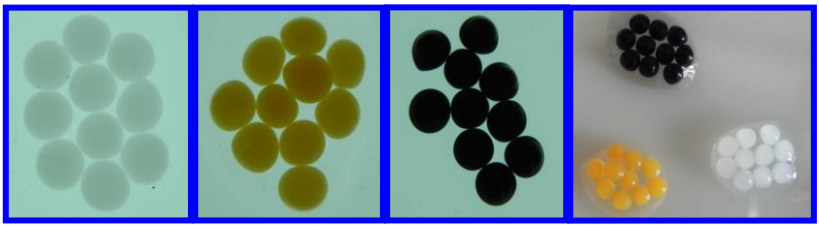

Figure 1: CS particles (~2 mm; translucent white particles), CS particles crosslinked with glutaraldehyde (yellow particles) and CS particles crosslinked with genipin (dark blue particles). The pictures were made using a Nikon Coolpix P100, 10.3 megapixel camera.

Spectra of chitosan particles with and without crosslinking with genipin are presented in Figure 2. The spectrum of CS (c) shows absorptions at 1650 cm^{-1} and 1585 cm^{-1}, characteristics of N-H bending vibrations of primary amines (Lambert, 1987) present on chitosan structure. The peak at 1376 cm^{-1} was attributed to −C−O−H stretching of a primary alcoholic group in chitosan. The absorption bands between 1000 and 1100 cm^{-1} were attributed to C-O and C-N stretching vibrations, and C-C-N bending vibrations (Lambert, 1987). The three spectra show a broad band between 3000 and 3600 cm^{-1} that was

attributed to the O−H stretching vibration, mainly from water, which probably overlaps the amine stretching vibrations (N−H) in the same region (Lambert, 1987), and the bands between 2800 and 3000 cm^{-1} were attributed to the C-H stretching vibration (Colthup, 1975).

The crosslinking of genipin with chitosan involves a fastest reaction that is the nucleophilic attack by the amino group of chitosan on the olefinic carbon atom of genipin which results in the opening of the dihydropyran ring and the formation of a tertiary amine, i.e. a genipin derivative linked to a glucosamine unit. The subsequent slower reaction is the formation of amide through the reaction of the amino group on chitosan with the ester group of genipin (Mi et al., 2001). At the same time, polymerization can take place between genipin molecules already linked to amino groups of chitosan, which could lead to the crosslinking of amino groups by short genipin copolymers (Butler et al., 2003; Muzzarelli, 2009).

Then, after crosslinking with genipin, the amide II band at 1546 cm^{-1}, characteristic of N-H deformation (Lambert, 1987), is probably due to the formation of secondary amides as a result of the reaction between the genipin ester and hydroxyl groups and the chitosan amino groups. The peak at 1633 cm^{-1} were attributed to C=O stretch in secondary amides (Lambert, 1987). Furthermore, the increase observed in the peaks at around 1400 cm^{-1} and 1000 cm^{-1} can be assigned to absorptions from C-N stretching vibrations and C-OH stretching vibrations (Lambert, 1987), respectively, more numerous after crosslinking with genipin.

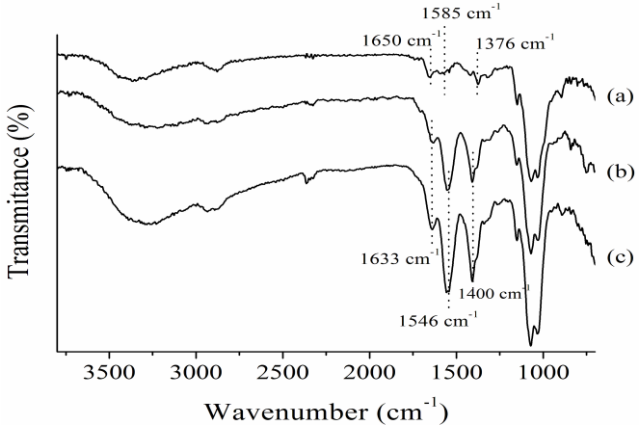

Figure 2. FTIR spectra of (a) CS, (b) CS-GEN and (c) CS-GEN with immobilized β-galactosidase.

As the enzyme was firstly adsorbed onto chitosan particles, it is quite probable that crosslinking with genipin occurred between amino groups of enzyme and chitosan, as well as between amino groups of different enzyme molecules and between different portions of the chitosan chain, being these interactions of covalent nature (Butler et al., 2003). Touyama and co-workers (1994) reported that only primary amines, rather than secondary or tertiary amines, can react with genipin. Moreover, in the work of Sung and co-workers (1998), it was found that the amino acid residues in the porcin pericardium that reacted with genipin are lysine, hydroxylysine, and arginine. Butler and co-workers (2003) reported lower ability of gel formation for BSA and gelatin as compared to chitosan due to the lower number of amine groups available for participating in the crosslinking reaction in these systems. It was also pointed out that for a globular protein such as BSA, the secondary and tertiary structures are also important because it may be assumed that the lysine or arginine residues involved in the crosslinking reaction must be near the outside surface of the molecule for them to be effective (Butler et al., 2003). Then, it could be inferred that the terminal amino and others free amino groups on the enzyme surface are the mainly involved in the reaction with genipin.

The spectra of CS-GEN with immobilized β-galactosidase showed no changes in comparison with the spectra of CS-GEN probably because the mechanisms involved in the crosslinking reaction in the presence of the enzyme are the same involved in the crosslinking of chitosan particles (CS). The increase in the intensity of characteristic bands is presumable due to the increase of amino groups available (from the adsorbed enzyme), which react with genipin, and, in turn, contributes to the increase of groups from crosslinking, as amide linkages.

The thermal stability of chitosan particles was measured using thermogravimetric analysis. The changes in sample weight with the increase of the temperature are shown in Figure 3 and Table 1. In all samples it was observed a weight loss up to 100 ºC due to elimination of adsorbed water. It can be seen that chitosan particles (CS) shows a lower weight loss in this region indicating lower hydrophilic character compared to the CS-GEN particles. It was also observed in Figure 3 that chitosan is thermally stable up to 270 ºC, and from 270 ºC up to 500 ºC, it showed a significant weight loss as can be seen in Table 1. This decomposition step can be assigned to the complex dehydration of the saccharide rings, depolymerization, and pyrolytic decomposition of the polysaccharide structure with vaporization and elimination of volatile

products (Penichecovas et al., 1993; Zohuriaan & Shokrolahi, 2004). However, for the CS-GEN particles and CS-GEN with immobilized enzyme it was observed a continuous weight loss from 100 ºC up to 270 ºC, being of 25.8 % and 30.8 %, respectively, indicating a lower thermal stability compared to CS. These high values for the weight loss at this range of temperatures can be ascribed to a possible weakening of part of the chitosan structure caused by the crosslinking with genipin. It is important to observe that the total weight loss increases for CS-GEN and CS-GEN with immobilized *β*-galactosidase, confirming the crosslinking and the enzyme immobilization, respectively. Moreover, TGA curves indicate that the obtained chitosan particles would be thermally stable at the temperature range used in most enzymatic reactions (up to 100 °C).

Table 1: Thermogravimetric analysis of chitosan particles.

	Weight loss (%)		
	CS	CS-GEN	CS-GEN-*β*-gal*
0-100 ºC	5.1	12.4	12.8
100-270 ºC	4.1	25.8	30.8
270-500 ºC	50.5	31.4	29.4
Total	59.7	69.6	73.3

*CS-GEN-*β*-gal: chitosan particles with adsorbed *β*-galactosidase crosslinked with genipin.

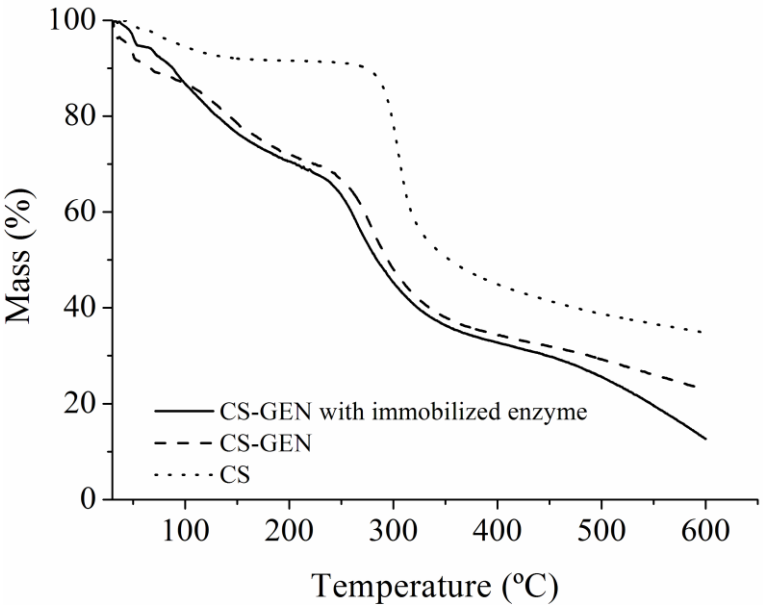

Figure 3. TGA curves of chitosan particles (CS), chitosan particles crosslinked with genipin (CS-GEN) and CS-GEN particles with immobilized *β*-galactosidase.

Enzyme immobilization

Crosslinking with glutaraldehyde involves its functional groups (−CHO), which simultaneously react with the binding sites of chitosan ($-NH_2$) and the amino terminal group from the enzyme, forming the Schiff's base (Chiou & Wu, 2004). On the other hand, crosslinking with genipin involves more complex reactions, as also explained above. Despite chemical reactions involved in the crosslinking with glutaraldehyde and genipin being distinct, the enzyme seems to have been affected in a similar way by both reagents, since values of activity retention (AR %) are similar when approximately the same load of enzyme activity was applied to the chitosan particles (Table 2). From these results, it can be concluded that both biocatalysts prepared by crosslinking presented no differences from a catalytic point of view. Fujikawa and co-workers (1988) reported slight differences using different crosslinking reagents, since 50 % and 63 % of activity effectiveness was found for *β*-glucosidase immobilized in alginate gel crosslinked with glutaraldehyde and genipin, respectively. On the other hand, Wang and co-workers (2011) reported very high activity

recoveries (98.67 % and 90.33 %) for lipase immobilized on two different mesoporous resins by crosslinking with genipin. The same authors pointed out that highest activity recoveries was achieved after 6 h of reaction, and longer crosslinking time gave the immobilized lipase a good strength, however leads to more loss of activity. Then, immobilization by crosslinking with genipin should be a compromise between adequate mechanical strength combined with relatively high enzyme activity.

Table 2: Adsorption yield and activity retention of *β*-galactosidase immobilized on chitosan particles using genipin and glutaraldehyde as crosslinking agents.

Sample	Applied Activity (U g^{-1}*)	Adsorbed Activity (U g^{-1})	Adsorption Yield (%)	Support Activity (U g^{-1})	Activity Retention (%)
CS-GEN	820	567	69	288	51
CS-GLU	865	615	71	301	49

* U g^{-1}: U per gram of dry support. Values are the mean between duplicate activity assays from two independent immobilization batches. Errors are lower than 8 %.

Optima pH and temperature

The effect of pH on the relative activity of the free and immobilized *β*-galactosidase was evaluated in the range of 2.3–8.0 (Figure 4A). The optimum pH for the free enzyme was found to be around 4.5-5.0, which agree with others works reporting the effect of pH on the activity of *β*-galactosidase from *A. oryzae* (Park et al., 1979; Tanaka et al., 1975). After immobilization on chitosan particles, the optimum pH shifted toward a more acidic region, being pH 4 considered the optimum for both, CS-GLU and CS-GEN. Moreover, the both immobilized enzymes showed to have higher activity also at pH 3, preserving more than 90 % of its activity, when compared to the free enzyme.

Generally, binding of the enzyme to a polycationic support would result in an acidic shift in the optimum pH (Goldstein et al., 1964). The pKa of the amino group of glucosamine residue on chitosan is about 6.3, hence chitosan is polycationic at acidic pH values, being extremely positively charged at pH 4.5 (Hwang & Damodaran, 1995;

Shahidi et al., 1999). Close to neutrality or at higher pHs, chitosan has free positive charges in smaller amounts, being neutralized if pH is too high (Berger et al., 2004). Then, it could be inferred that these positive free charges can influence in the changes of pH optimum observed after immobilization. Indeed, according to Chibata (1978), charged supports shift the enzyme activity/pH profile towards lower pHs when the concentration of hydroxyl ions in the immediate vicinity of the support surface is higher than in the bulk solution or towards higher pH values when the contrary occurs.

Figure 4B shows the effect of reaction temperature on the residual activities, in the range of 15-80 ºC, for free and immobilized *β*-galactosidase. The optimum temperature for free *A. oryzae β*-galactosidase was found to be around 55-60 ºC, assayed during 10 min. This result agrees with the findings of Park and co-workers (1979). After immobilization, the optimum temperature for the enzyme immobilized in both CS-GLU and CS-GEN was also found to be around 55-60 ºC, indicating that immobilization did not alter the optimum temperature of free *β*-galactosidase. In fact, the immobilized enzyme presents the same profile of residual activities above and below the optimum. Budriene and co-workers (2005) also reported no changes in the temperature profile after immobilization of *Penicillium canescens β*-galactosidase on chitosan.

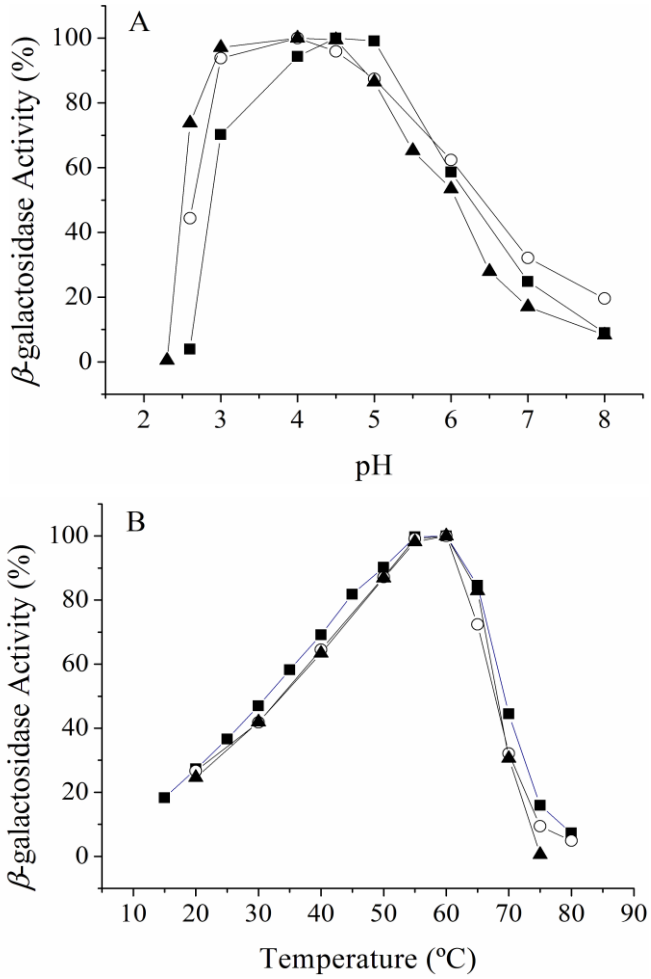

Figure 4: Effect of pH (A) and temperature (B) on the activity of free (!) and immobilized *β*-galactosidase on (–) CS-GLU and (7) CS-GEN.

Thermal stability

The thermal stability of immobilized *β*-galactosidase on CS-GEN, CS-GLU and CS-aGLU (*β*-galactosidase immobilized on the glutaraldehyde pre-activated chitosan particles) was determined by incubating each form in 0.1 M acetate buffer solution (pH 4.5) at 60 ºC. Moreover, in order to determine the thermal stability of CS-GEN immobilized *β*-galactosidase under operational conditions of

galactooligosaccharides synthesis, this biocatalyst was also incubated at 60 ºC in the presence of lactose buffer solution (40 % w/v lactose in 0.1 M acetate buffer pH 4.5). Figure 5 shows the residual activity of the different biocatalysts. After 60 min of incubation under non-reactive conditions, the immobilized enzyme on CS-GEN, CS-GLU and CS-aGLU presented 34 %, 44 % and 46 % of residual enzyme activity. It is noteworthy that all immobilized preparations were more stable than the free enzyme, which presents 16 % of residual enzyme activity after 60 min of incubation in the same conditions, according to our previous report (data not shown). The thermal stability of the immobilized enzymes on chitosan (CS-GLU and CS-aGLU) by using glutaraldehyde is very similar, which can be attributed to the same mechanism of binding enzyme-support in both cases, namely, through the amino terminal group from the enzyme (Chiou & Wu, 2004). On the other hand, the crosslinking with genipin involves many distinct reactions, and provides a different gel structure compared to glutaraldehyde (even less thermostable than the particles crosslinked with glutaraldehyde, as demonstrated by the TGA analysis); a factor that can provoke unwanted reactions at high temperatures and could explain the somewhat lower thermal stability of the CS-GEN particles.

Sugars and other osmolytes can improve the thermal stability of enzymes by reducing the enzyme movement due to the preferential exclusion of the osmolytes from the protein backbone, thus avoiding unfolding and denaturation (Kumar et al., 2012; Liu et al., 2010). Figure 5 also shows that, in the presence of lactose buffered solution (40 % w/v), the immobilized enzyme on CS-GEN particles presented increased thermal stability. After 540 min of incubation at 60 ºC the immobilized enzyme still presented 63 % of its residual activity, which means that, under operational conditions, the enzyme is much more stable than in buffer solution. Estimate β-galactosidase thermal stability in the presence of lactose is much important since it gives essential information about the real potential of this enzyme for dairy industry application. Moreover, it avoids underestimate enzyme stability.

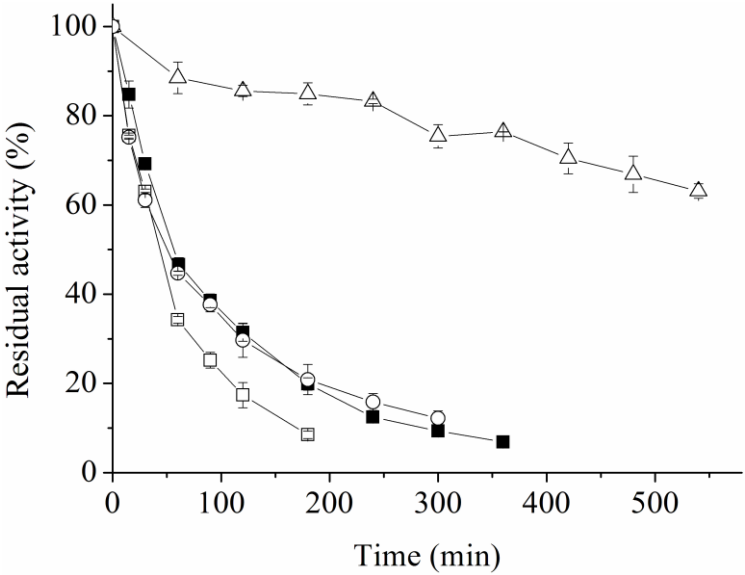

Figure 5: Thermal inactivation at 60 ºC of *A. oryzae* *β*-galactosidase immobilized on (∀) CS-GEN, (–) CS-GLU, (!) CS-aGLU, and (8) CS-GEN in the presence of lactose 40 % (w/v).

Operational Stability in the Lactose Hydrolysis

The operational stability of immobilized enzymes is an important criterion for their industrial application and makes their usage more advantageous. Then, operational stability of the CS-GEN biocatalyst was evaluated by the hydrolysis of buffered lactose solutions (5% w/v; pH 4.5) at 40 ºC. Lactose hydrolysis performed with twenty-five CS-GEN particles in 1.5 mL of lactose buffered solution resulted in 70 % of lactose conversion in 6 h from its first use (Figure 6). Repeated batch hydrolysis of buffered lactose solutions by the immobilized enzyme allowed 25 repeated cycles with maximum activity. From these results, it can be concluded that *A. oryzae* *β*-galactosidase immobilized on chitosan by crosslinking with genipin shows excellent operational stability in the lactose hydrolysis. This result is similar to that in the operational stability on the lactose hydrolysis obtained for *K. lactis* *β*-galactosidase immobilized on pre-activated chitosan particles using the traditional methodology with glutaraldehyde (Klein et al., 2012).

Although comparison is not appropriate, the observation of few others results found in the literature on the immobilization of enzymes via crosslinking with genipin, shows that the present results are among

the best ever reported. Fernandes and co-workers (2013) evaluated the genipin-crosslinked chitosan as a support for laccase biosensor and they found good stability after repeated use over 65 days, and the biosensor retained 85 % of activity in relation to the day of construction. On the other hand, Chiou and co-workers (2007), in a study about the immobilization of lipase on chitosan beads using genipin, reported that 74 % of the initial lipase activity was retained after 5 runs of reuse. Lastly, the chitosan-immobilized *β*-glucosidase by crosslinking with genipin retained 100 % of the initial activity after twelve repeated uses (Fujikawa et al., 1988).

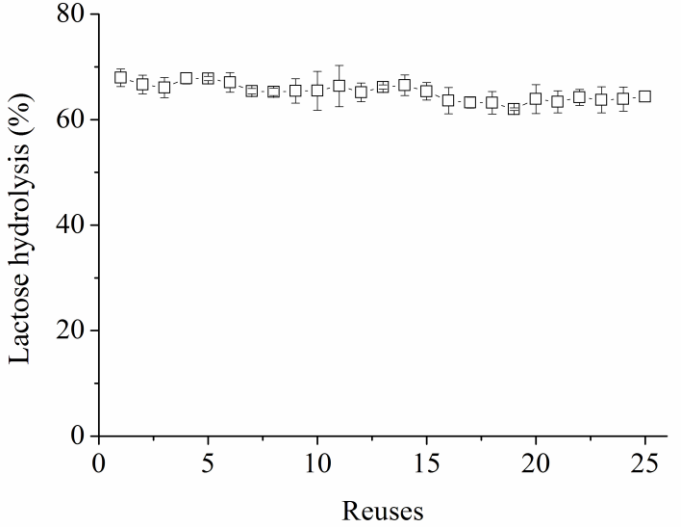

Figure 6: Operational stability on the lactose hydrolysis performed with *β*-galactosidase immobilized on CS-GEN at 40 °C.

Galactooligosaccharides synthesis

Given the interest of GOS synthesis from the *A. oryzae β*-galactosidase immobilized by means of a safe and natural crosslinker on a non-toxic support, the optimization of the enzymatic synthesis was carried out in order to obtain the maximum GOS concentration from the reaction since temperature, pH, time and substrate concentration were the main studied factors.

Effect of lactose concentration

The most significant factor on GOS formation is the initial lactose concentration (Grosova et al., 2008). To determine the influence

of substrate concentration on tri- and tetra-galactooligosaccharides (main GOS) synthesized by immobilized *A. oryzae* β-galactosidase on CS-GEN particles, experiments were performed with increasing lactose concentration (300, 400 e 500 g L^{-1}) at 45 ºC and pH 5.25, following a time course of reaction up to 420 min. Figure 7A shows that GOS synthesis increased with increasing lactose concentration. The maximum GOS concentration for initial lactose concentration of 300 g L^{-1}, 400 g L^{-1} and 500 g L^{-1} was 75 g L^{-1}, 114 g L^{-1} and 146 g L^{-1} after 180 min, 300 min and 420 min, respectively. In fact, β-galactosyl groups should have a higher probability of attaching to lactose than water at increasing lactose concentrations (Iwasaki et al., 1996). For the initial lactose concentration of 300 g L^{-1} and 400 g L^{-1}, the GOS synthesis decreased after achieving the maximum. This can be attributed to a preferential hydrolysis rather than GOS synthesis (Neri et al., 2009). The same decrease was not observed using an initial lactose concentration of 500 g L^{-1}, since there is more substrate to be hydrolyzed and to serve as acceptor for β-galactosyl groups from lactose hydrolysis, then the reaction appears to be still incomplete at 420 min. In terms of GOS yield, the values remained almost constant for the increasing lactose concentrations (25 %, 28.5 % and 29 %, respectively). Neri and co-workers (2009) observed the same behavior for GOS synthesis with *A. oryzae* β-galactosidase immobilized on magnetic polysiloxane-polyvinyl alcohol. Yields of around 28 % were also found by Huerta and co-workers (2011) on the synthesis of GOS from lactose 500 g L^{-1} using distinct concentrations of the enzyme (*A. oryzae* β-galactosidase) immobilized on glyoxyl-agarose.

Effect of pH

The effect of pH on the GOS synthesis was investigated at 45 ºC for pH values of 4.5, 5.25 and 7, at an initial lactose concentration of 400 g L^{-1}. Figure 7B shows the time course of GOS synthesis at different pH values. The rate of the transgalactosylation reaction increased as the pH decreased, since the maximum GOS concentration was achieved in less time at pH 4.5 (116 g L^{-1} in 180 min), than at pH 5.25 (114 g L^{-1} in 300 min) and at pH 7 (121 g L^{-1} in 420 min). The corresponding yields are 29 % at pH 4.5, 28.5 % at pH 5.25, and 30 % at pH 7. Since the optimum pH was found to be between 3.5-4.5 (Figure 4A), it seems clear that lactose hydrolysis occurs faster at acidic conditions than at more basic ones. In these conditions there is more galactose liberated from lactose hydrolysis that will serve as substrate for the transgalactosylation reaction, than increasing its rate. At pH 7, the opposite occurs: since hydrolysis activity is not favored, the rate of

liberated galactose is slower and the maximum GOS synthesis is achieved in longer times. The reaction at pH 4.5 has the advantage of provide higher productivity (38.7 g L^{-1} h^{-1}) than at pH 7 (17.3 g L^{-1} h^{-1}). Our results are comparable with the obtained by Cardelle-Cobas and co-workers (Cardelle-Cobas et al., 2008). Using *β*-galactosidase from *Aspergillus aculeatus*, the authors showed that the rate 6-*β*-galactosyl-lactose formation (tri-GOS) increased with pH from 4.5 to 6.5, furthermore, higher concentration of tri-GOS was obtained at pH 6.5 in comparison with pH 4.5. On the other hand, Neri and co-workers (2009) have not found significant changes in GOS synthesis when pH ranged from 3.5 to 5.5 using the *β*-galactosidase from *A. oryzae*.

Effect of temperature

To determine the influence of temperature on GOS synthesis, experiments were performed at 40, 47.5 and 55 ºC at initial lactose concentration of 400 g L^{-1} and pH 5.25, following a time course of reaction up to 420 min. Temperature normally has a pronounced effect on enzyme reaction rates but showed to have a minimal effect on GOS yield. From the Figure 7C it can be seen that the maximum GOS concentration, at 40 ºC, 47.5 ºC and 55 ºC was 120 g L^{-1}, 114 g L^{-1} and 108 g L^{-1} after 420 min, 300 min and 180 min, respectively. These concentrations represent GOS yields of 30 %, 28.5 % and 27 % at 40 ºC, 47.5 ºC and 55 ºC, respectively. Besides GOS yield remained almost unchanged at this temperature range, the time needed to reach maximum GOS synthesis was shorter at 55 ºC than at 40 ºC. Then, in terms of productivity, the GOS synthesis at 55 ºC is advantageous since the productivity was of 36 g L^{-1} h^{-1} in comparison to the productivity at 40 ºC (17.1 g L^{-1} h^{-1}). Meanwhile, although the immobilized enzyme presents good thermal stability in the presence of concentrated lactose (Figure 6), it is slowly losing its activity along the reaction, making impossible the long term reuse. From these results we could suggest that an adequate range of temperature for GOS synthesis with the obtained biocatalyst is around 47 ºC, since it gives good productivity (22.8 g L^{-1} h^{-1}) and allows more numbers of reuses. Vera and co-workers (Vera et al., 2011) also reported that the transgalactosylation activity of *A. oryzae* *β*-galactosidase increased with temperature in the range of 40 to 55 ºC, and this is reflected in the corresponding increase in productivity for the synthesis, both of GOS and lactulose. Despite this, almost the same concentration of reaction products was achieved, although in different moments. Others authors also reported little effect, if any, of different

temperatures on the GOS production by *A. oryzae* *β*-galactosidase (Albayrak & Yang, 2002; Neri et al., 2009).

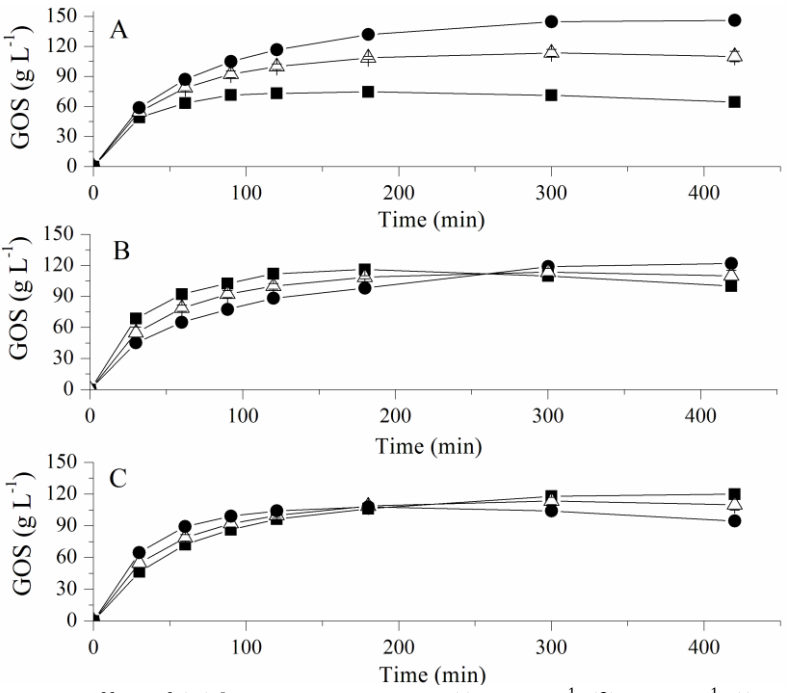

Figure 7: Effect of (A) lactose concentration: (!) 300 g L^{-1}, (**8**) 400 g L^{-1}, (,) 500 g L^{-1}, (B) pH: (!) 4.5, (8) 5.25, (,) 7, and (C) temperature: (!) 40 ºC, (8) 47.5 ºC, (,) 55 ºC, on the galactooligosaccharides synthesis using *β*-galactosidase immobilized on CS-GEN.

Optimization of GOS synthesis reaction related to the initial lactose concentration, pH and temperature was also carried out for the enzyme immobilized on the pre-activated chitosan particles (CS-aGLU) and the results were very similar to the obtained with the CS-GEN biocatalyst. The Figure 8 illustrates the time-course of GOS synthesis up to 420 min, at 400 g L^{-1} initial lactose concentration, 47.5 ºC and pH 5.25. It can be noted that the reaction achieved approximately the same GOS concentration (113.5 g L^{-1} after 120 min of reaction) compared to the GOS synthesis with CS-GEN in the same conditions (114 g L^{-1} after 300 min of reaction), however, in less time due to the increased enzyme concentration used for the reactions with CS-aGLU.

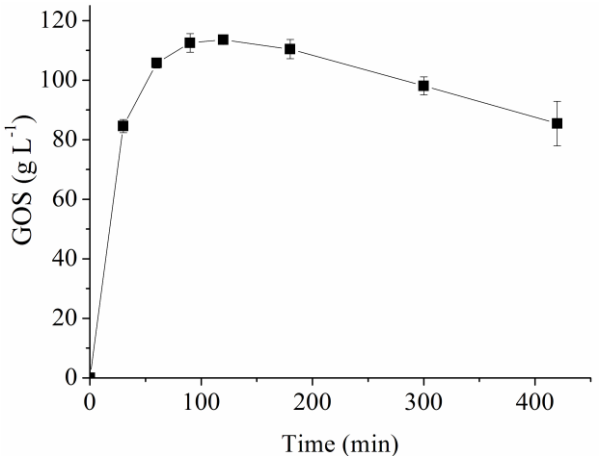

Figure 8: GOS synthesis using *β*-galactosidase immobilized on chitosan pre-activated with glutaraldehyde (CS-aGLU) using lactose 400 g L^{-1}, pH 5.25, at 47.5 ºC.

CONCLUSIONS

To make feasible the industrial application of immobilized enzymes, they have to present, at least, reusability and stability. Notwithstanding, the support used should be both cheap and safe, especially when applied in the food industry. The biocatalyst obtained in the present work satisfies these requirements, since it was prepared from chitosan, a nontoxic polysaccharide, and crosslinked with genipin, a naturally occurring and proven safe bi-functional crosslinking reagent. From a kinetic point of view, the *β*-galactosidase immobilized on this support showed to have satisfactory activity, thermal stability, reusability on the lactose hydrolysis and yields on the synthesis of galactooligosaccharides. From a practical point of view, the obtained particles were resistant to acid pH, easy to handle and appeared to be more resistant mechanically than the particles prepared with glutaraldehyde, since no fractures were observed in all batches of lactose hydrolysis or galactooligosaccharides synthesis.

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CAPÍTULO 5: KINETICS AND THERMODYNAMICS OF THERMAL INACTIVATION OF *β*-GALACTOSIDASE FROM *ASPERGILLUS ORYZAE*

Neste trabalho estudou-se a estabilidade térmica da *β*-galactosidase de *Aspergillus oryzae*, na sua forma livre, em diferentes temperaturas. Avaliou-se também a utilização de diferentes modelos cinéticos de inativação. Os resultados estão apresentados no manuscrito a seguir, submetido para publicação na revista *Applied Biochemistry and Biotechnology*.

Kinetics and thermodynamics of thermal inactivation of *β*-galactosidase from *Aspergillus oryzae*

ABSTRACT: For optimization of biochemical processes in food and pharmaceutical industries, the evaluation of enzyme inactivation kinetic models is necessary to allow its adequate use. Kinetic studies of thermal inactivation of *β*-galactosidase from *Aspergillus oryzae* were conducted in order to critically evaluate mathematical equations presented in the literature. Statistical analysis showed that Weibull model presented the best adequacy to residual enzymatic activity data through the processing time and its kinetic parameters as a function of the temperature, in the range of 58-66 ºC. The investigation suggests the existence of a non-sensitive heat fraction on the enzyme structure, which is relatively stable up to temperatures close to 59 ºC. Thermodynamic parameters were evaluated and showed that such *β*-galactosidase presents activation energy of 277 kJ mol^{-1} and that the enzyme inactivation is due to molecular structural changes. Results showed that the enzyme is quite stable for biotechnological applications.

Keywords: *β*-Galactosidase, Biocatalysis, Enzyme Inactivation, Modeling, Protein Denaturation, Weibull.

INTRODUCTION

β-Galactosidase (EC 3.2.1.23) is an important commercial enzyme used in the food and pharmaceuticals processes. It is mainly applied to produce low lactose dairy products for lactose intolerant people, and also to produce prebiotics like galactooligosaccharides (GOS) (Gekas & Lopezleiva, 1985; Ladero et al., 2000; Ladero et al., 2006; Ladero et al., 2001). *β*-galactosidase from *Aspergillus oryzae* has

been shown a particular importance in GOS production (Gaur et al., 2006; Neri et al., 2009; Vera et al., 2012). This enzyme can produce trisaccharides and higher saccharides, while *β*-galactosidases from *Kluyveromyces* spp. produces, mainly trisaccharides (Boon et al., 2000).

GOS are formed by a glucose molecule attached to two to six galactose molecules through different glycosidic linkages, or only by galactose molecules linked by glycosidic bonds (Gosling et al., 2010; Ito, 1990), and its structure is primarily controlled by the identity of the enzyme used (Gosling et al., 2010). Beyond the enzyme source, the GOS synthesis can be influenced by the temperature, since at higher temperatures it is possible to achieve higher concentrations of lactose (600 g L^{-1}), increasing the GOS yield (Gosling et al., 2010). Moreover, the possibility of operating at higher temperatures offers the advantage of avoiding potential microbial contamination, which is greatly desirable in industrial scale. Then, in this case, thermal stability evaluations are essential in order to know the temperatures in which it is possible to work without important losses in enzyme activity, enabling the development of the galactooligosaccharides synthesis reaction with higher efficiency.

The term "stability" refers to a protein's resistance to adverse influences such as heat or denaturants, that is, to the persistance of its molecular integrity or biological function in the face of high temperatures or other deleterious influences (Fagain, 1995). Inactivation of an enzyme can be of inter- or intramolecular nature. Intermolecular inactivation mechanisms may include autolysis and aggregation, whereas the intramolecular phenomena are due to the interaction of the protein with irreversible inhibitors, solvents, surfactants, salts, among others, or to extremes of pH and temperature. The mathematical simulation of the effect of these agents on the activity of the enzymes is an important approach to understand and to improve the stability of proteins as biocatalysts (Ladero et al., 2006; Sadana & Henley, 1988).

Some studies on the inactivation of the *Aspergillus oryzae β*-galactosidase are available in the literature. However, the kinetic modelling of thermal inactivation of this enzyme, and the determination of its thermodynamic parameters, that is of great interest for the GOS synthesis, is still lacking. Ladero and co-workers (2006) studied the thermal and operational stability of a commercial *β*-galactosidase from *Kluyveromyces fragilis* (Lactozym) in several buffered solutions by testing different kinetic models for the thermal inactivation data of the enzyme. In the same way, Jurado and co-workers (2004) have evaluated the influence that different experimental conditions (pH, temperature

and ionic concentration) have on the activity of two commercial *β*-galactosidases (Lactozym - *Kluyveromyces fragilis* and Maxilact - *Kluyveromyces lactis*) by using two kinetic models. More recently, Guidini and co-workers (2011) evaluated the thermal stability of the immobilized *Aspergillus oryzae β*-galactosidase during lactose hydrolysis; however, thermodynamics parameters were not determined.

In this sense, the objective of the present work was to analyze the thermal stability of the *Aspergillus oryzae β*-galactosidase in different inactivating temperatures and to evaluate several proposed mathematical models to predict enzyme residual activity as a function of time. Moreover, temperature dependence and thermodynamic parameters for thermal inactivation were determined.

MATERIALS AND METHODS

Chemicals

β-Galactosidase and *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) were obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade.

Enzymatic assay

β-Galactosidase activity was determined using *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) as substrate. The reaction contained 0.45 mL of ONPG (15 mM, final concentration) dissolved in acetate buffer (0.1 M, pH 4.5) and 50 μL of conveniently diluted enzyme. After incubation (40 °C for 3 min), the reaction was stopped by adding 1.5 mL of 0.1 M sodium carbonate buffer (pH 10) and the absorbance was measured at 415 nm. One unit (U) of *β*-galactosidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol per minute at the defined assay conditions.

Thermal inactivation studies

Aliquots of buffered *β*-galactosidase solutions (0.5 mL; 0.015 mg mL^{-1} in 0.1 M acetate buffer, pH 4.5) were heated in sealed tubes with 1 mm of thickness, 9 mm of internal diameter and 4 cm of length in a thermostatically controlled water bath (Thermomix BM-S, B. Braun Biotech International, Melsungen, Germany) at temperatures ranging from 58 to 66 °C during up to 300 min. In order to avoid the effects of heating-up and cooling-down, the enzyme activity after 30 s of heating-up time ($t = 0$) was considered to be the initial activity, and after the exposure to heat, tubes were immediately immersed in an ice bath.

Assays were done in duplicate and the average residual hydrolitic activities with respect to processing time at different temperatures were fitted to several kinetic models using non-linear regression by Statistica 7.0 (StatSoft Inc., Tulsa, OK).

Kinetic analysis

The mechanisms of the reactions involved in enzyme inactivation are complex, thus several inactivation equations have been proposed to mathematically express this kinetic behavior. In the equations, A represents residual β-galactosidase activity at time t (min) and A_0 is the initial enzyme activity.

First-order kinetics (Eq. 1) suggests that the reaction happens at one inactivation rate (k-value) in a single step. It has been reported to model heat degradation of several enzymes, including β-galactosidase (Daroit et al., 2011; Mercali et al., 2013; Ustok et al., 2010)

$$\frac{A}{Ao} = \exp(-kt) \tag{1}$$

Models that suggest the existence of more than one enzyme with similar activity but presenting different heat sensitivities (Eq. 2-6) can be described by the combination of exponential behaviors of the different fractions. The distinct isoenzymes model (Eq. 2) describes the sum of two exponential decays. A_L and A_R represent the residual activities for the labile and the resistant isoenzymes, respectively. k_L and k_R are the correspondent first-order reaction rate constants for each fraction, respectively (Weemaes et al., 1998).

$$\frac{A}{Ao} = A_L \exp(-k_L t) + A_R \exp(-k_R t) \tag{2}$$

The two-fraction model, represented by Eq. 3, also describes the inactivation as a combination of two distinct groups of enzyme fractions, a stable and other sensitive to heat, where the coefficient a represents the active fraction of the heat labile group in relation to the total activity (Chen & Wu, 1998).

$$\frac{A}{Ao} = a \times \exp(-k_L t) + (1-a) \times \exp(-k_R t) \tag{3}$$

When there is an extremely thermal resistant fraction in the enzyme (A_r), fractional conversion model (Eq. 4) is employed to describe the residual activity as function of the processing time. It refers to a first-order degradation reaction and considers the non-zero enzyme activity upon prolonged heating (Rizvi & Tong, 1997).

$$\frac{A}{Ao} = A_r + (1 - A_r) \times \exp(-kt) \tag{4}$$

Multi-component model (Eq. 5), equation proposed by Fujikawa and Itoh (1996) uses the concept of cumulative of two distinct resistance fraction of the enzymes, where *r*-value is related to the combination of the initial activity of the resistant and labile form of the enzyme.

$$\frac{A}{Ao} = \frac{\left[\exp(-k_1 t) + r\exp(-k_2 t)\right]}{1 + r} \tag{5}$$

The series-type model (Eq. 6) is based on a succession of first-order steps (Henley & Sadana, 1985). In the first step the protein unfolds (irreversibly or reversibly) from the native structure to yield an inactive or partially active intermediate, which is followed by an irreversible step that converts the intermediate in an inactive enzyme.

$$\frac{A}{Ao} = \alpha_2 + \left[1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right]\exp(-k_1 t) - \left[\frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_1}{k_2 - k_1}\right]\exp(-k_2 t) \tag{6}$$

Applications of nth-order equation (Eq. 7) for the heat degradation of enzymes suggests that the activity decay through time is not necessarily exponential (Decordt et al., 1992; Shalini et al., 2008).

$$\frac{A}{Ao} = \left\{A_o^{\,1-n} + (n-1) \times kt\right\}^{1/(1-n)} \tag{7}$$

Weibull distribution pattern (Eq. 8) (Weibull, 1951) is based on the assumption that, under the conditions examined, the momentary rate of thermal sensitivity to heat is only a factor of the transient heating

intensity and residual activity, but not of the rate at which the residual activity has been reached (Corradini & Peleg, 2004). Weibull model is characterized by the values *n* and *b*-values; the former determines the shape of the distribution curve, whereas the later determines its scaling (Corradini & Peleg, 2004).

$$\frac{A}{Ao} = \exp\left(-bt^{n}\right) \tag{8}$$

Comparison of kinetic models

Residual activities with respect to heating time were fitted to the kinetic models, using the Quasi-Newton method for non-linear regression from Statistica 7.0 (StatSoft Inc., Tulsa, OK). For comparison of fits obtained, statistical and physical criteria were considered.

A physical criterion for rejection of a model is the estimation of negative kinetic parameters at a given temperature. The statistical criteria include coefficient of determination (r^2), chi-square (χ^2) and Akaike's optimization criterion (AIC), like proposed by Sant'Anna and co-workers (2010).

Chi-square, used to compare the models, is mathematically given by Eq. 9:

$$\chi^2 = \frac{\sum\left(a_{mensured} - a_{predicted}\right)^2}{\left(n - p\right)} \tag{9}$$

When models with different numbers of parameters are compared, the residual sum of squares does not give enough information to discriminate between these models. The AIC produces ranking of parsimonious models when the number of experimental data is small, or when the number of fitted parameters is a moderate to large fraction of the number of data. This optimization criterion compares models by their sum of squares, corrected for the number of parameters and observations (Hurvich & Tsai, 1989). AIC is defined as:

$$AIC = n\ln\left(\frac{\sum(a_{measured} - a_{predicted})^2}{n}\right) + \frac{n(n+p)}{n-p-2} \quad (10)$$

where n is the number of observations and p the number of parameters.

The model with the lowest χ^2, AIC, and higher r^2 for the adequacy of the experimental data to the kinetic equations was considered as the best choice from a statistical point of view.

Thermodynamics analysis

The Arrhenius equation is the most common mathematical expression to describe the temperature effect on the inactivation rate constants and the dependence is given by the activation energy (*Ea*):

$$\ln(k) = \ln(k_0) - \frac{E_a}{R.T} \quad (11)$$

where k_0 is the Arrhenius constant, E_a the activation energy, R (8.31 J mol^{-1} K^{-1}) the universal gas constant and T is the absolute temperature. The activation energy can be estimated by linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature.

Inactivation enthalpy ($8H^{\#}$), free energy of inactivation ($8G^{\#}$) and inactivation entropy ($8S^{\#}$) can be calculated according to the expressions:

$$\Delta H^{\#} = E_a - R.T \quad (12)$$

$$\Delta G^{\#} = -R.T.\ln\left(\frac{k.h}{K_B T}\right) \quad (13)$$

$$\Delta S^{\#} = \frac{\Delta H^{\#} - \Delta G^{\#}}{T} \quad (14)$$

were h (6.6262 x 10^{-34} J s) is the Planck´s constant and K_B (1.3806x10^{-23} J K^{-1}) is the Boltzmann´s constant.

Data analysis

Statistical analysis of the data was performed using the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA) and plots were made using Microsoft Excel 2000 (MapInfo Corporation, Troy, NY, USA). Obtained *k*-values were compared using Tukey's approach, and a $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Kinetic analysis for β-galactosidase thermal inactivation

Residual hydrolytic activities in temperature range of 58-66 ºC are shown in Fig. 1, where an exponential behavior can be observed. Eight inactivation kinetic models were tested to fit the experimental data for heat treatments of *Aspergillus oryzae β*-galactosidase. The statistical performance of these models is summarized in Table 1. For multi-component, two-fractions and series models, negative parameter values were estimated, which is a physical criterion for rejection of the equations. For the distinct isoenzymes model, equal inactivation rate parameters, at 63 ºC and 66 ºC, were calculated, excluding this model for these temperatures. Fractional conversion, first order and Weibull distributions gave satisfactory description of the inactivation kinetics, with higher r^2-values and low values of χ^2 and AIC. From this, fractional conversion and Weibull equations presented the better adequacy for the experimental data, with the r^2-values higher than 0.99, and similar χ^2 and AIC values. The r^2 values for fractional conversion model ranged from 0.9912 and 0.9978, while the Weibull distribution's r^2 values ranged from 0.9916 and 0.9991. The AIC values for Weibull varied from -100 to -57 and from -82 and -51 for the fractional conversion model. The χ^2 ranged from 0.000299 and 0.001223 to fractional conversion and from 0.0001 to 0.001 for Weibull pattern, indicating a similar fit for both models. According to Schokker and co-workers (1997), for predictive modeling, it is recommendable to choose the equation with fewer parameters to be estimate, because it is more stable, due to the parameters being less correlated, and easier to use the model. Then, it is suggested that, in the temperatures range studied here, the Weibull model is the best model to explain the thermal inactivation for the *β*-galactosidase from *A. oryzae*.

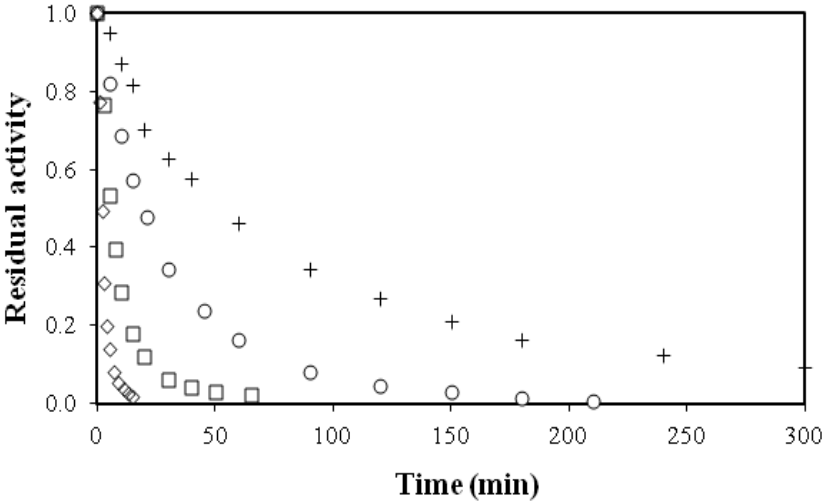

Figure 1. Thermal inactivation of *β*-galactosidase at 58 (+), 60 (o), 63 (□), and 66 ºC (◊). Data presented are average values of two independent experiments.

Table 1: Statistical error analysis for fitting experimental data to different models.

Model (Equation)	r^2	χ^2	AIC
First-order (1)	[0.9784;0.9952]	[9.2E-4;1.0E-3]	[-80;-60]
Isoenzymes (2)	[0.9929;0.9998]	[5.9E-5;2.4E-3]	[-111;-46]
Two-fraction (3)	[0.9916;0.9998]	[2.2E-5;1.1E-3]	[-117;-52]
Fractional conversion (4)	[0.9912;0.9978]	[2.9E-4;1.2E-3]	[-82;-51]
Multi-component (5)	[0.9916;0.9998]	[2.2E-5;1.2E-3]	[-116;-55]
Series (6)	[0.6701;0.9912]	[3.2E-3;5.1E-1]	[-59;-23]
nth-Order (7)	[0.3541;0.9975]	[3.1E-04;1.7E-3]	[-90;2.9]
Weibull (8)	[0.9916;0.9992]	[1.0E-4;1.0E-3]	[-100;-57]

Discussion and validation of the applicability and usefulness of Weibull model to explain the heat inactivation of enzymes like peroxidase (Shalini et al., 2008) and protease P7 (Sant'Anna et al., 2013) have been recently published. The mathematical characteristics of enzyme populations during heat inactivation can be adequately described by continuous functions, and an alternative approach is to consider the survival curve as the cumulative form of a temporal distribution of lethal events. In heat processing, it is common to characterize Weibull reaction mechanisms in terms of the reliable life *(t_R)* (thermal death time concept) (van Boekel, 2002). t_R (Eq. 15) is the necessary time to the enzyme activity decays 90% of its initial activity.

$$t_R = \left(\frac{2.303}{b} \right)^{1/n} \tag{15}$$

Table 2 shows the estimated values for *b*, *n*, t_R and *z* kinetic parameters for the *β*-galactosidase for heat treatments between 58 and 66 °C. The inactivation rate constants ranged from 0.3033 and 0.0272 min^{-1}, increasing with the higher processing temperatures, meanwhile t_R-values are between 103.15 and 6.72 minutes at temperatures between 58 ºC and 66 °C, indicating faster inactivation at higher temperatures.

Table 2: Kinetic parameter values for thermal inactivation of *β*-galactosidase to Weibull model.

Temperature (ºC)	r^2	b (min^{-n})	n	t_R (min)	z' (º C)
58	0.9945	0.0272	0.8089	103.15	2.96
60	0.9992	0.0527	0.8666	49.65	
63	0.9916	0.1171	0.9959	19.73	
66	0.9945	0.3033	1.1726	6.72	

A Weibull distribution with *n* higher than 1 indicates that the semilogarithmic inactivation curve has a downward concavity, an upward concavity when *n* is lower than 1, and an exponential distribution when *n* = 1 (Corradini & Peleg, 2004; Peleg, 2003). The *n*-values estimated for the inactivation of *β*-galactosidase ranged between 0.809 and 1.173. *n*-values lesser than 1 indicate the 'tailing' phenomena, which suggests that enzyme molecules showed different inactivation susceptibilities during heat treatment, corroborating to the idea of the isoenzyme and fractional conversion models, that also satisfactorily described the inactivation behavior (Table 1). Fig. 2 shows that the increasing of the heating temperature implied in the linear enhance of the *n*-values. These results indicate that the shoulder behavior is attenuated with the increasing of the processing temperature, leading to an exponential behavior when the temperature process was 66 ºC. This is possibly because the temperature is high enough to the stable fraction of the enzyme to be degradated so faster as the labile fraction, leading to a single inactivation step.

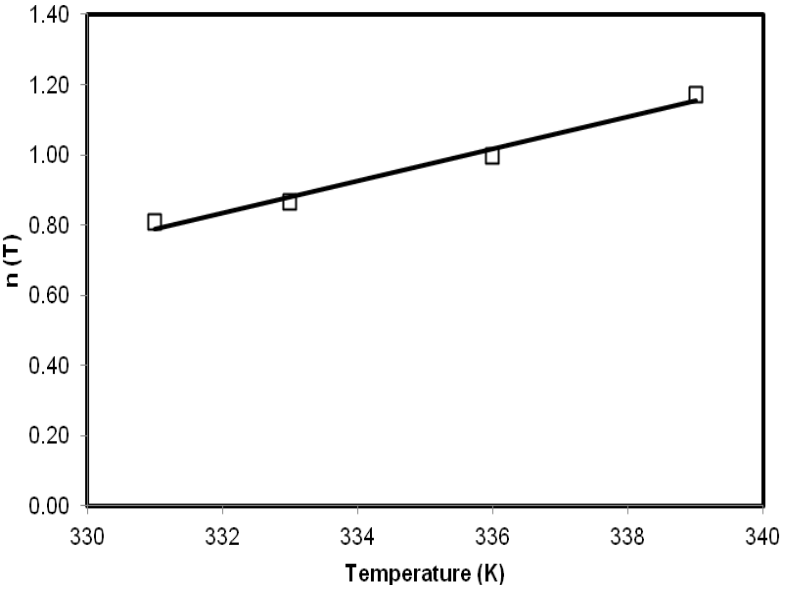

Figure 2. Temperature dependence of *n*-values in Weibull model. The regression equation was determined as $n(T) = 0.0457\ T - 1.859$ ($r^2 = 0.9823$).

Dependence of enzyme activity with time and temperature

Time-temperature is an important binome in industrial applications. On this basis, the combination of the Weibull model, the Arrhenius equation and the linear behavior between the *n*-values and the processing temperature allow the relation among the enzymatic residual activity, time and temperature. Fig. 3 shows the three-dimensional graphic of the enzymatic activity of the *β*-galactosidase as function of the two variables based on Eq. 16.

$$\frac{A}{A_0} = \exp\left[-1.514\times10^{42}\exp\left(-\frac{277027}{8.314T}\right)t^{0.046T-1.859}\right] \quad (16)$$

The 3D representation is an innovative approach in the enzyme thermal inactivation field, offering the possibility to evaluate the interaction of time and temperature on enzyme activity. It can be observed that *β*-galactosidase is relatively stable at 332 K (59 ºC) for up to 50 min maintaining about of 50 % of its initial activity. Also, Fig. 3 shows that for temperatures higher than 334 K (62 ºC) the enzyme becomes very sensitive to heat leading to a reduction of the catalytic capacity in few minutes.

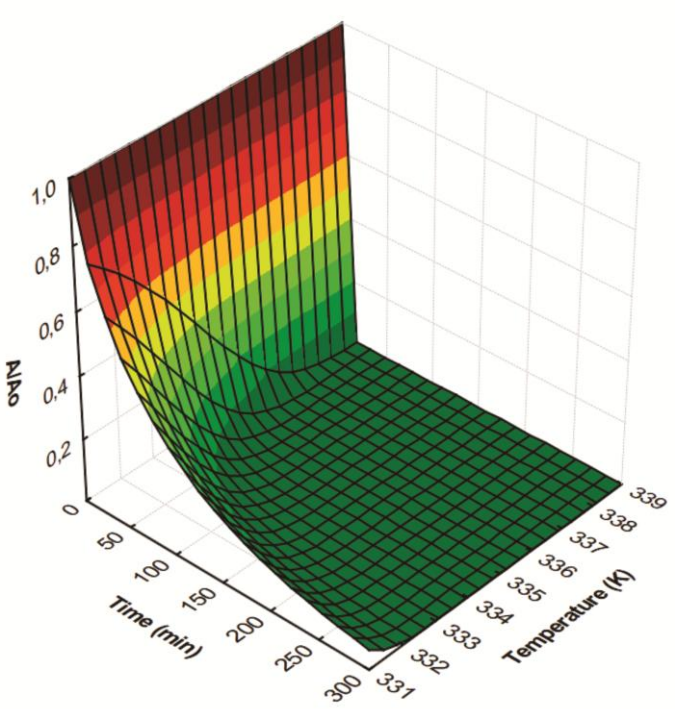

Figure 3. Representation 3D of the residual enzymatic activity of *β*-galactosidase as function of time and temperature, mathematically described by Eq. 16.

Thermodynamic analysis for β-galactosidase thermal inactivation

Estimation of thermodynamic parameters is an important issue to determine biotechnological potential of enzymes and their structure-stability relationships. Activation energy (E_a), activation enthalpy ($8H^{\#}$), activation entropy ($\delta S^{\#}$) and free energy of inactivation ($\delta G^{\#}$), calculated by the transition state theory, for the inactivation of *β*-galactosidase, are presented in Table 3. E_a can be defined as the energy barrier that molecules need to cross in order to be able to react, and the proportion of molecules able to do that, usually increases with temperature, qualitatively explaining the effect of temperature on rates (van Boekel, 2008). Therefore, the higher the E_a values, the higher the energy barrier to be transposed for enzyme inactivation, indicating an increased

stability (Daroit et al., 2011). For the thermal inactivation of commercial *β*-galactosidase from *A. oryzae*, E_a was 277 kJ mol^{-1}, which is close to results observed by Ustok and co-workers (2010). These authors studied the inactivation of *β*-galactosidases from different strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, and their E_a values ranged from 200 to 215 kJ mol^{-1}.

Table 3: Thermodynamic parameter values of thermal inactivation of *β*-galactosidase activity.

Temperature (K)	E_a (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
331	277.03	274.28	79.97	587.04
333		274.26	78.63	587.46
336		274.24	77.14	586.59
339		274.21	75.17	587.13

$\delta H^{\#}$ and $\delta S^{\#}$ are activation enthalpy and entropy, and are mainly related to the break of non-covalent bonds in enzymes, including hydrophobic interactions, and to the disorder change of molecules in the system, respectively (Tanaka & Hoshino, 2002). Positive $\delta H^{\#}$ values indicate that enzyme inactivation is an endothermic process (Viana et al., 2010) and, in turn, a positive $\delta S^{\#}$ indicate that there is an increase in the molecule disorder during the exposure to high temperatures, as expected, and peptide chain unfolding might be the factor determining for the inactivation step. Positive values of $\delta G^{\#}$, that decrease with increasing the incubation temperature, indicate that the destabilization of the enzyme molecule is more spontaneous and faster (Riaz et al., 2007). Since $\delta H^{\#}$ and $\delta S^{\#}$ values are positive, there is an indication of the breakage of weak, non-covalent bonds and changes in the *β*-galactosidase structure (to a disordered polypeptide) (Daroit et al., 2011; Ortega et al., 2004).

CONCLUSION

Commercial *β*-galactosidase from *A. oryzae* presented distinct active fractions with different heat sensibilities. The knowledge on the thermal stability is essential in evaluating the enzyme suitability for biotechnological applications. For *A. oryzae β*-galactosidase, this information is notably important, since this enzyme is recognised for their propensity to form GOS, which in turn, is favored at high temperatures. Weibull model showed to be the best equation to describe the changes on the residual activity through the incubation time and kinetic parameters as function of the temperature. Thermodynamic approach shows an enzyme relatively stable and suggests that inactivation mechanism is based on molecular structural changes.

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CAPÍTULO 6: HIGH IMPROVEMENT OF *β*-GALACTOSIDASE THERMAL STABILITY BY GALACTOOLIGOSACCHARIDES AND LACTOSE: MODELING HEAT INACTIVATION KINETICS

Neste trabalho, estudou-se a estabilidade térmica da *β*-galactosidase de *A. oryzae* imobilizada em macropartículas de quitosana pré-ativadas com glutaraldeído, em diferentes temperaturas e em diferentes condições: em tampão, em presença de lactose e em presença de galactooligossacarídeos. Diferentes modelos cinéticos de inativação foram avaliados. Os resultados estão apresentados no manuscrito a seguir, a ser submetido para publicação na revista *Food and Bioprocess Technology*.

High Improvement of *β*-galactosidase Thermal Stability by Galactooligosaccharides and Lactose: Modeling Heat Inactivation Kinetics

ABSTRACT: Thermal inactivation kinetics of chitosan-immobilized *β*-galactosidase from *Aspergillus oryzae* was investigated in buffer and in the presence of lactose and galactooligosaccharides (GOS), at different temperatures, thereby simulating GOS synthesis. After 60 min of inactivation at 66 ºC, 49 % and 62 % of enzyme activity was retained in presence of GOS and lactose, respectively, while the immobilized enzyme in buffer was fully inactivated. Seven kinetic models were fitted to the data, and the Weibull model was accepted as the best model to describe thermal inactivation in the three evaluated conditions (presence and absence of GOS and lactose). The kinetic and thermodynamic parameters demonstrated the protective effect that GOS and lactose, besides immobilization, has in the enzyme stabilization at different temperatures. As biocatalysts stability is fundamental for industrial and commercial applications, the knowledge about the stabilizing effect of GOS and lactose on *β*-galactosidases is of great interest and could be exploited aiming the improvement of the GOS synthesis reaction.

Keywords: β-Galactosidase, Enzyme immobilization, Kinetic modeling, Thermal inactivation, Galactooligosaccharides

INTRODUCTION

The *β*-Galactosidase (*β*-D-galactoside galactohydrolase, EC 3.2.1.23) is one of the most important enzymes used in food processing. This enzyme, which are also referred to as lactase, hydrolyze the *β*(1→4) linkage of lactose (galactosyl *β*(1→4) glucose) to glucose and galactose, allowing the consumption of milk and other dairy products by lactose intolerant people. Moreover, in the presence of excess lactose, this enzyme can transfer the released galactose from lactose hydrolysis to the galactose moiety of another lactose to yield galactooligosaccharides (GOS) (Panesar et al. 2006). GOS are considered prebiotic food ingredients, since they are indigestible and promote the growth of intestinal bifidobacteria, with the subsequent healthy effects (Grosova et al. 2008). Among the *β*-galactosidases suitable for GOS synthesis, the one from *Aspergillus oryzae* outstands because of its high specific activity and relatively high thermal stability (Vera et al. 2012).

Temperature directly affects enzyme reactions, as increasing temperatures lead to increased catalytic activity; however further increment can result in activity loss. Thermal inactivation of enzymes can involves denaturation, aggregation, and dissociation into subunits of the polypeptide structure (O'Fagain 2003). Considering these possibilities, kinetic models are based on different mechanisms such as: first-order, consecutive reactions, and parallel reactions. Regardless of the specific biophysical mechanisms that actually cause inactivation of enzymes, residual activities indicate the total destructive effect of heat on the enzymes (Shalini et al. 2008). In order to counteract mechanisms of enzyme thermal inactivation, many techniques of enzyme stabilization have been developed (O'Fagain 2003), and modeling the thermal inactivation kinetics of enzymes could provide a better understanding of the enzyme functionality (Ladero et al. 2006).

The protein stability characteristics can be improved by strategies as protein engineering through site-specific mutagenesis, and other simpler stabilization alternatives, including chemical modification, the use of additives (usually solutes), enzyme immobilization, among others (O'Fagain 2003). In a recent work about the effect of the support size on the properties of a *Kluyveromyces lactis β*-galactosidase immobilized on chitosan, the enzyme thermal stability was considerably improved after immobilization on chitosan macroparticles compared to the free enzyme and to the enzyme immobilized on chitosan nanoparticles. The protector effect on the protein structure was

attributed to the characteristics of the chitosan matrix (size and porosity) and to the immobilization process itself (Klein et al. 2012). Moreover, in the case of many enzymes, including *β*-galactosidases, it was seen that the presence of substrates and products (lactose, glucose and galactose) in the reaction medium could also stabilize the overall structure of the enzyme (Ladero et al. 2006; Klein et al. 2013). On the other hand, evaluation of thermal inactivation is frequently carried out under non-operational conditions and this does not reflect the real behavior of enzymes during catalysis.

In this context, evaluating enzyme inactivation under operational conditions is interesting for enzyme characterization from both scientific and technological perspectives. This study was therefore undertaken in order to evaluate the thermal stability of a *β*-galactosidase from *Aspergillus oryzae*, immobilized on chitosan macroparticles, in the presence of lactose and galactooligosaccharides, thereby simulating operational conditions of GOS synthesis. Moreover, kinetic and thermodynamic parameters for thermal inactivation were determined.

Materials and Methods

Materials

Aspergillus oryzae β-galactosidase and *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) were obtained from Sigma-Aldrich (St. Louis, USA). Vivinal GOS® syrup was kindly donated by Friesland Foods (Amersfoot, The Netherlands). All other chemicals were of analytical grade.

Methods

β-Galactosidase Immobilization on Chitosan Macroparticles

A. oryzae β-galactosidase was covalently immobilized on glutaraldehyde-activated chitosan macroparticles as described in a previous work (Klein et al., 2012). The amount of protein attached on chitosan macroparticles was 6.25 mg g^{-1} of dry support, presenting an activity of 400 U g^{-1} of dry support. The chitosan-immobilized *β*-galactosidase was stored at 7 ºC in 0.1 M of sodium acetate buffer (pH 4.5).

Enzyme Activity Assay

β-Galactosidase activity was determined using *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) as substrate. The reaction contained 1 mL of ONPG 15 mM (pH 4.5) and an adequate amount of free or

immobilized enzyme. After incubation (40 °C for 3 min), the reaction was stopped by adding 3 mL of 0.1 M sodium carbonate buffer (pH 10) and the absorbance was measured at 415 nm. One unit (U) of *β*-galactosidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol per min at the defined assay conditions.

Enzyme Inactivation at Different Temperatures

β-Galactosidase (free or immobilized), without added sugars, or containing Vivinal GOS® syrup or 400 g L^{-1} lactose (in order to simulate concentrations conditions of GOS synthesis), were heated in sealed tubes (0.5 mL; 1 mm of thickness, 9 mm of internal diameter and 4 cm of length) at temperatures ranging from 58 to 74 °C, in a thermostatically controlled water bath (Thermomix BM-S, B. Braun Biotech International, Melsungen, Germany). Exposure time varied between 12 and 660 min, and the tubes were immediately immersed in an ice bath, stopping heat inactivation. In order to avoid the effects of heating-up, the enzyme activity after 30 s of heating-up time ($t = 0$) was considered to be the initial activity, since this time was enough to achieve the inactivation temperature. Residual *β*-galactosidase activities with respect to processing time at different temperatures were fitted to several models (Table 1) through nonlinear regression using the software Statistica 7.0 (StatSoft, Tulsa, OK, USA).

Kinetic Modeling

Seven possible decay models usually reported to describe enzyme inactivation processes have been examined in this investigation (Table 1). In the model equations, A/A_0 represents residual enzyme activity at time t (min), and k (min^{-1}) is the reaction rate constant at a given temperature. First-order kinetics (Eq. 1) has usually been reported to describe thermal inactivation of enzymes and others compounds (Bai et al. 2013; Zhu et al. 2013; Ludikhuyze et al. 1999). Parallel models are presented in the Eqs. (2), (3) and (4). These models take into account that the enzyme is in fact a mixture of at least two active forms (isoforms, isoenzymes) with different heat sensitivities, represented by the 'labile' (A_L) and 'resistant' (A_R) fractions, each following its own first order, with its proper velocity constant (k_L and k_R, for the labile and resistant fractions, respectively) (Aymard and Belarbi 2000). In the Eq. (3), the coefficient α represents the activity fraction of the thermal labile group in relation to the total activity (Chen and Wu 1998). Fractional conversion, Eq. (4), refers to a first-order inactivation process and takes

into account of the non-zero enzyme activity upon prolonged heating due to the presence of an extremely heat-resistant enzyme fraction (Rizvi and Tong 1997). The multi-component first-order model (Eq. 5) is expressed as the sum of the kinetics of its components, and each component is supposed to follow first-order inactivation during heating (Fujikawa and Itoh 1996). Fujikawa and Itoh (1996) discussed the characteristics of the multi-component first-order model, for thermal inactivation of microorganisms and enzymes, under various simulation conditions and have described the estimation method of the parameters values of the model from an inactivation pattern. For reactions of *n*th-order (n≠1), where *n* is the order of the reaction, the residual enzyme activity is given by the Eq. (6) (Shalini et al. 2008). The Weibull distribution function has been used in several research studies (Igual et al. 2013; Shahni et al. 2008; Sant'Anna et al. 2013; Torres et al. 2008) to characterize the enzyme inactivation kinetics after distinct processes and was used to fit experimental data by using the Eq. (7). This equation represents the cumulative form of the Weibull distribution, where the rate parameter *b* is characteristic of each reaction and emulates the thermal reaction rate.

Table 1: Kinetic equations used to analyze the thermal inactivation of immobilized *A. oryzae β*-galactosidase under different conditions.

Model		Equation
First-order	(1)	$\frac{A}{Ao} = \exp(-kt)$
Distinct isoenzymes	(2)	$\frac{A}{Ao} = A_L \exp(-k_L t) + A_R \exp(-k_R$
Two-fraction	(3)	$\frac{A}{Ao} = a \times \exp(-k_L t) + (1-a) \times \exp$
Fractional conversion	(4)	$\frac{A}{Ao} = A_r + (1-A_r) \times \exp(-kt)$
Multi-component	(5)	$\frac{A}{Ao} = \frac{[\exp(-k_1 t) + r \exp(-k_2 t)]}{1+r}$

*n*th-order	(6)	$\frac{A}{Ao} = \left\{A_o^{\,1-n} + (n-1)\times kt\right\}^{1/(1-n)}$
Weibull	(7)	$\frac{A}{Ao} = \exp\left(-bt^{n}\right)$

Comparison of Kinetic Models

For comparison of fits, statistical and physical criteria were considered. Statistical criteria used were coefficient of determination (r^2), chi-square (χ^2), and standard error of means (S.E.M.). Generally, low R^2 and high S.E.M. means that the model is not able to explain the variation in the experimental data. Moreover, a physical criterion for rejection of a model is the estimation of negative kinetic parameters at a given temperature (Shalini et al. 2008; Sant'Anna et al. 2013).
Calculation of χ^2 is done by the equation:

$$\chi^2 = \frac{\sum\left(\alpha_{measured} - \alpha_{predicted}\right)^2}{m-p} \tag{8}$$

and S.E.M. is defined as:

$$S.E.M = \frac{\sum\left(\alpha_{measured} - \alpha_{predicted}\right)^2}{\sqrt{m}} \tag{9}$$

where m is the number of observations, p is the number of parameters, and α is the residual enzyme activity.

Thermodynamic Analysis
The inactivation rate in each model can be described as dependent of the temperature used, which can be expressed by the Arrhenius equation (Eq. 10).

$$\ln(k) = \ln(A) - \frac{E_a}{RT} \tag{Eq. 10}$$

where A is the Arrhenius constant, E_a is the activation energy, R is the universal gas constant (8.31 $Jmol^{-1}K^{-1}$), and T is the absolute temperature. The E_a can be estimated by the slope of linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature.

The activation enthalpy ($\Delta H^{\#}$) and the free energy of inactivation ($\Delta G^{\#}$) at each temperature studied were obtained using Eqs. (11) and (12), respectively:

$$\Delta \mathrm{H}^{\#} = E_a - RT \qquad \text{(Eq. 11)}$$

$$\Delta \mathrm{G}^{\#} = -RT \ln \frac{(kh)}{K_B T} \qquad \text{(Eq. 12)}$$

where h (6.6262 x 10^{-34} J s) is the Planck's constant and K_B (1.3806 x 10^{-23} J K^{-1}) is the Boltzmann's constant. From Eqs. (11) and (12), it is possible to calculate the activation entropy ($\Delta S^{\#}$), Eq. (13):

$$\Delta \mathrm{S}^{\#} = \frac{\Delta H^{\#} - \Delta G^{\#}}{T} \qquad \text{(Eq. 13)}$$

RESULTS AND DISCUSSION

Influence of lactose and GOS on the thermal inactivation of the chitosan-immobilized β-galactosidase

Figure 1 shows the profile of thermal inactivation kinetics, at four different temperatures, for free and chitosan-immobilized *β*-galactosidase from *A. oryzae*, respectively. It is clearly seen the improved enzyme thermal stability provided by enzyme immobilization on chitosan macroparticles. After 60 min of exposure at 58 ºC the free enzyme remained with 46 % of its initial activity while the chitosan-immobilized counterpart still presented 71 % of its initial activity.

The stabilization derived from immobilization is a well-known topic and has been reported for many authors for the more varied sort of combinations of enzymes and supports. Compared to enzymes in solution, immobilized enzymes display remarkable stability due to the large decrease of the molecular, submolecular motions, and of the

molecular vibrations. Due to the structural rigidity, the high kinetic barrier prevents transition to the unfolded state, thus the natural state is preferred (Bromberg et al. 2008). Such rigidification can be achieved, mainly, by covalent attachment of enzymes onto activated supports, being the numbers of covalent bounds established between enzyme and support closely related to enzyme thermal stability. The immobilization of enzymes inside the porous structure of a solid support is another way to get better enzyme thermal stabilization. This effect can be explained as the result of molecular confinement: the enzyme molecules that match the pore size of the support are probably confined into the pores, thus being less flexible than the free enzyme (Cao 2005). The thermal stabilization observed in the present study can be probably the effect of both, the molecular confinement of the enzyme molecules into some pores of the chitosan particles (Klein et al. 2012) and the covalent attachment of the enzyme molecule, via glutaraldehyde, to the chitosan structure.

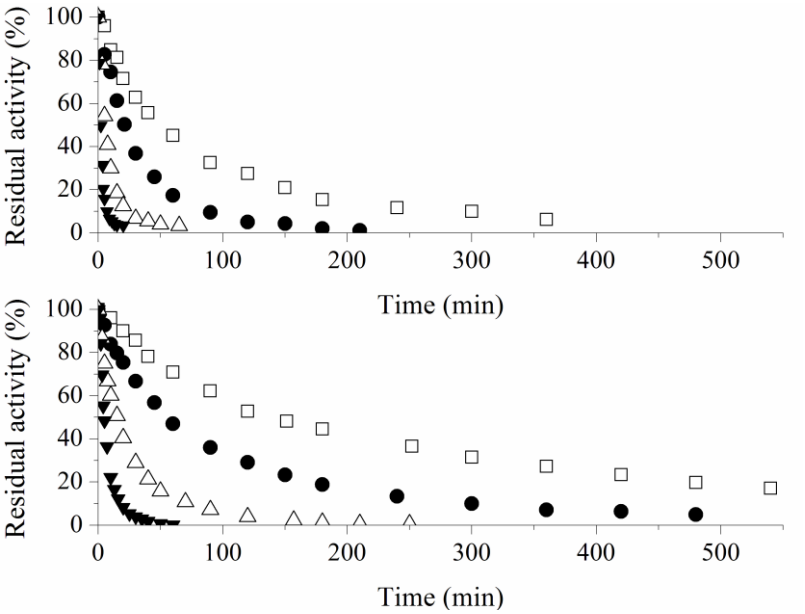

Figure 1: Thermal inactivation kinetics of free (top) and chitosan-immobilized *β*-galactosidase (bottom) at 58 ºC (□), 60 ºC (●), 63 ºC (△) and 66 ºC (▼).

In order to study the thermal stability of the chitosan-immobilized *β*-galactosidase from *A. oryzae* under operational

conditions of galactooligosaccharides synthesis, thermal inactivation was performed in the presence of 400 g L^{-1} lactose (a commonly used concentration for GOS synthesis) or in the presence of the commercial product Vivinal GOS® syrup, appropriately diluted. In this case, the concentration of sugars from the syrup in the inactivation reaction was adjusted to: 150 g L^{-1} of GOS, 47.5 g L^{-1} of lactose, 50 g L^{-1} of glucose and 3.5 g L^{-1} of galactose, totaling about 250 g L^{-1} of total sugars concentration, determined from the supplier information and from HPLC analysis. This concentration of GOS (150 g L^{-1}) was chosen once it is around the concentration achieved in most studies reporting GOS synthesis using *β*-galactosidase from *A. oryzae* (Vera et al. 2012; Huerta et al. 2011; Neri et al. 2009).

From the kinetics of thermal inactivation on Figures 2 and 3, it can be seen that the addition of both, lactose and GOS syrup, can provide a useful stabilization effect on the enzyme structure, proving that under reaction conditions of GOS synthesis the enzyme became less susceptible to thermal inactivation. After 60 min of exposure at 66 ºC the immobilized enzyme remained with 49 % and 62 % of residual activity in presence of GOS syrup and lactose, respectively, while for the immobilized enzyme without sugar addition (Figure 1), the enzyme was fully inactivated in the same conditions.

Such a phenomenon can be mainly ascribed to the preferential exclusion of osmolytes from the protein backbone, which forces the polypeptide to adopt a completely compact folded structure with minimum exposed surface area. Moreover, osmolytes enhances water structure and forms a hydration layer with water molecules (Kumar et al. 2012). In other words, the solvophobic effect of osmolytes on the enzyme structure makes the unfolded state very unfavorable relative to the folded state (Baskakov and Bolen 1998; Bolen and Rose 2008; Liu et al. 2010). Consequently, the folded structure of the immobilized *β*-galactosidase is more stable in lactose or GOS syrup solutions than in buffer solution.

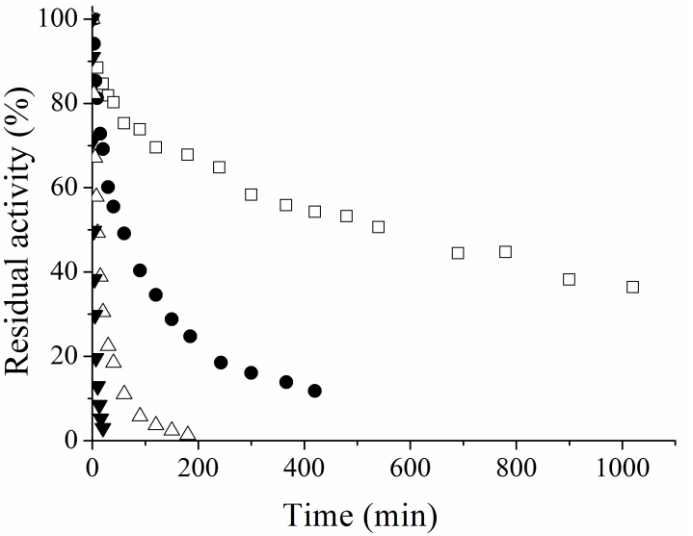

Figure 2: Thermal inactivation kinetics of chitosan-immobilized *β*-galactosidase from *A. oryzae* in the presence of Vivinal GOS® syrup (250 g L^{-1} total sugars concentration) at 63 ºC (□), 66 ºC (●), 69 ºC (△) and 72 ºC (▼).

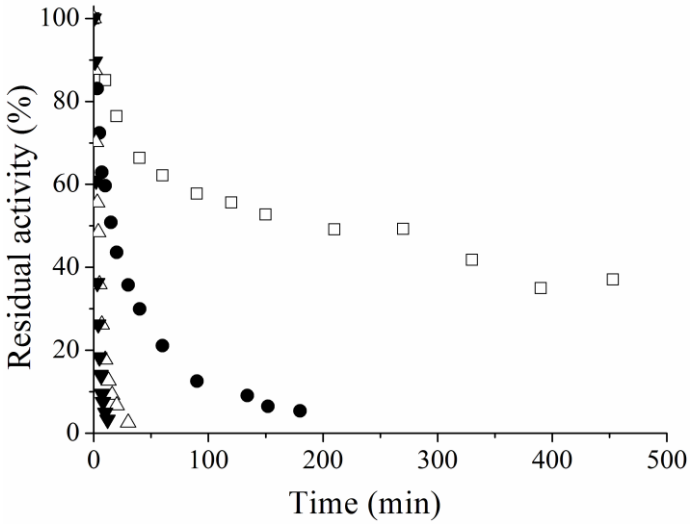

Figure 3: Thermal inactivation kinetics of chitosan-immobilized *β*-galactosidase from *A. oryzae* in the presence of lactose (400 g L^{-1}) at 66 ºC (□), 69 ºC (●), 72 ºC (△) and 74 ºC (▼).

Concerning the differences of thermal stability observed with the addition of lactose and GOS syrup, from a comparative point of view, these results appear to be inconclusive, since the total sugar concentration of the lactose solution (400 g L^{-1}) and the total sugar concentration in GOS syrup (250 g L^{-1}) are not equal. Liu and co-workers (2010), through molecular dynamic simulation studies, showed that the capacity of the osmolyte-induced protein stability increases with their molecular weights or volumes being trehalose ≈ sucrose > sorbitol > xylitol > glycerol. It occurs because the steric exclusion effect of osmolytes increases with its volume. Therefore, an osmolyte of larger volume has stronger preferential exclusion effect by the protein. Moreover, large osmolytes tend to self aggregate into a big cluster through intermolecule H-bonds, thus enhancing the extent of preferencial exclusion and contributing to the stabilizing effect (Liu et al. 2010). In order to check this statement (since GOS syrup are constitute mainly of sugars with high degree of polymerization, tri- and tetra-galactooligosaccharides) thermal inactivation of immobilized *A. oryzae* *β*-galactosidase was also carried out with the addition of more concentrated GOS syrup (~ 400 g L^{-1} total sugar concentration, being 240 g L^{-1} of GOS, 76 g L^{-1} of lactose, 80 g L^{-1} of glucose and 5.6 g L^{-1} of galactose) to compare with the inactivation in the presence of 400 g L^{-1} lactose solution (Figure 4).

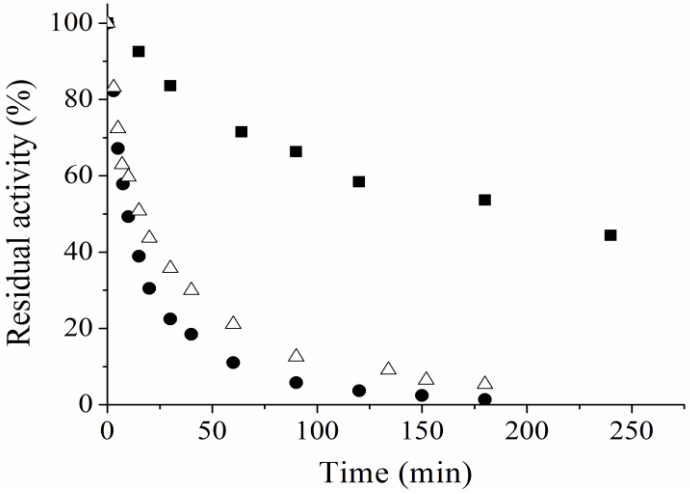

Figure 4: Thermal inactivation kinetics of chitosan-immobilized *β*-galactosidase from *A. oryzae*, at 69 ºC, in the presence of GOS syrup 400 g L^{-1} total sugar concentration (■), lactose 400 g L^{-1} (△) and GOS syrup 250 g L^{-1} total sugar concentration (●).

In the presence of more concentrated GOS syrup (400 g L^{-1} total sugar concentration), the immobilized enzyme presented a higher thermal stability (53 % of residual enzyme activity after 180 min at 69 ºC) compared with the immobilized enzyme in the presence of lactose 400 g L^{-1} (almost fully inactivated). This pronounced difference, can be assigned, as explained above, to the presence of sugars of high molecular mass, as tri- and tetra-galactooligosaccharides, that contribute more effectively to the stabilization of enzymes.

Modeling thermal inactivation of immobilized β-galactosidase

Although the thermal stability of several *β*-galactosidases is described, few studies deal with mathematical modeling of kinetic thermal inactivation of these enzymes in its immobilized form or in the presence of substances that can improve thermal stability. Moreover, most studies simply assume a first-order kinetics from semi-logarithmic plots of activity versus time. The choice of the best equation for process modeling is essential from both engineering and economical points of view, minimizing errors and improving the effectiveness of the process, ultimately resulting in lower costs (Daroit et al. 2011). Thereby, seven inactivation kinetic models were tested to fit the experimental data for heat treatments of the immobilized *A. oryzae β*-galactosidase with buffer or in the presence of lactose 400 g L^{-1} or GOS syrup (Table 1).

The performance of these models for the inactivation of chitosan-immobilized *β*-galactosidase without added sugars is summarized in Table 2. The models based on the presence of isoenzyme populations inactivating by first-order mechanism (distinct isoenzymes, two-fraction and multi-component first-order models) were statistically applicable, presenting high r^2 and low values of χ^2, AIC and S.E.M. However, the distinct isoenzymes and two-fractions models were not physically feasible because the estimated initial activity of the thermostable enzyme (A_S) and thermolabile enzyme (A_L), both of which must be constant, varied as a function of the temperature (data not shown). Moreover, for the two-fraction model, negative parameter values were obtained for the inactivation temperature of 60 ºC, which is a physical criterion for rejection of the equations. For the *n*th-order model, no convergence was observed, indicating that the equation is not stable to modulate the experimental data. The models 4, 5 and 7 yielded high adjusted coefficient regressions, lower values of residuals and lower values of χ^2 and AIC. Thus, from a statistical point of view, these models can be chosen to describe the thermal inactivation pattern of

chitosan-immobilized β-galactosidase. Related to the first-order model, it did not yield as good statistical criteria as the others models.

Table 2: Summary of performance of models selected to describe inactivation of chitosan-immobilized *β*-galactosidase in buffer.

Model	r^2	χ^2	AIC	S.E.M
1	[0.96;0.98;0.98;0.99]	[29;22;22;12]	[-82;-83;-83;-93]	[119;85;85;46]
2	[0.99;0.99;NC;0.99]	[1;0.77;NC;10]	[-136;-133;NC;-88]	[3.4;2.4;NC;34]
3	[0.99;0.98;0.99;0.99]	[1.2;25;0.94;13]	[-136;-76;-132;-87]	[4;85;3;45]
4	[0.99;0.99;0.99;0.99]	[4;7;8.6;13]	[-111;-98;-94;87]	[17;23;29;45]
\5	[0.99;0.99;0.99;0.99]	[1.2;0.8;0.94;13]	[-136;-134;-132;-87]	[4;3;3;45]
6	[NC]	[NC]	[NC]	[NC]
7	[0.99;0.99;0.99;0.99]	[4.8;2.2;2;10]	[-114;-119;-122;-93]	[17;8;7;39]

(NC) No convergence. Numbers in 2nd, 3rd, 4th and 5th columns correspond to temperatures 58, 60, 63 and 66 ºC. χ^2 and S.E.M. values ($x10^{-4}$). Models: (1) First-order, (2) Distinct isoenzymes, (3) Two-fraction, (4) Fractional conversion, (5) Multi-component, (6) *n*th-order, (7) Weibull.

Statistical analysis of the models for thermal inactivation of chitosan-immobilized β-galactosidase in the presence of lactose is presented in Table 3. In agreement with thermal inactivation in buffer, the first-order model did not yield good values of adjusted R^2 at all applied temperatures and it had higher values of residuals compared to the others models. For the models based on the presence of distinct isoenzyme populations inactivating by first-order mechanism (models 2, 3 and 4), negative parameters were estimated. Therefore, these models were ruled out. On the other hand, models 5 and 7 had highest adjusted coefficient regressions, lowest values of residuals, χ^2 and AIC. Therefore, these two models could be chosen to explain the thermal inactivation pattern of chitosan-immobilized β-galactosidase in the presence of lactose 400 g L^{-1}.

Table 3 Summary of performance of models selected to describe inactivation of chitosan-immobilized *β*-galactosidase in the presence of lactose 400 g L^{-1}.

Model	r^2	χ^2	AIC	S.E.M
1	[0.53;0.92;0.99;0.97]	[180;66;8.6;34]	[-38;-52;-68;-46]	[625;230;27;103]
2	[0.99;0.99;0.99;0.97]	[3.2;2.8;11;37]	[-85;-87;-53;-33]	[8.6;7.6;27;79]
3	[0.99;0.99;0.99;0.97]	[2.9;2.6;5.7;38]	[-90;-92;-67;-39]	[8.6;7.6;14.8;91.9]
4	[0.91;0.96;0.99;0.97]	[39;34;5.9;40]	[-54;-56;-66;-38]	[116;101;15;97]
\5	[0.99;0.99;0.99;0.97]	[2.9;2.6;5.7;38]	[-90;-92;-67;-39]	[8.6;7.6;14.8;91.9]
6	[0.97;0.99;0.99;0.97]	[9.3;5.8;7.2;33]	[-74;-81;-64;-40]	[27;17;18;80]
7	[0.98;0.99;0.99;0.98]	[6.5;3.4;8.8;16]	[-82;-91;-65;-52]	[21;11;25;43]

Numbers in 2nd, 3rd, 4th and 5th columns correspond to temperatures 66, 69, 72 and 74 ºC. χ^2 and S.E.M. values (x 10^{-4}). Models: (1) First-order, (2) Distinct isoenzymes, (3) Two-fraction, (4) Fractional conversion, (5) Multi-component, (6) *n*th-order, (7) Weibull.

Finally, for chitosan-immobilized β-galactosidase inactivation under the presence of GOS, from the criteria showed in Table 4, it was concluded that models 6 and 7 were the best models to describe the inactivation in these conditions. Although the two-fraction and the multi-component models have shown high r^2 and low χ^2, AIC and SEM, these models gave negative estimated parameters at 72 ºC, which is a physical criterion for rejection of these models. For the distinct isoenzyme model, equal inactivation rate parameters were calculated at 72 ºC, excluding this model. For the first-order and fractional conversion models, low values of r^2 and high values of χ^2, AIC and SEM were obtained in comparison with the other models.

Table 4: Summary of performance of models selected to describe inactivation of chitosan-immobilized *β*-galactosidase in the presence of GOS.

Model	r^2	χ^2	AIC	S.E.M
1	[0.65;0.89;0.97;0.98]	[112;92;27;20]	[-62;-58;-64;-52]	[463;358;96;61]
2	[0.92;0.99;0.99;0.98]	[29;2.4;1.3;24]	[-81;-113;-98;-38]	[99;7.6;3.5;51]
3	[0.99;0.99;0.99;0.98]	[2.4;2.3;1.3;26]	[-131;-117;-102;-43]	[8.9;7.7;3.8;63]
4	[0.88;0.97;0.98;0.98]	[42;29;16;25]	[-77;-73;-67;-43]	[155;101;47;61]
5	[0.99;0.99;0.99;0.98]	[2.4;2.3;1.3;26]	[-131;-117;-102;-43]	[8.9;7.7.;3.8;63]
6	[0.96;0.99;0.99;0.98]	[13;4.7;2.2;26]	[-99;-105;-95;-43]	[49;16;6.4;63]
7	[0.99;0.99;0.99;0.98]	[2.8;3.2;6.7;18]	[-130;-113;-82;-50]	[11;11;21;51]

Numbers in 2nd, 3rd, 4th and 5th columns correspond to temperatures 63, 66, 69 and 72 ºC. χ^2 and S.E.M. values (x 10^{-4}). Models: (1) First-order, (2) Distinct isoenzymes, (3) Two-fraction, (4) Fractional conversion, (5) Multi-component, (6) *n*th-order, (7) Weibull

Mathematical modeling of the kinetics of heat inactivation of enzymes can be used for predicting the residual activity after a heat treatment and for elucidating the mechanism of the inactivation. When the purpose is predictive modeling, it is recommendable to choose the model in which the fewest parameters are estimated, because it is the easiest model to use. Moreover, the model with the fewest parameters has the largest number of degrees of freedom, which can be important when the number of measurements is small (Schokker and van Boekel 1997). In the three cases discussed here, the statistically acceptable model with the fewest parameters is model 7.

The Weibull model is a pure empirical model, more flexible due to its non-linear nature (Yu et al. 2011). Empirical (or phenomenological) models are based on a purely mathematical description of observed data without any presumed mechanism, and aims to reproduce the thermal degradation behavior with a certain degree of accuracy. Meanwhile, mechanistic or theoretical models are based on some presumed molecular or physical mechanisms, i.e; a mono- or bimolecular reaction, or an enzyme-catalyzed reaction, or heat/mass transfer (Heldman and Newsome 2003; Ferrer et al. 2009). Although descriptions for *A. oryzae* β-galactosidase were not found in the literature, the Weibull pattern was well studied in area of microbial thermal inactivation (Peleg et al. 2008) and several studies also confirmed its effectiveness in modeling thermal degradation of nutrients and enzymes such as peroxidase (Shalini et al. 2008), pectin methyl esterase (Elez-Martinez et al. 2007) and protease (Sant'Anna et al. 2013).

In the case of free enzymes, the irreversible inactivation proceeds, mainly, via the unfolded state (Fagain 1995; Lumry and Eyring 1954; O'Fagain 2003). However, for immobilized enzymes (or enzymes in the presence of additives), where the unfolding is slowed down or even frozen, other mechanisms may exist. Bromberg and co-workers (2008), for the thermal inactivation of cholinesterase enzymes trapped within a rigid matrix, have suggested that thermal inactivation proceeds directly from the native state to the inactivated state.

In view of the above arguments, the Weibull model appear to be a good alternative to describe thermal inactivation of the immobilized β-galactosidase, since it is not based in any pre-conceived mechanism of inactivation. Moreover, it yielded good statistical parameters for the three cases analyzed, allowing appropriate comparisons of their kinetic and thermodynamic parameters.

Estimation of the kinetic and thermodynamics parameters

Weibull distribution is characterized by two parameters *b* and *n*. The value of *n* determines the shape of the distribution curve while *b* determines its scaling. Consequently, *b* and *n* are called the scale and shape factors, respectively (Shalini et al. 2008). The parameters *b* and *n* obtained at different temperatures are reported in Table 5. A Weibull distribution with $n > 1$ indicates that the semilogarithmic inactivation curve has a downward concavity, an upward concavity when $n < 1$, and an exponential distribution when $n = 1$ (van Boekel 2002). The *n* values ranged between 0.421 and 1.333. Although the Weibull model is of an empirical nature, a link can be made with physiological effects (van Boekel 2002). The *n* values less than 1 indicate the 'tailing' phenomena, suggesting that enzyme molecules showed different inactivation susceptibilities during heat treatment. For the higher temperatures, in each of the three treatments, *n* values was higher than 1, indicating that enzyme molecules become increasingly inactivated (van Boekel 2002).

b-Values were observed to increase as the temperature increased, ranging between 0.014 and 0.197 min^{-n}. The Weibull rate parameter *b* is characteristic to each reaction and reflects the thermal reaction rate (Shalini et al. 2008). The higher is the temperature, the higher is *b* and faster inactivation occurs.

In the Weibull model, the reliable life (t_R), which is the time needed to reduce the number of microorganisms by a factor of 10 (analogous to the *D*-value employed in first-order analysis), is given by Eq. (14) (van Boekel 2002; Peleg 1999):

$$t_R = \left[(2.303)/b\right]^{1/n} \tag{14}$$

The t_R values ranged from 203.79 to 18.98 min in the temperature range of 58-66 ºC for the inactivation in buffer. In the presence of GOS, t_R values varied from 157.39 to 11.76 min in the temperature range of 63-72 ºC, and, for the inactivation in the presence of lactose, t_R values varied from 93.04 to 9.44 min in the range of 66-74 ºC. As expected, the t_R values decreased as the temperature increased, indicating faster inactivation at higher temperatures, in the three cases analyzed. When comparing the t_R values for the different conditions at the same temperature (66 ºC), it can be noted that the t_R values increased from 18.98 min for inactivation without added sugars to 66.65 min for inactivation in the presence of GOS, and 93.04 min in the presence of

lactose, demonstrating the potential protector of these sugars on the enzyme stability.

The *z*-value is the temperature needed to reduce the *D*-value in one log unit, and it is obtained by plotting the t_R values on a log-scale against the corresponding temperatures (Cobos and Estrada 2003). The equivalent of the *z*-values (here named as *z*') for the thermal inactivation of chitosan-immobilized *β*-galactosidase also increased from 3.34 ºC for inactivation in buffer to 3.42 ºC and 3.41 ºC, for the inactivation with added GOS and lactose, respectively. This slight but significant difference once again reiterates the protective effect of these sugars against *β*-galactosidase thermal inactivation.

Table 5: Kinetic parameters for the inactivation of chitosan-immobilized *β*-galactosidase.

Condition	*T* (ºC)	r^2	*b* (min^{-n})	*n*	t_R (min)	*z'* (º C)
Buffer	58	0.994	0.014	0.765	203.79	
	60	0.998	0.029	0.775	99.36	3.34
	63	0.998	0.077	0.805	36.15	
	66	0.991	0.112	1.097	18.98	
Buffer plus GOS	63	0.991	0.038	0.459	157.39	
	66	0.996	0.059	0.603	66.65	3.42
	69	0.993	0.124	0.725	4.35	
	72	0.986	0.175	1.146	11.76	
Buffer plus lactose	66	0.984	0.077	0.421	93.04	
	69	0.996	0.123	0.622	31.03	3.41
	72	0.992	0.195	0.954	12.27	
	74	0.987	0.197	1.333	9.44	

To determine the industrial potential of enzymes, it is essential to understand their structure-stability relationships. Therefore, estimation of thermodynamic parameters may provide valuable information regarding thermal inactivation of chitosan-immobilized *β*-galactosidase and the mechanism involved in the process. Activation energy (E_a), free energy of inactivation ($\Delta G^{\#}$), activation enthalpy ($\Delta H^{\#}$) and activation entropy ($\Delta S^{\#}$), calculated by the transition state theory (Eqs. 11-13), are presented in Table 6.

Table 6: Thermodynamic parameters for the inactivation of chitosan-immobilized *β*-galactosidase.

Condition	T (°C)	E_a (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
Buffer	58	242.61	81.68	239.86	477.89
	60		80.24	239.84	479.29
	63		78.28	239.82	480.78
	66		77.96	239.79	477.38
Buffer plus GOS	63	148.4	80.21	145.6	194.62
	66		79.75	145.58	194.19
	69		78.39	145.56	196.4
	72		78.12	145.53	195.4
Buffer plus lactose	66	120.7	79	117.88	114.69
	69		78.43	117.86	115.29
	72		77.82	117.83	115.99
	74		78.25	117.81	114.03

$\Delta G^{\#}$ represents the difference between the activated state and reactants (Al-Zubaidy and Khalil 2007), and the positive values means that enzyme inactivation is not a spontaneous reaction. $\Delta H^{\#}$ is a measure of the energy barrier that must be overcome by the reacting molecules

and is related to the strength of the bonds, which are broken and made in the formation of the transition state from the reactant (Vikram et al. 2005), and $\Delta S^{\#}$ measures the disorder change of molecules in the system (Ortega et al. 2004).

Comparing the values of thermodynamic parameters for the three different conditions at the same temperature (66 ºC), it can be seen that $\Delta H^{\#}$ and $\Delta S^{\#}$ values were lower and $\Delta G^{\#}$ was higher, in the presence of GOS and lactose (Table 6). As $\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$, large values for $\Delta H^{\#}$ are associated with increased enzyme stability unless coupled with a large compensatory increase in the value of $\Delta S^{\#}$, which is destabilizing (Ortega et al. 2004). This destabilizing effect occurs since the increase of $\Delta S^{\#}$ compensates the high inactivation barrier, which causes the $\Delta G^{\#}$ to be low enough, resulting in an overall less energy requirement for the inactivation process to occur relatively fast (Bromberg et al. 2008). Comparing the values of $\Delta G^{\#}$ at 66 ºC, it is higher in the presence of GOS and lactose, indicating that it is necessary more energy for inactivation to occur in these conditions. In a recent work, we have modeled the thermal inactivation of free *A. oryzae* β-galactosidase (Klein submitted), and the $\Delta G^{\#}$ value, at 66 ºC, was 75.17 kJ mol^{-1} (against 77.96 kJ mol^{-1} for the immobilized enzyme - Table 6), indicating the stabilizing effect of immobilization has on the enzyme molecules. Bromberg and co-workers (2008) also found a higher value of $\Delta G^{\#}$ for cholinesterase enzymes immobilized in solid matrices in comparison with the enzyme in solution.

The remarkable difference between the values of $\Delta S^{\#}$ (associated with the disorder change of the molecules) obtained in the presence of sugars in comparison to those in buffer solution can be an indication that in the presence of GOS and lactose the unfolding processes can be blocked and the major deactivation route is the irreversible step from the native form to the inactivated form. The lower values of $\Delta S^{\#}$ in the presence of sugars is probably due to the fact that enzyme molecules adopt a completely compact folded structure with minimum exposed surface area in these conditions, making the unfolding very unfavorable. The decrease on values of $\Delta S^{\#}$ was also observed in comparison with the free enzyme. The entropy change for free *A. oryzae* β-galactosidase was 587.13 J mol^{-1} K^{-1}, at 66 ºC (data not shown) while for the chitosan-immobilized form it was 477.38 J mol^{-1} K^{-1} (at 66 ºC, Table 6), also indicating an advantage that immobilization causes against the unfolding process.

The activation energy (E_a) can be seen as the energy barrier that molecules need to cross in order to be able to react (van Boekel 2008).

Therefore, the higher the E_a values, the higher the energy barrier to be transposed for enzyme inactivation, indicating an increased stability. Meanwhile, although the value of E_a for inactivation of chitosan-immobilized β-galactosidase was higher in buffer than in the presence of sugars (Table 6), the enzyme was inactivated at slower rates with added GOS and lactose (*b* values, Table 5), probably due to the usual dominant role of $\Delta S^{\#}$. Therefore, it is suggested that the decrease in the inactivation rate constant (*b*), or the increase in $\Delta G^{\#}$ values, are more reliable criteria to observe the enhancement of enzyme stability than the increase in the E_a for inactivation (Kazan and Erarslan 1997).

CONCLUSIONS

This work studied the thermal inactivation kinetics of chitosan-immobilized β-galactosidase under reaction conditions simulating GOS synthesis. Weibull model provided a good description of the kinetics of inactivation of the immobilized enzyme for the three conditions evaluated: in buffer and in the presence of added sugars (GOS and lactose), therefore, it is appropriate for predictive purposes. All results indicated a much higher stability of the immobilized enzyme in the presence of sugars, and GOS syrup was able to protect the enzyme against thermal inactivation in a greater extent compared to lactose. Analysis of the thermodynamic parameters also support the findings on the protector effect provided by GOS and lactose on the folded structure of the enzyme, since $\Delta S^{\#}$ decreased and $\Delta G^{\#}$ increased in the presence of these sugars. From the results obtained in the current study, it can be concluded that the determination of enzyme thermal inactivation under conditions that reflect the real behavior of the enzyme during catalysis, is essential in order to do not underestimate the potential industrial application of enzymes. Thus, the GOS synthesis, which is normally carried out at 40 ºC, can be improved by increasing the reaction temperature, what is, ultimately, the result of an increased lactose solubility and higher reaction rates.

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CAPÍTULO 7: CONSIDERAÇÕES FINAIS

As enzimas são catalisadores muito versáteis e podem ser usadas no setor de laticínios e de ingredientes para produtos lácteos, na obtenção de diferentes produtos de alto valor agregado e com características dietéticas e/ou funcionais. O aproveitamento de enzimas comerciais já disponíveis, aliado aos avanços científicos no campo da modificação de enzimas, possibilita a obtenção de biocatalisadores com aplicações industriais específicas, com propriedades (atividade, especificidade) novas e/ou melhoradas. Adicione-se a isto o uso de técnicas de imobilização enzimática e podemos produzir catalisadores com alta atividade catalítica e estabilidade.

Assim, a presente Tese de Doutorado teve como objetivo estudar a hidrólise da lactose e a síntese de galactooligossacarídeos (GOS) catalisada pela enzima *β*-galactosidase imobilizada em diferentes suportes à base de quitosana. Dentro do tema proposto, investigou-se a imobilização da *β*-galactosidase em suportes à base de quitosana como forma de estabilização da enzima. A possibilidade de aplicação industrial da enzima imobilizada foi avaliada através da hidrólise da lactose e da síntese de GOS, em um reator de leito fixo, em regime de operação contínua. Tendo em vista a necessidade de utilização de materiais seguros do ponto de vista toxicológico na indústria de alimentos, investigou-se também a imobilização da *β*-galactosidase em macropartículas de quitosana utilizando genipina como agente de entrecruzamento. Com este suporte, as condições para a síntese de GOS e o reuso da enzima para a hidrólise da lactose foram avaliadas. Além disso, a modelagem cinética da inativação térmica da *β*-galactosidase de *Aspergillus oryzae*, livre e imobilizada em macropartículas de quitosana, foi estudada em presença de lactose e de galactooligossacarídeos para simular as condições operacionais de síntese de GOS.

Inicialmente, a imobilização da *β*-galactosidase de *Kluyveromyces lactis* foi estudada em suportes de características distintas, à base de quitosana: as macropartículas e as nanopartículas. A fim de obter mais informações acerca dos suportes a serem utilizados para a imobilização da enzima, realizou-se a caracterização em função de suas propriedades físico-químicas utilizando técnicas como infravermelho, análise termogravimétrica (TGA), calorimetria diferencial (DSC), microscopia eletrônica de transmissão (TEM), medida de área superficial e espalhamento de luz. Através destas análises, provou-se que os dois suportes à base de quitosana, apesar de

terem sido obtidos de maneiras distintas, apresentaram similaridades no que diz respeito à sua estrutura molecular, tanto antes quanto depois de sua ativação com glutaraldeído. Da mesma forma, não foram observadas diferenças de estabilidade térmica dos materiais. Apesar das macropartículas terem apresentado maior área superficial em relação às nanopartículas, o grau de ocupação de sua superfície foi menor, devido, provavelmente, à existência de poros internos inacessíveis para imobilização de enzimas. Também em função disso e/ou à sobrecarga do suporte, uma menor atividade específica e retenção de atividade (%) foram observadas para as macropartículas em relação às nanopartículas, uma vez que nem toda atividade enzimática pode ser expressa quando a enzima está confinada no interior de poros e/ou bloqueada por outras moléculas de enzima. Em relação à estabilidade térmica da enzima, as macropartículas de quitosana exerceram um efeito protetor maior sobre as moléculas de enzima em relação às nanopartículas de quitosana. Por fim, a estabilidade operacional de ambos biocatalisadores foi satisfatória, já que a taxa de hidrólise da lactose se manteve alta após 50 bateladas de reuso. De um modo geral, nas condições avaliadas, as macropartículas apresentam diversas características interessantes e, em particular, são de fácil manipulação. Assim, de um ponto de vista prático, o uso das macropartículas é vantajoso, uma vez que simples procedimentos como filtração ou peneiramento são suficientes para separar o biocatalisador do meio reacional, ao passo que as nanopartículas necessitam força centrífuga para sua adequada separação. Além disso, as macropartículas permitem seu uso em diversas configurações de reatores, o que seria mais difícil no caso das nanopartículas, devido a complicações como a perda de suporte, colmatação e formação de caminhos preferenciais.

Em função de tais características, as macropartículas foram escolhidas para avaliar um protótipo de reator enzimático com *β*-galactosidase imobilizada. Assim, montou-se um reator de leito fixo recheado com a enzima imobilizada no qual foram testadas a hidrólise contínua de uma solução de lactose e também de soro de leite, em duas temperaturas distintas, a 37 ºC e a 7 ºC, em diferentes fluxos. Além disso, a síntese de GOS foi avaliada variando-se o fluxo de alimentação do reator. Tanto para a hidrólise da lactose como para síntese de GOS, o reator mostrou-se efetivo. Aproximadamente 90 % de conversão de lactose foi alcançada, a 37 ºC, num fluxo de 2,6 mL min^{-1}, a qual se manteve nestas condições durante mais de 15 dias de operação contínua. A produtividade máxima de GOS foi de 484,5 g L^{-1} h^{-1} a 37 ºC num fluxo de 15 mL min^{-1}. Cabe ressaltar que a produtividade poderia ser

incrementada utilizando uma *β*-galactosidase com maior atividade de transgalactosilação, como as de *Aspergillus oryzae*, que também foi utilizada neste trabalho, ou de *Bacillus circulans.* A enzima imobilizada foi também caracterizada em função de seu pH e temperatura ótimos e em função de sua estabilidade térmica. Embora a imobilização em quitosana não tenha modificado o pH e temperatura ótimos, em comparação à enzima livre, os derivados enzimáticos apresentaram uma modificação desejável, a ampliação da faixa de pH e temperatura com maior atividade. Uma vez que reatores são sistemas de operação contínua, ou seja, que estão sempre em presença de altas concentrações de substratos e produtos, achou-se pertinente avaliar a estabilidade térmica da enzima imobilizada em presença de lactose, glicose e galactose. De fato, observou-se uma maior estabilidade térmica em presença de lactose altamente concentrada devido ao fenômeno de exclusão preferencial dos açúcares da superfície da proteína. Porém, observou-se também que a galactose, como inibidora competitiva, é capaz de se ligar ao sítio ativo da enzima e promover uma maior estabilidade térmica em relação à glicose, inibidor não-competitivo da enzima. Assim, pode-se sugerir que, como a lactose também se liga ao sítio ativo da enzima, em baixas concentrações seu efeito protetor advém da sua ligação ao sítio ativo e, em altas concentrações, seu efeito protetor é resultado da hidratação preferencial da molécula de enzima (exclusão preferencial dos açúcares).

Em função da hidrólise da lactose e síntese de galactooligossacarídeos em reator contínuo ter sido bem sucedida, buscou-se uma metodologia para obter macropartículas de quitosana estáveis e aplicáveis industrialmente sem a utilização do glutaraldeído como agente de reticulação, dada sua relativa toxicidade. Assim, avaliou-se a genipina, um composto natural e seguro, como agente de entrecruzamento da quitosana com a enzima adsorvida. A análise de infravermelho demonstrou que o entrecruzamento com genipina apresenta um perfil de bandas distinto da quitosana sem tratamento, o qual também apresentou-se diferente da ativação com glutaraldeído. Ao mesmo tempo, apresentou bandas características da formação de ligações amida entre o suporte, a genipina, e a enzima. Pelos resultados obtidos através da análise por TGA, pode-se concluir que as macropartículas entrecruzadas com genipina diminuíram sua estabilidade térmica em relação às macropartículas de quitosana sem tratamento e às ativadas com glutaraldeído. De qualquer maneira, essa menor estabilidade térmica do suporte não se caracteriza como um problema, já que as temperaturas alcançadas na maioria das reações

enzimáticas são inferiores a 100 ºC. Em função da utilização da *β*-galactosidase de *Aspergillus oryzae*, procedeu-se novamente às caracterizações da enzima imobilizada em relação à carga aplicada ao suporte, pH e temperatura ótimos e estabilidade térmica, tanto da enzima livre, da enzima imobilizada por ativação com glutaraldeído e da enzima imobilizada por entrecruzamento com genipina. Em relação à carga aplicada, a quitosana não ativada parece ter sido capaz de adsorver, a pH 7, uma menor quantidade de proteína do que a quitosana ativada com glutaraldeído, provavelmente devido ao fato de que, a pH 7, a quitosana apresenta pouca quantidade de grupamentos amino carregados positivamente, já que o pK dos grupos amino é em torno de 6,3-6,5. Durante a ativação com glutaraldeído, os grupos amino são substituídos por grupamentos aldeído e, provavelmente por isso, há um aumento na capacidade de carga do suporte. Em relação ao pH ótimo, este deslocou-se de 4,5 (enzima livre) para uma faixa mais ácida, ao redor de 4, tanto para a enzima imobilizada utilizando glutaraldeído, como para enzima imobilizada utilizando genipina, devido à natureza catiônica do suporte. Em relação à temperatura ótima da enzima, não foram observadas diferenças entre a enzima livre e suas formas imobilizadas. A imobilização da *β*-galactosidase de *Aspergillus oryzae* utilizando genipina também foi avaliada em função de sua estabilidade operacional na hidrólise da lactose e da síntese de GOS. A enzima imobilizada foi completamente estável durante 25 bateladas de hidrólise da lactose a 40 ºC, com retenção de 100 % de sua atividade inicial. Além disso, as esferas não apresentaram nenhum tipo de dano físico, o que se caracteriza como uma vantagem em relação às esferas de quitosana ativadas com glutaraldeído, as quais se apresentaram mais frágeis e quebradiças. A síntese de GOS foi otimizada em relação ao pH, temperatura e concentração inicial de lactose. O parâmetro que mais apresentou influência no rendimento em GOS foi a concentração de substrato. O pH mais alto (pH 7) deixou a reação mais lenta (já que retardou o aparecimento da máxima concentração de GOS), uma vez que a atividade hidrolítica é mais acentuada em pH ácido. Por outro lado, temperaturas mais altas aceleraram a reação e a máxima concentração de GOS foi obtida em tempos mais curtos. De modo geral, os rendimentos em GOS obtidos foram semelhantes aos reportados na literatura para esta enzima. Por fim, da mesma maneira que para os experimentos anteriores, a estabilidade térmica da enzima imobilizada em quitosana entrecruzada com genipina foi bastante superior quando em presença de lactose concentrada.

Uma vez que em todos os casos observou-se uma maior estabilidade térmica em presença de lactose, a estabilidade térmica dos biocatalisadores imobilizados foi avaliada também em presença de GOS, simulando assim as reais condições de obtenção deste composto na indústria. Desse modo, avaliou-se a estabilidade térmica da enzima de *A. oryzae* em sua forma livre e imobilizada, e desta última em presença de tampão, solução de lactose e solução de GOS. A maior estabilidade térmica foi observada para a enzima imobilizada em presença de GOS. O efeito protetor advém da imobilização e da presença de osmólitos de maior massa molecular. O fenômeno de exclusão preferencial de osmólitos da superfície da proteína (ou hidratação preferencial) é mais acentuado quando estes apresentam maior massa ou volume molecular, como é o caso dos GOS. Modelos matemáticos também foram avaliados para caracterizar a enzima em função de seus parâmetros cinéticos e termodinâmicos.

Para concluir, o presente trabalho abordou vários aspectos de fundamental importância relativos à hidrólise da lactose e a síntese de galactooligossacarídeos (GOS) catalisada pela enzima β-galactosidase imobilizada. Assim, foram obtidos e caracterizados suportes para imobilização de enzimas à base de quitosana, um polissacarídeo seguro e de relativo baixo custo. Da mesma forma, o entrecruzamento da quitosana com genipina rendeu biocatalisadores biocompatíveis, estáveis e adequados para aplicação em processos em laticínios. De fato, foi possível utilizar satisfatoriamente o biocatalisador imobilizado em um reator de leito fixo para a hidrólise da lactose e para síntese de GOS. Por fim, o estudo da estabilidade térmica da enzima em presença de GOS mostrou que é possível incrementar a temperatura em processos de obtenção de GOS, que geralmente é realizado a 40 ºC, sem prejuízo da atividade enzimática, o que facilita os processos de dissolução da lactose e evita a contaminação microbiana.

Portanto, com os resultados obtidos nesta Tese de Doutorado, pretende-se contribuir no desenvolvimento tecnológico da imobilização de enzimas, em particular para os processos utilizando a enzima β-galactosidase, já que foi possível obter biocatalisadores fáceis de usar, tanto em batelada como em processos contínuos, termicamente e operacionalmente estáveis, biocompatíveis e seguros para utilização em indústrias de alimentos.

Contudo, os resultados obtidos e estudo dos aspectos relacionados aos experimentos realizados, apontam para várias possibilidades de trabalhos futuros. Assim, como **perspectivas** de continuidade deste trabalho, têm-se:

- A aplicação da *β*-galactosidase de *A. oryzae* imobilizada em macropartículas de quitosana entrecruzadas com genipina em diferentes reatores para avaliar tanto a hidrólise da lactose como a síntese de GOS;
- A utilização de outras *β*-galactosidases com maior atividade de transgalactosilação, como a *β*-galactosidase de *Bacillus circulans*, para aumentar o rendimento da síntese de GOS;
- A utilização de *β*-galactosidases de microrganismos probióticos, como os lactobacilos, que rendem GOS com ligações específicas, que "retroestimulam" seletivamente o crescimento/atividade destes mesmos microrganismos;
- A utilização da engenharia de proteínas para obter *β*-galactosidases com mutações específicas para auxiliar na imobilização por regiões escolhidas das enzimas, ou para melhorar estabilidade térmica, ampliar as faixas de pH e temperatura de atividade ótima, bem como para incrementar o rendimento de síntese de GOS através de modificações dirigidas em determinados resíduos de aminoácidos próximos ao sítio ativo, o que torna o ambiente mais hidrofóbico ou mais propenso ao encaixe da lactose ao invés da água como receptor da molécula de galactose;
- A obtenção de GOS a partir de açúcares como a sacarose, para formação de lactosacarose, ou de lactulose, para formação de GOS com distintas propriedades prebióticas;
- Estudo e a obtenção de novos suportes com propriedades desejáveis para aplicação na indústria, como por exemplo, as nanopartículas de quitosana com núcleo magnético que facilitem sua separação do meio reacional.