UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

ESCOLA DE ENGENHARIA

DEPARTAMENTO DE ENGENHARIA QUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

IMOBILIZAÇÃO E ENGENHARIA DE PROTEÍNAS DE GLUCANSUCRASES

TESE DE DOUTORADO

NATÁLIA GUILHERME GRAEBIN

Engenheira de Alimentos

Mestre em Ciência e Tecnologia de Alimentos

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Tese de doutorado submetida ao Programa de Pós-graduação em Engenharia Química como requisito parcial para obtenção do grau de Doutora em Engenharia Química

Orientador: Prof. PhD Marco Antônio Záchia Ayub

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A Comissão Examinadora, abaixo assinada, aprova a defesa da Tese de Doutorado, cujo projeto é intitulado “IMOBILIZAÇÃO E ENGENHARIA DE PROTEÍNAS DE GLUCANSUCRASES”, elaborado por Natália Guilherme Graebin, como requisito parcial para obtenção do grau de Doutora em Engenharia Química.

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DEDICATÓRIA

“Dedico esse trabalho à minha família!

Àqueles que me apoiam todos os dias com muito amor!”

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SUMÁRIO

LISTA DE FIGURAS

CAPÍTULO II

CAPÍTULO III

LISTA DE TABELAS

CAPÍTULO II

CAPÍTULO III

CAPÍTULO IV

CAPÍTULO V

RESUMO

Glucansucrases são enzimas que atuam em reações de síntese de polissacarídeos e oligossacarídeos. Para que esses biocatalisadores sejam aplicados em escala industrial, é desejável ótimas estabilidades térmica e operacional, o que pode ser alcançado com a imobilização de enzimas. Como alternativa aos suportes sólidos amplamente estudados, está a quitosana, polímero que não apresenta toxicidade e possui alta biocompatibilidade e alta afinidade com proteínas. Outra possibilidade promissora na imobilização de enzimas, é a síntese dos agregados enzimáticos entrecruzados (CLEAs), os quais apresentam alta atividade catalítica e alta estabilidade. Contudo, uma peculiaridade das glucansucrases quando produzidas em meio contendo sacarose é a camada de polímero que as envolve, e que bloqueia o acesso aos grupos reativos na superfície da proteína. No caso da expressão heteróloga das glucansucrases em *Escherichia coli* essa dificuldade pode ser contornada. Além disso, o uso da mutagênese sítio-dirigida pode proporcionar modificações de aminoácidos na superfície da enzima, tais como os resíduos Lys, Cys, His, com o intuito de que melhorias na imobilização sejam alcançadas. Sendo assim, na primeira etapa desse trabalho, uma extensa discussão é apresentada em relação às metodologias de imobilização de dextransucrase encontradas na literatura. A seguir, estudos referentes à imobilização da dextransucrase de *Leuconostoc mesenteroides* B-512 F em esferas de quitosana ativadas com glutaraldeído foram realizados. Esse imobilizado apresentou alta atividade catalítica (197 U/g) quando utilizada a carga de proteína de 400 mg/g de suporte. Além disso, observou-se que a imobilização covalente e os açúcares maltose e glicose promoveram proteção à enzima em temperaturas de 40 ºC e 50 ºC. Na etapa seguinte, a produção e a caracterização de CLEAs de dextransucrase de *L. mesenteroides* B-512 F foram investigados. Demonstrou-se que o tratamento com a dextranase foi essencial para a imobilização da glucansucrase e que o isopropanol foi o melhor agente precipitante. Os CLEAs apresentaram pH e temperatura ótimos de 3,0 e 60 ºC, respectivamente, enquanto que a dextransucrase imobilizada nas esferas de quitosana funcionalizada com glutaraldeído apresentaram os valores de 4,5 e 20 ºC. Ambas formas imobilizadas apresentaram boa estabilidade operacional na síntese de oligossacarídeos uma vez que após 10 ciclos, 40 % de atividade residual foi observada. Por fim, estão apresentados estudos sobre a modelagem das estruturas tridimensionais e a mutagênese sítio-dirigida das glucansucrases DSR-S vardel Δ4N and ASR C-APY del. Os modelos preditos demonstraram boa qualidade e a mutagênese sítio-dirigida não promoveu perdas significativas na atividade enzimática dos mutantes. Somente o mutante DSR_S326C mostrou-se inativo. Os resultados obtidos sugerem que a imobilização da dextransucrase foi satisfatória e que cada técnica possibilita diferentes características ao imobilizado. Além disso, os imobilizados foram adequados para síntese de dextrana e oligossacarídeos.

Palavras-chave: Glucansucrases; imobilização de enzimas; mutagênese sítio-dirigida; oligossacarídeos.

ABSTRACT

Glucansucrases are enzymes that catalyze the synthesis of polysaccharides and oligosaccharides. In order to assure continuous processing and reuse of the biocatalyst in industrial applications, enzyme immobilization techniques are required to promote good thermal and operational stabilities. Among the several solid supports for enzyme immobilization, chitosan shows interesting properties because it is non-toxic, it is biocompatible, and it has high protein affinity. Other possibility is the production of cross-linked enzyme aggregates (CLEAs), which presents high catalytic activity and good stability. However, glucansucrases have a particularity when produced in sucrose medium, since a polymer layer surrounds the protein, blocking the access to reactive groups on the enzyme surface. To overcome this problem, it is possible to make the heterologous production of glucansucrases in *Escherichia coli*. Likewise, the site-directed mutagenesis may promote changes in the amino acids located on the surface to improve immobilization parameters. Therefore, this work aimed to discuss the several techniques applied for dextransucrase immobilization, and to design new immobilized biocatalysts. In a first step, it is presented a review about the distinct immobilization methodologies for dextransucrase. In a second study, an investigation about dextransucrase from *Leuconostoc mesenteroides* B-512 F immobilized on glutaraldehyde-activated chitosan particles was carried out. The novel immobilized biocatalyst showed 197 U/g (400 mg/g dried support) of catalytic activity. The covalent immobilization promoted protection against enzyme damages at 40 ºC and 50 ºC, whereas maltose and glucose acted as stabilizers. Furthermore, it was studied the production and characterization of CLEAs dextransucrase from *L. mesenteroides* B-512 F. It was demonstrated that dextranase treatment was crucial for immobilization. Isopropanol was chosen as the best precipitant agent. CLEAs presented optimal pH and temperature of 3.0 and 60 ºC, respectively, whereas it was found values of 4.5 e 20 ºC for dextransucrase immobilized on glutaraldehyde-activated chitosan particles. Both immobilized biocatalysts showed good operational stability in the oligosaccharides synthesis, exhibiting 40 % of residual activity after 10 cycles. Finally, the study concerning the homology modeling and site-directed mutagenesis of glucansucrases DSR-S vardel Δ4N and ASR C-APY del is presented. The predicted models showed good quality and it has been demonstrated that the site-directed mutagenesis did not promote significant losses in the variant enzyme activities. Only one mutant (DSR_S326C) had shown no dextransucrase activity. The results obtained in this work suggest that the immobilization of dextransucrase was satisfactory, also showing that each technique promotes different characteristics to the immobilized biocatalyst. Besides, these immobilized enzymes were feasible for the synthesis of dextran and oligosaccharides.

Keywords: Glucansucrases; enzyme immobilization; site-directed mutagenesis; oligosaccharides.

INTRODUÇÃO

As glucansucrases são enzimas que catalisam, principalmente, reações de polimerização para síntese de polissacarídeos e, em presença de aceptores, para síntese de oligossacarídeos (Robyt e Walseth, 1978; Robyt, 1996). Essas enzimas são pertencentes à família GH 70 e são produzidas por bactérias ácido-lácticas dos gêneros *Leuconostoc*, *Lactobacillus,* e *Weissella*, reconhecidas como GRAS (*Generally Recognized as Safe*) (Bourdichon *et al*., 2012). Os produtos dessas reações são de interesse para as indústrias de alimentos e cosmética (Koepsell *et al*., 1953; Eggleston e Côté, 2003), tendo em vista seu papel texturizante, estabilizante e emulsificante, além de sua ação prebiótica (Valette *et al.*, 1993; Gibson e Roberfroid, 1995; De Vuyst e Degeest, 1999; Fernando *et al.*, 2011; Madhukumar e Muralikrishna, 2012).

A síntese desses compostos pode ser realizada por via química ou por via enzimática. Entretanto, a síntese enzimática proporciona algumas vantagens, tais como maior seletividade e maior eficiência do processo, bem como menor oneração em processos *downstream*. Além disso, tendo em vista a produção industrial desses compostos, é de interesse que o biocatalisador possa ser facilmente recuperado e que possa ser aplicado diversas vezes na reação. Desse modo, a imobilização das glucansucrases apresenta-se como uma solução capaz de promover vantagens para aplicação em larga escala (Sheldon e van Pelt, 2013).

Na literatura, os estudos sobre imobilização de glucansucrases em sua maioria versam sobre a dextransucrase, a qual é mais imobilizada com a técnica de encapsulamento (Reischwitz *et al.*, 1995; Alcalde *et al.*, 1999; Berensmeier *et*

al., 2006; Gómez de Segura *et al.*, 2006; Kothari *et al.*, 2012). Altos rendimentos de imobilização e simplicidade de protocolo são descritos como algumas das vantagens dessa metodologia. Entretanto, problemas como restrições difusionais e perda de atividade catalítica também são destacados (Dols-Lafargue *et al.*, 2001; Tanriseven e Doğan, 2002; Gómez de Segura *et al.*, 2004; Parlak *et al.*, 2013). Dentre os suportes mais utilizados estão a agarose e o alginato (Brady e Jordaan, 2009). Como alternativa aos suportes comerciais, que são geralmente muito caros, surge a síntese de suportes sólidos a partir de resíduos da indústria, como a quitosana. Essa é um polímero proveniente da quitina obtido de exoesqueletos de crustáceos e de paredes celulares de fungos (Santos *et al.*, 2002), capaz de ser funcionalizada para que seja um suporte sólido empregável na imobilização covalente de enzimas (Krajewska, 2004). Lisozima (Liburdi *et al.*, 2016), lipase (Liu *et al.*, 2016), β-frutofuranosidase (Lorenzoni *et al.*, 2015) e β-galactosidase (Klein *et al.*, 2012) são alguns exemplos de biocatalisadores aplicados na indústria de alimentos, que já foram imobilizados nesse polímero e que apresentaram boa estabilidade operacional.

Outra possibilidade é o desenvolvimento de agregados enzimáticos entrecruzados (*Cross-linked enzyme aggregates* – CLEAs). Uma vez que para a síntese dos CLEAs não há necessidade do uso de suportes sólidos e são descritas na literatura, alta atividade catalítica e alta estabilidade do biocatalisador (Sheldon *et al.*, 2005), essa técnica apresenta-se como promissora. Alguns CLEAs já foram desenvolvidos a partir de diferentes enzimas, tais como lipases e pectinases (Cruz *et al.*, 2012; Goetze *et al.*, 2017).

Contudo, umas das peculiaridades dessas enzimas quando produzidas de forma nativa, isto é, pelas bactérias do gênero *Leuconostoc*, é que uma

camada de polissacarídeo acaba também sendo sintetizada no entorno da proteína, uma vez que o meio de cultivo contém sacarose, substrato do biocatalisador. Para o caso da dextransucrase, a dextrana que a envolve, acaba promovendo estabilidade ao biocatalisador (Robyt e Walseth, 1978; Miller e Robyt, 1984), porém impossibilita ligações dos grupos reativos da enzima àqueles do suporte, ou até mesmo entre os próprios grupos reativos da enzima, como no caso para imobilização via CLEAs. Para o caso da dextransucrase, o uso de dextranase, enzima capaz de hidrolisar a dextrana, tem sido empregado para permitir sua imobilização covalente ao suporte (Alcalde *et al.*, 1999; Gómez de Segura *et al.*, 2004). Contudo, para a alternansucrase, o uso da enzima hidrolítica não é uma solução, visto que sua ação é ineficaz (Côté, 1992).

Sendo assim, faz-se necessário estudos que minimizem essas dificuldades, como é o caso da expressão heteróloga das glucansucrases em *Escherichia coli*, visto que o crescimento celular e, consequentemente a produção da enzima, dá-se em meios de cultivos sem sacarose (Joucla *et al.*, 2006; Moulis *et al.*, 2006). Além disso, o uso da mutagênese sítio-dirigida pode proporcionar modificações de aminoácidos na superfície da enzima, tais como os resíduos Lys, Cys, His ou até mesmo, para inclusão de caudas His na sequência proteica, com o intuito de que melhorias na imobilização sejam alcançadas (Hemdan *et al.*, 1989; Abian *et al.,* 2004; Ovsejevi *et al.*, 2009).

Desta forma, o presente trabalho teve como objetivo principal investigar a imobilização de glucansucrases por diferentes metodologias e o emprego da mutagênese sítio-dirigida. Além disso, pretendeu-se como objetivos específicos:

- Estudar a imobilização da dextransucrase de *L. mesenteroides* B-512 F covalentemente em esferas de quitosana funcionalizadas com glutaraldeído;
- Desenvolver *cross-linked enzyme aggregates* (CLEAs) com a dextransucrase de *L. mesenteroides* B-512 F;
- Avaliar a influência das diferentes técnicas de imobilização em relação à pH e T ótimos, estabilidades térmica e operacional;
- Investigar a influência da enzima dextranase na imobilização da dextransucrase de *L. mesenteroides* B-512 F;
- Estudar a síntese de oligossacarídeos com os dois tipos de imobilizados da dextransucrase de *L. mesenteroides* B-512 F em batelada;
- Investigar a mutagênese sítio-dirigida da dextransucrase DSR-S vardel Δ4N e da alternansucrase ASR C-APY del, a fim de melhorar as propriedades de imobilização;

A presente Tese de Doutorado foi desenvolvida no Laboratório de Biotecnologia do Instituto de Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul e no *Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés*, do *Institut National des Sciences Appliquées* em Toulouse na França.

O trabalho está apresentado na forma de artigos científicos de acordo com as normas estabelecidas pelo Programa de Pós-Graduação em Engenharia Química. No Capítulo I, tem-se uma revisão bibliográfica em português abordando os principais pontos propostos da tese. No Capítulo II, uma extensa

revisão sobre as diferentes formas de imobilização de glucansucrases está exposta. A seguir nos Capítulos de III a V, estão apresentados os resultados, na forma como foram submetidos à publicação em periódicos internacionais. Por fim, no Capítulo VI, tem-se as considerações finais do trabalho, com as principais conclusões obtidas e as perspectivas para estudos futuros.

CAPÍTULO I – REVISÃO BIBLIOGRÁFICA

1.1 Glucansucrases

Glucansucrases, também conhecidas como glicosiltransferases, são enzimas extracelulares, membros da família GH 70, as quais possuem domínios catalíticos semelhantes àquelas pertencentes às famílias GH 13 e GH 77 (Robyt e Walseth, 1978; Henrissat e Davies, 1997). A família GH 70 compreende as enzimas dextransucrase (EC 2.4.1.5), alternansucrase (EC 2.4.1.140), reuteransucrase, α-4,6-glucanotransferase, dextransucrase α-1,2-ramificada, e α-4,3-glucanotransferase, e sua tríade catalítica é composta por dois ácidos aspárticos e um ácido glutâmico (http://www.cazy.org/GH70.html/). Essas enzimas são produzidas, majoritariamente, por bactérias ácido-láticas, tais como aquelas pertencentes aos gêneros *Leuconostoc*, *Lactobacillus* e *Weissella,* consideradas GRAS (*Genereally Recognized as Safe*), isto é, seguras para aplicação nas indústrias de alimentos e farmacêutica (Bourdichon *et al.*, 2012). Como produtoras de dextransucrase, destacam-se as bactérias *L. mesenteroides* NRRL B-512 F, *L. mesenteroides* B-512 FMCM, *L. mesenteroides* NRRL B-1299 e *L. citreum* KACC 91348P (Neely e Nott 1962; Kobayashi e Matsuda, 1977; Ryu *et al.*, 2000; Otgonbayar *et al.*, 2011). As espécies *L. mesenteroides* NRRL B-1355, *L. mesenteroides* NRRL B-1501 e *L. mesenteroides* NRRL B-1498, por sua vez, são reconhecidas produtoras de alternansucrase (Jeanes *et al.*, 1954; Seymour e Knapp, 1980).

Esses biocatalisadores são responsáveis pela catálise de 4 tipos de reação: (i) polimerização (Ebert *et al.*, 1968; Buchholz e Monsan, 2003), (ii)

hidrólise, em altas concentrações de água no meio reacional, (iii) reação com aceptores (Robyt e Walseth, 1978) e (iv) desproporcionalização (Binder *et al.*, 1983), dentre as quais as reações (i) e (iii) possuem mais destaque na literatura.

Nas reações de polimerização, quando o biocatalisador é a dextransucrase, o polímero a ser formado é denominado dextrana, e esse possui maioria de ligações α-1,6, com ramificações em ligações α-1,2, α-1,3, α-1,4. Particularmente, a espécie *L. mesenteroides* NRRL B-1299 é capaz de sintetizar dextranas compostas com, aproximadamente, 30 % de ligações α-1,2 (Jeanes *et al.*, 1954; Seymour e Knapp, 1980), o que confere potencial prebiótico (Valette *et al.*, 1993). Já quando a alternansucrase é responsável pela catálise da reação, alternana é sintetizada e essa possui em sua cadeia principal ligações α-1,6 e α-1,3 alternadas, com ramificações ligadas por α-1,3. Esses polímeros possuem distintas características. As dextranas possuem massa molar de 10^5 a 10^9 Da e são facilmente hidrolisadas por dextranases. Já as alternanas possuem massa molar de até 10^7 Da (Joucla *et al.*, 2006), são resistentes à ação de enzimas hidrolíticas (amilases e dextranases) e ainda, possuem grande solubilidade em água (Côté, 1992).

Em relação às reações com aceptores, os produtos têm sua composição variada de acordo com os substratos utilizados na reação de síntese, como, por exemplo, os açúcares glicose, frutose, maltose, D-manose, celobiose (Côté e Leathers, 2005; Rabelo *et al.*, 2006; Erhardt *et al.*, 2008; Kim e Day, 2008; Lee *et al.*, 2008; Da Silva *et al.*, 2012). Além disso, diferentes ligações são descritas nesses oligossacarídeos, o que é dependente da enzima que os produz.

Os polissacarídeos produtos das glucansucrases devido às suas características são relatados como emulsificantes, estabilizantes e texturizantes.

Além disso, são compostos utilizados como substitutos de plasma sanguíneo e como suporte de cromatografia (De Vuyst e Degeest, 1999). Os oligossacarídeos, por sua vez, são referidos por seu potencial prebiótico, isto é, não são fermentados no estômago e, portanto, seletivamente digeridos por *Bifidobacteria* e *Lactobacillus* no intestino, o que estimula o crescimento da microbiota intestinal benéfica (Valette *et al.*, 1993; Gibson e Roberfroid, 1995; Fernando *et al.*, 2011; Madhukumar e Muralikrishna, 2012). Diferentes sucos de frutas, como laranja, mandarim, e caju, já foram utilizados como substrato para a produção de oligossacarídeos por dextransucrase, em virtude de suas altas concentrações de açúcares, o que torna esses produtos menos calóricos e benéficos à saúde daqueles que os consomem (Da Silva *et al.*, 2012; Nguyen *et al.*, 2015; Nguyen *et al.*, 2015). Sendo assim, vê-se que há amplo espectro de aplicação desses produtos nas indústrias farmacêutica e de alimentos (Koepsell *et al.*, 1953; Eggleston e Côté, 2003).

1.2 Imobilização de enzimas

A imobilização de enzimas consiste na interação e/ou no confinamento da enzima em um suporte. Essa técnica tem como objetivos promover a reutilização dos biocatalisadores, e, consequentemente facilitar a recuperação de produtos, além de minimizar a produção de efluentes, mantendo o potencial catalítico encontrado na enzima livre (Guisán, 2006).

De acordo com o tipo de imobilização, é possível promover maior atividade catalítica do biocatalisador, assim como maiores especificidade e estabilidade (Guisán, 2006; Mateo *et al.*, 2007). Além disso, enzimas

imobilizadas podem ser aplicadas em biorreatores, em processos em batelada ou em processos contínuos (López-Gallego *et al.*, 2005; Guisán, 2006). Contudo, algumas desvantagens devem ser consideradas, tais como a alteração da conformação da enzima nativa, o custo do suporte e a perda da atividade catalítica durante o processo de imobilização (Arroyo, 1998).

A imobilização por encapsulamento consiste no confinamento da molécula de enzima em uma matriz física. Embora seja um excelente método para manter a conformação da molécula de enzima, bem como para proteger fisicamente o biocatalisador de condições adversas do meio reacional, tem como desvantagem os recorrentes problemas de transferência de massa, o qual está relacionado com a difícil difusão de substrato e/ou produto, através dos poros do suporte (Nunes e Marty, 2006). Agarose, alginato, quitosana e celulose são exemplos de polímeros utilizados para o encapsulamento de enzimas (Krajewska, 2004; Brady e Jordaan, 2009).

Outros tipos de imobilização são aqueles nos quais a enzima é ligada por ligação covalente – unipontual ou multipontual – ou é adsorvida. Os grupos reativos, geralmente, envolvidos na imobilização por ligação covalente são o aldeído, o epóxi e o glioxil. Os grupos glioxil são aldeídos lineares que formam bases de Schiff com os resíduos aminados das proteínas (Blanco e Guisán, 1989). Como exemplo, há o glioxil-agarose que possui a vantagem de direcionar a proteína a partir da ligação com sua área rica em resíduos Lys, o que permite intensa interação multipontual entre a enzima e o suporte, garantindo maior estabilidade (Mateo *et al.*, 2005; Grazu *et al.*, 2006; Mateo *et al.*, 2006). Os suportes com os grupamentos epóxi, por sua vez, apresentam a possibilidade

de interação com a Lys, como também outros resíduos de aminoácidos na superfície da enzima (Guisán, 1988; Mateo *et al.*, 2000).

A adsorção de enzimas em resinas iônicas é amplamente utilizada, por ser uma técnica rápida, simples, de baixo custo e, ainda, permitir fácil regeneração do suporte quando há perda da atividade catalítica do biocatalisador imobilizado (Girelli e Mattei, 2005; Mateo *et al.*, 2007). Suportes como MANAE-agarose, DEAE-agarose, Q-Sepharose adsorvem ionicamente enzimas (Cunha *et al.*, 2008; Filho *et al.*, 2008), por possuírem grande número de cargas positivas ou negativas. Uma vez que esses suportes sólidos interagem fortemente com o biocatalisador, modulam sua atividade catalítica, protegendo-o de distorções conformacionais, geralmente ocasionadas pelo uso de solventes e de temperaturas inadequadas (Mateo *et al.*, 2007).

Dentre os métodos de imobilização de biocatalisadores, um dos mais recentes é o desenvolvimento de agregados enzimáticos entrecruzados (*cross-linked enzyme aggregates* – CLEAs). Esses agregados enzimáticos são preparados a partir da precipitação das moléculas de enzima, por solventes orgânicos, polímeros não-iônicos ou sais inorgânicos, e logo após, pela ligação dessas moléculas utilizando um reagente bifuncional, a fim de se obter macropartículas sem o uso de suportes sólidos. Como vantagens, destaca-se a obtenção de alta atividade catalítica, além de alta estabilidade, aliado ao baixo custo de produção dos CLEAs em virtude da não oneração pela ausência de suportes. Além disso, a partir da precipitação concomitante de duas ou mais diferentes enzimas, é possível a obtenção de combi-CLEAs, os quais surgem como solução para reações enzimáticas em cascata (Sheldon *et al.*, 2005, Sheldon e van Pelt, 2013).

1.3 Imobilização sítio-dirigida

A imobilização sítio-dirigida é o direcionamento da proteína frente ao suporte a ser utilizado, diferentemente da técnica convencional de imobilização na qual a enzima é randomicamente ligada à matriz. Conforme já mencionado no item 1.2, a imobilização possui inúmeras vantagens em relação à especificidade e estabilidade enzimática, entretanto é possível que haja alteração da conformação da enzima e mal posicionamento da proteína, levando à perda de atividade catalítica (Arroyo, 1998). Além disso, a orientação das enzimas é crítica quando o sítio-ativo da enzima deve estar em uma posição específica no sistema imobilizado, como é o caso das enzimas redox (Freire *et al.*, 2003) e daquelas que utilizam substratos de tamanho elevado, como enzimas do grupo das ATPases e enzimas do coalho (Pessela *et al.*, 2004; Jeong *et al.*, 2007).

Estudos relatam que uma determinada orientação ou até mesmo o aumento da intensidade da reação entre enzima/suporte podem promover a estabilidade da proteína, o que é de extremo interesse no caso de aplicações industriais (Mateo *et al.*, 2007). Para que seja possível o direcionamento da enzima no sistema imobilizado, diferentes técnicas de imobilização podem ser utilizadas. Como exemplo, estão os suportes capazes de imobilizar covalentemente proteínas, tais como aqueles compostos por glioxil. Em um primeiro momento, em pH neutro, as enzimas se imobilizam via o terminal amino, se esse estiver exposto na superfície. Logo após, é possível que os grupos ε-aminos dos resíduos Lys reajam com o suporte. Outra possibilidade de orientação é para aquelas enzimas que possuem alta densidade de grupos

reativos em determinada parte de sua superfície, a qual acaba direcionando a imobilização em suportes de troca iônica, de adsorção hidrofóbica e de metais quelados (Pessela *et al*., 2004). Da mesma forma, as condições de imobilização, como alta força iônica, são capazes de levar a uma orientação específica da proteína (Pessela *et al.*, 2007). Suportes com grupamentos glioxil e epóxi são alternativas para ligações multipontuais, quando o sistema enzima/suporte já tem interação estabelecida após primeira aproximação por grupos reativos (Klibanov, 1979; Mateo *et al.*, 2006).

A mutagênese sítio-dirigida apresenta-se com uma maneira versátil para permitir que mesmos suportes e técnicas sejam empregados para uma mesma enzima, gerando diferentes orientações possíveis no momento da imobilização. Entretanto, alguns requisitos são necessários para utilização dessa técnica, tais como conhecimento da estrutura tridimensional da enzima ou análoga e conhecimento das ferramentas de manipulação genética do microrganismo a ser utilizado (Hernandez e Fernandez-Lafuente, 2011). Além de modificações de aminoácidos na superfície enzimática que possam orientar a imobilização, resíduos Lys, Cys, His ou até mesmo, caudas His podem ser incluídos na sequência proteica (Hemdan *et al.*, 1989; Abian *et al.*, 2004; Ovsejevi *et al.*, 2009).

Na literatura, muitos trabalhos versam sobre essa técnica no estudo do papel de alguns aminoácidos das glucansucrases importantes para aumento de estabilidade da enzima e de seu desempenho na reação de transglicosilação (Moulis *et al*., 2006). Particularmente para melhorias na imobilização, a dextransucrase de *L. mesenteroides* B-512 FMC truncada foi fusionada à enzima glutationa S-transferase no N-terminal para que os aminoácidos acrescidos à

estrutura por essa fusão fossem capazes de se imobilizar aos grupos reativos do suporte Eupergit® C (Parlak *et al.*, 2014). Enquanto que para a dextransucrase truncada, o rendimento de imobilização foi de 100 %, a atividade recuperada foi de somente 3 % (Parlak *et al.*, 2013). Já para a enzima truncada fusionada, o rendimento de imobilização manteve-se 100 %, porém a atividade recuperada chegou a 83,3 % (Parlak *et al.*, 2014).

1.4 Imobilização de glucansucrases

Na literatura, os relatos sobre imobilização de glucansucrases em sua maioria são sobre a dextransucrase, sendo o encapsulamento o mais encontrado na literatura. Para esse método, rendimentos de imobilização de 57 a 98 % são apresentados (Reischwitz *et al.*, 1995; Alcalde *et al.*, 1999; Berensmeier *et al.*, 2006; Gómez de Segura *et al.*, 2006; Kothari *et al.*, 2012). LentiKats® (Gómez de Segura *et al.*, 2003), esferas, cápsulas, fibras de alginato de cálcio (Kubik *et al.*, 2004; Kothari *et al.*, 2012), carreadores de celulose (Petronijević *et al.*, 2007) são exemplos de alguns suportes sólidos utilizados para o encapsulamento de dextransucrases.

Dentre as vantagens desse tipo de técnica, estão a facilitada retenção do complexo dextransucrase-dextrana nos géis, em virtude de seu tamanho (Reischwitz *et al.*, 1995), e a simplicidade do protocolo. Como desvantagens, há os problemas difusionais, efeito ocasionado pela camada do polissacarídeo ligado à enzima e a consequente redução da atividade catalítica (Berensmeier *et al.*, 2004). Em operação contínua, verifica-se baixa estabilidade operacional,

uma vez que há aumento do tamanho das esferas, em virtude da produção de dextranas e dos oligossacarídeos aprisionados dentro do suporte, podendo haver ruptura dos mesmos, bem como perda de proteína para o meio reacional (Dols-Lafargue *et al.*, 2001; Tanriseven e Doğan, 2002; Gómez de Segura *et al.*, 2004; Parlak *et al.*, 2013).

Alguns autores investigaram a imobilização covalente de dextransucrases em Bio-Gel P-2 (Robyt *et al.*, 1974), gel de poliacrilamida, membranas de acetato de celulose, fibras ocas de polisulfona (Robyt e Taniguchi, 1976; Robyt e Corrigan, 1977), e sílicas porosas ativadas (Kaboli e Reilly, 1980; Monsan e Lopez, 1981; Monsan *et al.*, 1987). Esses estudos apresentaram baixos rendimentos de imobilização, além de baixa estabilidade operacional, provavelmente em virtude do complexo dextransucrase/dextrana, o qual impede a ligação dos grupos reativos da enzima com os grupos reativos dos suportes (Funane *et al.,* 2005; Robyt *et al.,* 2008; Parlak *et al.*, 2013). Com o objetivo de disponibilizar os grupos reativos desses aminoácidos presentes na superfície das dextransucrases para que a imobilização covalente seja obtida, autores avaliaram a hidrólise desse complexo utilizando dextranase (Alcalde *et al.*, 1999; Gómez de Segura *et al.*, 2004). Tal técnica permitiu a imobilização covalente da dextransucrase em suportes epóxi comerciais, como Eupergit® C e Eupergit® C 250L, e em sílica X030 ativada (γ-aminopropil trimetoxisilano/glutaraldeído) (Alcalde *et al.*, 1999; Gómez de Segura *et al.*, 2004).

Estudos sobre a imobilização das glucansucrases alternansucrase, reuteransucrase, α-4,6-glucanotransferase, dextransucrase α-1,2-ramificada, e α-4,3-glucanotransferase não são relatados na literatura. As enzimas α-4,6-glucanotransferase, dextransucrase α-1,2-ramificada, e α-4,3-

glucanotransferase vêm sendo caracterizadas e ainda não possuem número EC definido.

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CAPÍTULO II – IMMOBILIZATION OF GLYCOSIDE HYDROLASES FAMILIES GH 1, GH 13, AND GH 70: STATE OF THE ART AND PERSPECTIVES

O Capítulo II, intitulado *Immobilization of glycoside hydrolases families GH 1, GH 13, and GH 70: state of the art and perspectives*, está apresentado na forma de artigo científico, publicado no periódico *Molecules* (DOI: 10.3390/molecules21081074).

Nesse trabalho, uma ampla revisão acerca das diferentes técnicas de imobilização de β-glicosidases (família GH 1), ciclodextrina-glicosiltransferase (família GH 13), α-amilases (família GH 13) e dextransucrases (família GH 70) foi realizada. Aspectos como vantagens, desvantagens e particularidades em relação à cada metodologia foram pontuados.

Immobilization of glycoside hydrolases families GH 1, GH 13, and GH 70: state of the art and perspectives

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ABSTRACT

Glycoside hydrolases (GH) are enzymes capable to hydrolyze the glycosidic bond between two carbohydrates or even between a carbohydrate and a non-carbohydrate moiety. Because of the increasing interest for industrial applications of these enzymes, the immobilization of GH has become an important development in order to improve its activity, stability, as well as the possibility of its reuse in batch reactions and in continuous processes. In this review, we focus on the broad aspects of immobilization of enzymes from the specific GH families. A brief introduction on methods of enzyme immobilization is presented, discussing some advantages and drawbacks of this technology. We then review the state of the art of enzyme immobilization of families GH 1, GH 13, and GH 70, with special attention on the enzymes β-glucosidase, α-amylase, cyclodextrin glycosyltransferase, and dextransucrase. In each case, the immobilization protocols are evaluated considering their positive and negative aspects. Finally, the perspectives on new immobilization methods are briefly presented.

Keywords: Enzyme immobilization; glycoside hydrolases; β-glucosidase; α-amylase; cyclodextrin glycosyltransferase; dextransucrase.

1. Introduction

Carbohydrates are important natural molecules presented in their free moieties or in association in glycoproteins, glycolipids, and polysaccharides, playing fundamental roles in the cell physiology and development of all organisms [1]. The enzymes that cleave or, inversely, mediate the ligation of glycosidic bonds of glycoconjugates, oligosaccharides, and polysaccharides can be classified by two different systems. The IUBMB Enzyme Nomenclature of the Enzyme Commission (EC) is based on distinct enzymatic activities, substrate specificity and occasionally on their molecular mechanism [2]. In this system, the EC 3.2.1 comprises the enzymes that hydrolyze O- and S-glycosyl compounds and comprises enzymes from EC 3.2.1.1 through EC 3.2.1.196, with some deletions and reclassifications. The second system of enzyme classifications is based on the carbohydrate-active enzymes (CAZy) database (http://www.cazy.org/), where enzymes are classified into several families based on their amino acid sequence similarities [3, 4]. Currently, the Glycoside Hydrolases (GH) family classification from CAZy extends from GH 1 to GH 135, with 190 different enzyme activities (based on the EC code), mainly glycosidases from EC 3.2.1, but also some glycotransferases from EC 2.4.1.

Among the enzymes from the GH families, some, such as amylase, cellulase, pectinase, hemicellulase, glucansucrase, lactase, invertase, and β-glucosidase, are of great interest to various industries. These enzymes are well studied in the literature and there are several reviews dealing with their catalytic mechanism and properties [5-9].

Because of their biological origin and function in live organisms, enzymes

are sometimes unsuitable for direct industrial process applications. In natural systems, enzymes are usually soluble, are inhibited by substrates and products, show low stability, and do not possess an ideal catalytic characteristic when applied to non-natural substrates. These disadvantages can be overcome by their immobilization in appropriate supports and matrices [10, 11]. Enzyme immobilization is defined as the process of confinement of the enzyme molecule into or onto a phase (matrix/support) different from that of substrates and products [12]. Immobilized enzymes can be applied in different reactor configurations, allowing easy reaction control, avoiding product contamination of the enzyme, which is an important property in food technology allowing their reuse over many reaction cycles [10]. Additionally, immobilization can improve biocatalyst stability and can modify the enzymatic activity, specificity, as well as enantio and regioselectivities [13].

Considering all these aspects, the objective of this review is to discuss on the protocols used for immobilization of enzymes from the GH families GH 1, GH 13, and GH 70, more specifically β-glucosidases, α-amylase, cyclodextrin glycosyltransferase, and dextransucrases. Initially, we present an overview on the types of immobilization techniques, and then proceed to cover the state of the art of enzyme immobilization of the aforementioned family of enzymes. Finally, we discuss on the perspectives of new developments in the field of enzyme immobilization for these three GH families.

2. Types of enzyme immobilization

Enzyme immobilization methods can be divided into three categories: enzyme molecule attachment to a solid support, entrapment into a matrix, and molecule cross-linking [11].

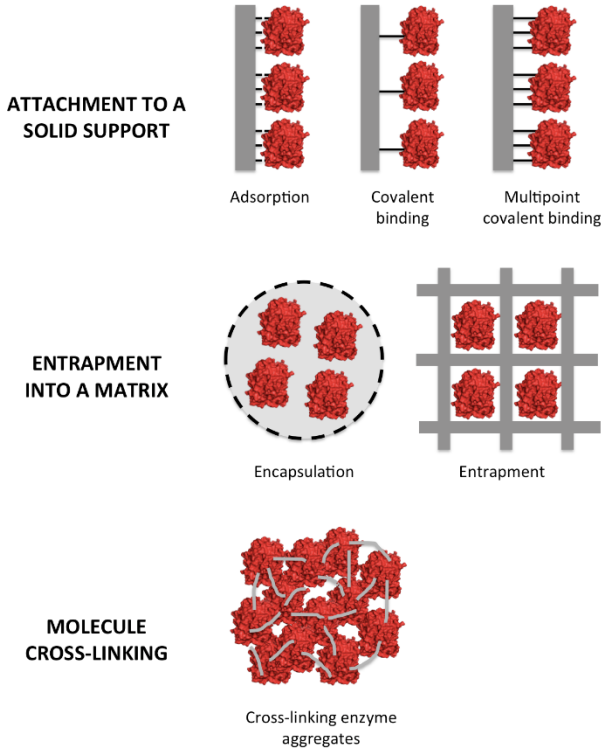

Fig. 1. Types of immobilization

The attachment to a solid support can be reversible or irreversible and it can be achieved using a broad variety of chemical and physical methods. Entrapment consists in the retention of enzyme molecules into a polymer matrix by covalent or non-covalent bonds. Cross-linking of enzymes employs bi-functional reagents to prepare carrier-free macroparticles [12, 14-22]. A schematic representation of the types of immobilization is presented in Fig. 1.

There are several reviews dealing with enzyme immobilization, explaining in details all advantages and disadvantages of each protocol [10-12, 17-19, 22-

24], chemical [25-27], and genetic [28, 29] modifications of enzymes in order to improve immobilization, one-step purification and immobilization of enzymes [30], stabilization of protein quaternary structure by immobilization [31], the effect of different supports on enzyme properties [32-36], as well as the different enzymatic reactors using immobilized enzymes [37, 38].

Considering that the focus of this review is to discuss on the immobilization of glycoside hydrolases and the fact that methods of immobilization have been extensively reviewed, in this paper we will attain to present the basic concepts of each type of immobilization, as mentioned before, in order to better elucidate the discussion that will follow.

2.1. Enzyme attachment to a solid support

The use of pre-existing supports for enzyme immobilization is possibly the most used technique. As mentioned before, the enzyme attachment to a solid support can be reversible or irreversible. The cost of the support will impact the overall cost of the final biocatalyst, thus must be considered during the design of the biocatalyst [10]. The reversible immobilization is achieved by adsorption of the protein on the surface of the support [14]. Adsorption uses physical interactions between the support and enzyme, including van der Waals forces, hydrophobic and ionic interactions, and hydrogen bonding. The binding is generally weak and does not change the native structure of the enzyme.

Irreversible immobilization is obtained by covalent linkage of the enzyme to the support. The reaction occurs between amino acids on the enzyme surface

and reactive groups placed on the support surface, being glutaraldehyde the reactive chemical most used for this covalent immobilization of the enzyme [39]. Glutaraldehyde is a bi-functional reagent that is used to activate the support and also to react with the enzyme, generally involving primary amine groups of the protein, although it may eventually also react with other groups (thiols, phenols, and imidazoles). Two other groups are frequently used to stabilize the 3D structure of the enzyme by multipoint covalent attachment: glyoxyl and epoxide [40, 41]. Both groups can react with primary amine groups of Lys at pH 10, promoting several linkages between enzyme and support, which produces high stabilization factors when compared to the soluble enzyme [13, 20].

The use of adsorption presents the advantage of support recovery after the loss of enzyme activity, allowing the immobilization of new enzyme load. However, there is the risk of enzyme leakage and product contamination by the enzyme. On the other hand, covalent attachment usually provides better stability than adsorption, specially using multipoint covalent attachment. Nevertheless, when the enzyme losses its activity, all preparation (enzyme plus support) should be discarded.

2.2. Entrapment

The technique of entrapment consists in the inclusion of an enzyme molecule into a polymer network, such as an organic polymer or a silica sol-gel, or a membrane device, such as a hollow-fiber or a microcapsule. The physical restraints are generally weak to prevent the enzyme leakage. Therefore, additional covalent attachment is often required. Entrapment generally requires

the synthesis of the polymeric matrix in the presence of the enzyme [11, 22]. Entrapment also protects enzymes from the direct contact with the environment, minimizing the effects of gas bubbles, mechanical shear, and hydrophobic solvents, but it has the drawback of mass transfer limitations and producing low enzyme loadings [17].

A common method of entrapment consists in the use of silica sol–gel matrices formed by hydrolytic polymerization. The polymer porosity, its network structure, surface functionalities, and particle size, can all be modified by adjusting the polymerization conditions [42].

Alginate and chitosan are other two natural polymers that are often used for enzyme entrapment. Chitosan can be applied to obtain covalent immobilization, however, the enzyme may also be entrapped into the gel if mixed with chitosan prior to the cross-linking reaction [43]. Finally, entrapment by nanostructured supports like electrospun nanofibers and pristine materials are being tested, making possible the application of immobilized enzymes in a wide-range of biocatalytic processes in the field of fine chemistry, biomedicine, biosensors, and biofuels [12].

The main advantage of the entrapment is the physical protection against harsh conditions, such as high temperature, extreme pH, gas bubbles, and the use of solvents in the reaction medium. In the opposite, the barrier formed between the enzyme and the carrier may promote diffusional limitations, leading to low productivities in the system.

2.3. Cross-linking of enzymes

Cross-linked enzyme crystals (CLECs) or aggregates (CLEAs) are prepared by using a bi-functional reagent to obtain carrier-free macroparticles. This approach offers some advantages: highly concentrated enzyme activity in the catalyst is achieved, showing high stability and with low production costs owing to the exclusion of an additional carrier [11, 22]. CLECs are based on the crystallization of pure enzymes, followed by their chemical cross-linking, whereas CLEAs preparation involves the enzyme aggregation before chemical cross-linking [19, 22, 44]. CLEAs are generally preferred over CLECs because CLEAs do not require the purification of enzymes and allow the ease co-immobilization of several enzymes [25]. CLEAs are prepared by the precipitation of the enzyme, or the mixture of enzymes, using organic solvents (e.g. acetone, ethanol, propanol, *tert*-butanol), salts (e.g. ammonium sulfate), or non-ionic polymers (e.g. polyethylene glycol) [11]. Next, the aggregates are cross-linked using bi-functional reagents, usually glutaraldehyde as it is inexpensive and commercially available [39], although other cross-linkers, such as dextran aldehyde, have been successfully used in cases where glutaraldehyde presented poor results [45].

In some instances, the addition of a proteic feeder, such as bovine serum albumin, can be used to improve the cross-linking when the target protein is poor in Lys residues [46-48]. Additionally, magnetic CLEAs (mCLEAs) can be prepared by performing the cross-linking in the presence of functionalized magnetic nanoparticles. These mCLEAs can be separated by magnetic precipitation or can be used in a magnetically stabilized fluidized-bed reactor [49-52].

CLEAs present the advantage of absence of solid support, where all solid particle is protein, increasing the productivity in terms of mass of protein by solid area, and consequently, decreasing the costs of immobilization process. However, the enzyme is more exposed to reaction medium compared to the other protocols, being more sensible to possible denaturation caused by high temperature, pH, gas bubbles or some other inactivation agent.

3. The GH 1 family of enzymes

The GH 1 glycoside hydrolase enzyme family includes, in the CAZy database, more than 9,900 enzymes from the eubacteria kingdom (approximately 90 %). There are also enzymes from archaea, fungi, plants, and animals. Belonging to the GH 1 family, 23 different EC numbers are found, represented by β-mannosidase (EC 3.2.1.25), β-glucuronidase (EC 3.2.1.31), β-xylosidase (EC 3.2.1.37), β-D-fucosidase (EC 3.2.1.38), 6-phospho-β-glucosidase (EC 3.2.1.86), 6-phospho-β-galactosidase (EC 3.2.1.85), and lactase (EC 3.2.1.108). However, the β-glucosidases (EC 3.2.1.21) and β-galactosidases (EC 3.2.1.23) represent the main enzymes in this family according to their importance [53].

In general, the GH 1 enzymes have a classical $(\alpha/\beta)_8$-TIM barrel fold structure that contains their active site. The hydrolysis of the glycosidic bond is catalyzed following the β-retaining action mechanism by two amino acid residues of the enzyme: a glutamate residue as catalytic proton donor and another glutamate residue as catalytic nucleophile/base [54, 55]. The catalytic residues are highly conserved among other families that constitute GH clans, such as families GH 13 and GH 70. In particular, glutamate acts as a nucleophile in

enzymes from GH 1 family, characterizing them in the GH-A clan [56, 57]. The family presents the nucleophile located close to the carboxy-terminus from β-strand 7 and a sequence of asparagine-glutamate (an asparagine residue preceding the general acid/base catalyst) close to the carboxy-terminus from β-strand 4, except for myrosinase, where the acid/base glutamate is replaced by glutamine [58, 59]. Henrissat et al. [60] suggested that the two key active site glutamic acids are about 200 amino acid residues apart from each other and these enzymes are able to hydrolyze a wide diversity of substrates with a similar disposition of their identical catalytic residues. The structure of β-glucosidases from GH 1 family is represented in Fig. 2 by the isoform A from *Phanerochaete chrysosporium* complexed with gluconolactone (PDB: 2E40) [61]. The catalytic residues are Glu365 (nucleophile) and Glu170 (acid–base), located in the center of the $(\alpha/\beta)_8$-TIM barrel structure (Fig. 2(a)). Additionally, the entrance of the substrate-binding pocket is formed primarily by four extended loops connecting strands and helices at the C terminal side of the barrel (Fig. 2(b)).

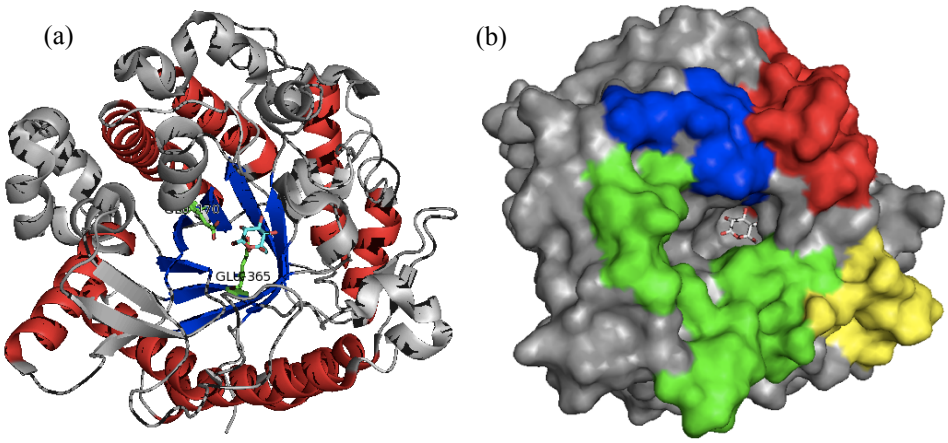

Fig. 2. Structure of GH 1 β-glucosidase complexed with gluconolactone; (a) Ribbon representation of the $(\alpha/\beta)_8$-TIM barrel structure and active site; α-Helices

are shown in red and β-sheet in blue. Glu170 and Glu165 are represented by green lines; (b) Molecular surface with representation of four loops on the entrance of active site. Loops A, B, C and D are shown in red, blue, green and yellow, respectively; The 3D structure was obtained from the Protein Data Bank (PDB) using PyMol vs. 0.99. The PDB code is 2E40.

Although the high diversity of enzymes in the GH 1 family, in this review, we will discuss aspects of the immobilization of β-glucosidases only, since these constitute some of the most important enzymes from the industrial point of view.

3.1. β-glucosidases features

The β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) are found in archaea, eubacteria, and eukaryotes, playing several functions in these organisms, as glycolipid and exogenous glycoside metabolism in animals, mechanism defense, cell wall lignification, release of aromatic compounds in plants, and biomass conversion in microorganisms [62, 63]. These enzymes catalyze the hydrolysis of β-glycosidic bonds between a monosaccharide and a moiety, which may be a carbohydrate or not [63, 64]. The hydrolysis reaction can be divided into two steps. The first one involves the nucleophile attack in the anomeric carbon (C-1) of the substrate resulting in a covalent glycosyl–enzyme intermediate with concomitant release of the aglycone after the protonation of the glucosidic oxygen by the acid catalyst, step called glycosylation. The second step corresponds to the hydrolysis of the covalent intermediate glycosyl–enzyme, with the acid catalyst acting as a base and a water molecule functioning as the

nucleophile, releasing the glucose and regenerating the nucleophile residue [58, 65].

Regarding the β-glucosidases active site, it is divided into several subsites. The subsite, denominated subsite -1 or glycone subsite, is responsible for binding the non-reducing end of the monosaccharide of the substrate, whereas the remaining part of the substrate interacts with the aglycone-binding site that may be formed by several subsites (+1, +2, +3, +n), also called aglycone subsite. Three-dimensional structures of β-glucosidases with different substrates and inhibitors showed that the substrate cleavage point is between the subsites -1 and +1 [64, 66, 67].

β-Glucosidases are extensively studied enzymes for the applications in food, feed, textile, and paper industries [68]. For example, β-glucosidases have been used for ethanol production in the process of saccharification [69], in the improvement of aromatic flavor compounds in juices and beverages [70], and for the hydrolysis of non-starch polysaccharides used in the feed of monogastric animals [71]. For these industrial applications, it is required that the enzymes possess some characteristics, such as resistance to environmental conditions, namely, pH and temperature, and multiple reusability. They also have to be economic viable. The immobilization process can promote these desired characteristics [43].

3.2. β-glucosidases immobilization

Several techniques have been developed for β-glucosidases immobilization, including adsorption, entrapment in gels and membranes, covalent linkage to insoluble supports, and cross-linking with bi-functional reagents [72]. One of the first reports about immobilized β-glucosidases was published in 1974. The enzyme was covalently immobilized on cyanogen bromide-activated cellulose, and its catalytic properties decreased after immobilization [73]. In the following sections, we will present and discuss the several methods used to immobilize β-glucosidases, discussing about their advantages and drawbacks.

3.2.1. Immobilization on chitosan particles

The covalent immobilization of β-glucosidases on chitosan is the most common method of immobilization for these enzymes (Table 1). This process was used to immobilize β-glucosidase in order to improve the aromatic potential of wines by cross-linking the enzyme using glutaraldehyde [74]. Immobilized β-glucosidases on chitosan beads for industrial application showed higher activities in a wider range of pH and temperatures, enhancing thermal stability, storage stability, and reusability, when compared to the free β-glucosidase [75]. Desai et al. [76] studied the immobilization of β-glucosidase from *Scytalidium lignicola* on chitosan. The authors prepared a biocatalyst showing higher resistance to temperature and pH values, without activity loss, and the immobilized enzyme retained 50 % of its residual activity after five use cycles. In another research, Bissett and Sternberg [77], immobilized the β-glucosidase from *Aspergillus phoenicis* QM 329 on chitosan using glutaraldehyde as cross-linker. The immobilized enzyme exhibited similar pH optimum, but was more active at lower

pH values, and improved thermal stability compared to free enzyme.

The cross-linked chitosan beads have innumerous advantages, such as an excellent hydrophilicity, high porosity, and large adhesion area. However, there are some operational limitations. Chitosan density is similar to water, causing it to easily float, and its texture is very soft, which limits its industrial applications. Aiming to eliminate these undesirable characteristics, it was performed the addition of activated clay to the wet chitosan (without freeze-drying) or dried chitosan (freeze-dried), followed by cross-linking [78]. In a similar way, activated carbon was added to chitosan beads before the cross-linking with glutaraldehyde [79]. The immobilization of β-glucosidase from *Exiguobacterium* sp. exhibited higher hydrolyzing activity of isoflavone glycoside, as well as higher pH and thermal stabilities in aqueous–organic two-phase system after these modifications of the support [79]. The same operational characteristics were obtained when chitosan was used as a base for magnetic carriers, when testing its potential recycling use in the hydrolysis of lignocellulosic biomass [80].

Table 1. Characteristics of different covalent binding based immobilizations of β-glucosidases

Reactive group	Immobilization support	Reference
Glutaraldehyde	Pore glass particles	[81]
	Chitosan and alginate beads	[70, 76]
	Bentonite, celite, silica gel, and nylon	[82]

	Mesoporous silica MCM-41	[83]
	SiO_2 nanoparticles	[84]
	chitin, loofa, sawdust coarse, sawdust fine, sponge, stainless steel, pumice, wool and agar, agarose and sodium alginate	[72]
	Chitopearl beads BCW-3001	[85]
	Amine agarose gel	[86]
	Silica gel	[87]
	Nylon powder	[88]
	Chitosan–clay composite	[78]
	Agarose matrix	[89]
	Chitosan	[74, 75, 77]
	Magnetic chitosan microspheres	[80]
	Chitosan-carbon beads	[79]
	Spent coffee grounds	[90]
	Iron oxide magnetic nanoparticle	[91]
	Polyvinylalcohol (PVA) nanofibrous membranes	[92]
	Mesocellular silica foams	[93]
L-lysine and glutaraldehyde	Chitosan microspheres	[94]

Hexamethylenediamine and glutaraldehyde	Chitin (IME-C) and calcium Alginate (IME-A)	[95]
Polyelectrolytes (PEI) and glutaraldehyde	κ-carrageenan beads	[96]
APTMS and glutaraldehyde	Cellulose PEI, alpha-alumina, gamma-alumina and chitosan	[97]
Epoxy	Eupergit® C 250L	[98]
	Polyacrylic matrices supports (Eupergit® C, Eupergit® C 250L, and cryogel)	[99]
	Eupergit® C	[100]
Nylon-hydrazide	Nylon pellets	[101]
CNBr	Sepharose gel beads 4B	[102]
Carbodiimide	Magnetic beads	[103]
Mercaptopropyl-functionalized	Mesoporous titanium dioxide	[104]
Aldehyde groups	Glyoxyl–agarose	[105]
Polyethyleneimine and glutaraldehyde	Magnetite (PAM) and (TiO_2)-coated magnetite (TAM)	[106]
Dextran dialdehyde and β-glucosidase-dextran conjugates	Silica and aminopropylsilica	[107]

Plasma immersion ion implantation (PIII)	Polystyrene films	[108]
	Polyethylene granules	[109]

APTMS: 3-Aminopropyl-trimethyoxysilan; CNBr: cyanogen bromide.

Another aspect concerning this support is the fact that the cross-linked chitosan microspheres exhibit reactive primary amino groups directly bonded with pyranoid rings, which causes large steric hindrances. The cross-linking reaction can consume a large portion of amino groups, reducing the enzyme load and the volumetric productivity. To improve these aspects, it was performed a cross-linking of chitosan modified by L-lysine (named LMCCR), where the Lys acted as flexible spacer arm to decrease the steric hindrances. The ε-NH_2 of Lys residues replaced the less reactive amino groups of cross-linked chitosan during the cross-linking reaction.

The immobilized β-glucosidase showed optimal pH in alkaline region, had 85 % of its residual activity after 13 cycles of use and efficiently produced resveratrol by the hydrolysis of polydatin in a continuous reactor [94, 110].

3.2.2. Covalent immobilization of β-glucosidases on other supports

The covalent immobilization of β-glucosidases by direct linkage or cross-linking was also performed using other supports than chitosan. In addition, the cross-linking using glutaraldehyde has also been extensively employed in solid supports. Ahmed et al. [72] immobilized β-glucosidase from *A. niger* on sponge,

which is safe, inexpensive, and readily available, by covalent binding it with glutaraldehyde as spacer group, obtaining high immobilization yields (95.7 %), high activity recovery (63.7 %), and a thermal stability 132-fold higher at 65 ºC, compared to the free enzyme. The immobilization of β-glucosidase using spent coffee grounds as the solid carrier, cross-linked with glutaraldehyde, was also studied for the conversion of isoflavone glycosides into their aglycones in black soymilk. The results showed that the immobilized enzyme could be used for more than 30 cycles, with the enzyme retaining its catalytic activity for 20 days. According to the authors, these advantages enabled a less costly process [90].

Another support that has been widely studied as carrier in the immobilization of β-glucosidase via covalent binding is silica gel. Jung et al. [87], studied the immobilization of β-glucosidase using silica gel. In order to prevent the formation of covalent bonds near to the active site, the authors bonded β-glucosidase with cellobiose and glucose, which resulted in 176 % higher enzyme activity, compared with the non-pretreated. In addition, the silica gel-immobilized enzyme kept 80 % of its relative activity after 20 reuses. Agrawal et al. [84] improved the overall storage, pH, and temperature stability, and the length of reuse to up to 10 cycles with 70 % of residual activity by immobilization of β-glucosidase from *Bacillus subtilis* on SiO_2 nanoparticles. Singh et al. [111] also reported an increase of 288-fold in the thermal stability at 65 ºC of this enzyme immobilized on SiO_2 nanoparticles. Similar results were also obtained when β-glucosidase from *A. niger* was immobilized on magnetic nanoparticles functionalized with glutaraldehyde [91].

The covalent immobilization of β-glucosidase onto epoxy activated Eupergit® C has also been reported. This macroporous carrier has the ability of

stabilizing protein conformation forming very stable covalent multipoint attachments with amino, hydroxyl, thiol and/or phenolic groups of amino acid side chains on the enzyme surface. The bonds between the enzyme and support are highly chemically and mechanically stable during storage over a pH range from 2 to 12 [20]. The immobilization process using these particles improved the pH and storage stabilities of β-glucosidase from *Issatchenkia terricola* [98]; it also improved stability at 65 ºC and the apparent K_m and V_{max} of β-glucosidase from *A. niger* [100]. One of the disadvantages of the covalent attachment is that the linkages can promote steric hindrances of the enzyme. In this way, a spacer arm can be added by modification of the support by ethylenediamine and glutaraldehyde to produce an aldehyde-activated support [112]. Khan et al. [99], showed that the reaction of epoxy group followed by reaction with glutaraldehyde in β-glucosidase from *Thermotoga neapolitana*, improved the thermal and the storage stabilities of the enzyme, and allowed extensive reuse of the biocatalyst, which kept 91 % of the initial enzyme activity after 10 batch cycles. The same improved enzymatic characteristics were observed when β-glucosidase was immobilized using amine-epoxy agarose support [89].

Spagna et al. [86] reported studies of the immobilization of β-glucosidase onto an amine agarose gel. This immobilization occurred via oxidation of the carbohydrate chains of the enzyme, since they are not directly involved in the catalysis, thus the aldehyde groups were capable of reacting with the amine groups of the matrix and with their subsequent reduction. Before the reduction reaction, the formation of covalent bonds by activation of carboxyl groups with carbodiimide was also tested in order to increase the density of the covalent bonds for each enzyme molecule immobilized, thus increasing the rigidity of the

secondary and tertiary structure of the enzyme. Although the immobilized enzyme exhibited high immobilization yields and enhanced stability, the activation of the biocatalyst using carbodiimide slightly negatively affected its activity.

In the light of these considerations, it is possible to propose that covalent binding provides the strongest attachment of the enzyme to the support, and it has been associated with high activities and the possibility of enzyme reuses. However, covalent attachment is generally achieved using complex chemicals as linkers, in multi-step reactions that require relatively long times to be completed [113]. Aiming to circumvent these disadvantages, Hirsh et al. [108] obtained a highly effective method for rapid covalent immobilization (within one minute reaction) using plasma immersion ion implantation (PIII) for activation of polystyrene film polymer surface. According to the authors, the immobilized thermophilic *Caldicellulosiruptor saccharolyticus* β-glucosidase showed an activity more than 20-fold higher than the commercial β-glucosidase, over five batch reuses. In another report, Nosworthy et al. [109], demonstrated that granules and particles of polystyrene treated with PIII were also successfully employed for immobilization, and the protein was strongly immobilized on the surface without the need of chemical treatments. This covalent binding enabled more robust linkages allowing high flow rates, high activity, large surface area and a broad operating pH range, making it possible its use into current reactor technologies, including batch, fluidized bed, and continuous flow reactors [108, 109].

3.2.3. Immobilization of β-glucosidases by adsorption

Although the immobilization by covalent bond could result in preparations showing high stability, the immobilization by physical adsorption can enhance the flexibility of the system. Table 2 presents the main reactive groups and supports used for β-glucosidase immobilization by adsorption.

Table 2. β-glucosidases immobilization by adsorption

Reactive group	Immobilization support	Reference
Physical adsorption	Kaolin	[114]
	Soil colloidal particles	[115]
	Towel gourd vegetable sponges	[116]
Cation exchanger	Duolite A-568 resin	[117]
	Hydroxyapatite (HTP)	[118]
	Resin Amberlite DP-1	[119]
	Eudragit S-100	[120]
	Polyacrylic resin	[105]
Anion exchanger	DEAE-sepharose	[121]
	DEAE-cellulose	[122, 123]
Anion exchanger and macroporous	Different ion exchange resins	[124]
Metal ionic binding	Magnetic Fe_3O_4 nanoparticles coupled with agarose	[125]
	Magnetic Fe_3O_4 nanoparticles	[75]

Hydrophobic polyaromatic	Amberlite XAD-4 resin	[126]
	Celite R-640	[127]
Physically immobilized by crossflow ultrafiltration	30 kDa cut-off capillary Polysulphone membranes	[128]
	Capillary membranes of polysulphone	[129]
Not declared	Cellulosic adsorbents: dewaxed, absorbent cotton, CF1 cellulose, Avicel^{TM} PH-101, and Cellufine	[130]

Non-covalent immobilization methods have the advantages of being simple and low-cost, and do not employ severe conditions. These advantages of adsorption were demonstrated in the immobilization of β-glucosidase on polyacrylic resin activated by carboxyl groups applied to the hydrolysis of sugarcane bagasse [105]. Similar results were obtained in the synthesis of n-octyl-β-D-glucopyranoside by β-glucosidase immobilized on Amberlite XAD-4 [126] and in water-soluble polymer Eudragit S-100 [120]. Furthermore, adsorbed-immobilized β-glucosidase was applied in packed and fluidized bed reactors to improve the aromatic quality of Muscat wine and to the hydrolysis of cellulose [117, 119]. In both cases, the enzyme was immobilized on ion exchange resins

Duolite A-568 [117] and Amberlite DP-I [119].

Another similar application was the preparation of a mixed bed ion exchanger hydroxyapatite (HTP) formed by calcium phosphate, which was very useful for the purification and immobilization in a one-step procedure of several proteins, including β-glucosidase. The HTP-bound β-glucosidase was active, stable, and easily recoverable from reaction medium. This preparation was used for the enhanced release of aromas in wine and fruit juices [118].

The β-glucosidase adsorption on DEAE-sepharose showed to be high, around 91 % of immobilization yields and 83 % of activity recovered [121]. Using similar supports, β-glucosidase adsorption on MANAE-agarose and DEAE-cellulose were approximately 75 and 120-fold more stable than the free enzyme [123], and were quickly immobilized showing a high residual activity after immobilization. Moreover, it was possible to modify the protein, by different reactions, such as acylation of the amino groups by pyromellitic dianhydride (PMDA), increasing the negative charges on the protein surface, minimizing the time of the immobilization. Tyagi and Gupta [122], were able to immobilize the β-glucosidase in DEAE-cellulose resin after modification with PMDA. This chemical modification improved the thermal stability, and this strategy may be useful for obtaining enzyme derivatives for reversible adsorption on anion exchangers.

Magnetic nanoparticles are other carriers that were studied for the non-covalent immobilization of enzymes. Chen et al. [125] synthesized magnetic Fe_3O_4 nanoparticles coupled with agarose using co-precipitation via alkaline conditions and span-80 surfactants in organic solvent. The enzyme bounded efficiently via metal ion affinity in alkaline amino groups of its surface and the Co^{2+}

chelated on the carriers, showing higher hydrolytic activity and higher thermal and operational stabilities than the free form. These nanoparticles can be easily separated from the reaction medium by magnetic field and it is possible to reuse them. In another study, β-glucosidase was immobilized onto Fe_3O_4 nanoparticles coated with sodium citrate and after was cross-linked with glutaraldehyde. This method presented extended ranges of pH and temperature activities, higher accessibility to the substrate (K_m value of immobilized β-glucosidase was lower than that of the free enzyme), high activity recovery (89 %), and improved thermal and storage stabilities [75]. However, one disadvantage of using chemical supports, as cited early, is their high cost and possible environmental concerns during their discharges. The use of natural carriers could minimize these problems. In this way, towel gourd vegetable sponges were tested to immobilize a marine *A. niger* β-glucosidase. This carrier is natural, biodegradable, has a low-cost and it is safe for humans [116]. Other interesting natural materials are fine soil colloidal particles with high surface area and content of iron oxides were employed to immobilize β-glucosidase via adsorption. The immobilized enzyme showed thermal stability at all tested temperatures and it was less sensitive to pH and temperature changes than the free enzyme, possibly because the support presented a protective effect [115].

3.2.4. Immobilization of β-glucosidases by entrapment

The entrapment in calcium alginate is the most frequently method used for β-glucosidase immobilization reported in the literature [70, 95, 131-135] (Table 3). This method showed good enzymatic recovered activities of 60 % [134], 66 %

[136], and 73 % [137]. However, when the whole cells from *Debaryomyces hanseniiwere* showing β-glucosidase activity were immobilized, the recovered activity was only 8 % [133]. Although this immobilization method generally provides low mechanical strengths, the immobilization process produces enhanced thermal properties and higher optimum temperature usage [133, 134, 137], the possibility of reuse [134, 137], and higher storage stability [133, 134], compared to the free enzyme. The leakage of the enzyme out of the alginate beads causing its loss increases the immobilization costs, which is a disadvantage. In order to overcome this problem, some studies have been carried out to cross-link the enzyme with glutaraldehyde prior to the immobilization [135, 138]. For instance, Su et al. [70] studied the calcium alginate as the carrier and cross-linking–entrapment–cross-linking as the immobilizing method to hydrolyze the glycosidic aroma precursors in tea beverages. The immobilized β-glucosidase exhibited optimum temperature 10 ºC lower, a recovered activity of 46 %, increased thermal and pH stabilities, and lower K_m than the free enzyme.

Table 3. β-glucosidases immobilized by entrapment methods

Immobilization support	Reference
Calcium alginate beads	[70, 131-134, 137, 139]
Calcium alginate beads and alumina	[95]
Calcium alginate beads and glutaraldehyde	[135, 138]

Calcium alginate beads in tetramethoxy-ortho-silicate (TMOS) and hexane	[140]
Calcium alginate and polyacrylamide gel	[136]
Polyacrylamide gel	[102]
Gelatin gel	[141]
Calcium alginate beads, gelatin, polyvinyl alcohol-(PVA-) based matrices, and sol-gel	[142]
Hydrogels of poly(2-hydroxyethyl methacrylate)	[143]
Nanoscale polymeric materials (polyurethane, latex and silicone)	[144]
Ionic liquid sol-gel matrices	[145]

In addition to calcium alginate, enzyme entrapment has also been reported using other carriers, such as the hydrogels of poly(2-hydroxyethyl methactylate) that showed excellent protective effect [143]. Recently, Javed et al. [144] studied the β-glucosidase immobilization within nanoscale polymeric materials (polyurethane, latex, and silicone) and obtained good results using latex (highest relative activity) and silicone matrix (highest entrapment efficiency). However, the latex immobilized enzyme leaked after each cycle, which did not occur when the entrapment was in silicone. Figueira et al. [142] obtained good results using

polyvinyl alcohol (PVA-) based matrices (LentiKats®) and in sol-gel (prepared with tetramethoxysilane), analyzing retention of the catalytic activity following immobilization. The immobilization in sol-gel resulted in higher stability under higher operational temperatures compared to the immobilization using only (LentiKats®), because these particles were not physically stable above 55 ºC.

The sol-gel matrices have the advantage of preventing leakage of enzyme from support during the reaction. However, the disadvantage of the gel contraction during condensation and drying process, causing possible enzyme denaturation, is known. To overcome this limitation, some mechanisms have been developed. For instance, Vila-Real et al. [119], studied the addition of ionic liquids in the sol-gel immobilization process. The authors improved the immobilization efficiency of the encapsulated enzyme, as well as the mechanical resistance against cracking, suggesting that the ionic liquids play an important role in enzyme performance [145].

One particularity of the β-glucosidases is that their industrial applications are usually associated with other enzymes to achieve a broader objective. In winemaking, for example, other enzymes can be co-immobilized in order to improve desirable effects, such as aroma and crystallinity. Co-immobilizations were studied and a simple and cost-effective procedure for the co-immobilization of β-D-glucosidase, α-L-arabinofuranosidase, α-L-rhamnopyranosidase, and β-D-xylopyranosidase was proposed by Ferner et al. [103]. The four enzymes were immobilized onto magnetic beads and showed good stability under winemaking conditions, with β-glucosidase showing the highest immobilization yields of 95 % between pH 3.5 and 4.0. According to the authors, the immobilization method was easy to obtain, it was effective and the commercial preparation did not

require cleanup steps [103].

Because β-glucosidase immobilization by adsorption or by entrapment causes a limited half-life of the biocatalyst due to progressive release of the enzyme into the reaction milieu, its applications is hampered. On the other hand, the covalent immobilization requires several chemical steps, which are frequently associated to substantial loss of enzyme activity [93]. In order to overcome these problems, Mateo et al. [20] studied the physical aggregation of enzymes followed by cross-linking (cross-linked enzyme aggregates, CLEAs) as a method to prepare solid biocatalysts. Using this method, Reshmi and Sugunan [93] obtained β-glucosidase immobilized onto mesocellular silica foams (MCFs) by formation of CLEAs of nanometer scale. The CLEAs retained activity over wider ranges of temperature and pH applications, and lower K_m than the free enzyme, and they were recyclable up to 10 cycles with more than 85 % residual activity, with high enzyme loadings.

In conclusion, it is possible to observe that β-glucosidases have been intensively investigated regarding possible carriers and immobilization methods. The choice of immobilization technique should be dictated by the enzyme application, since it has been demonstrated that different immobilization protocols lead to unique characteristics. The search for novel matrices and immobilization strategies can help to overcome these obstacles.

4. The GH 13 family of enzymes

The GH 13 enzyme family is the largest sequence-based family of glycoside hydrolases. It comprises a group of enzymes with different specificities, in which each one acts upon one type of substrate, composed by glucose residues linked through α-(1,1), α-(1,4), or α-(1,6) glycosidic bonds. This family can be divided into two subgroups: the starch-hydrolyzing enzymes, and the starch-modifying or transglycosylation enzymes [146]. Hydrolases and transferases from GH 13 family are multidomain proteins, sharing a common catalytic domain in the form of a $(\alpha/\beta)_8$ barrel fold: 8 parallel β-strands and 8 α-helices, being alternated along the protein sequence. The β-strands form the inner barrel, whereas the α-helices flank the exterior [147, 148]. Enzymatic hydrolysis of the glycosidic bonds takes place via general acid catalysis that requires two critical residues: a proton donor and a nucleophile/base [54]. These enzymes have a retaining mechanism (Fig. 3), acting in two steps: the glycosylation step, involving the formation of a covalent glycosyl-enzyme intermediate, and the deglycosylation, when hydrolysis occurs [54, 146, 147].

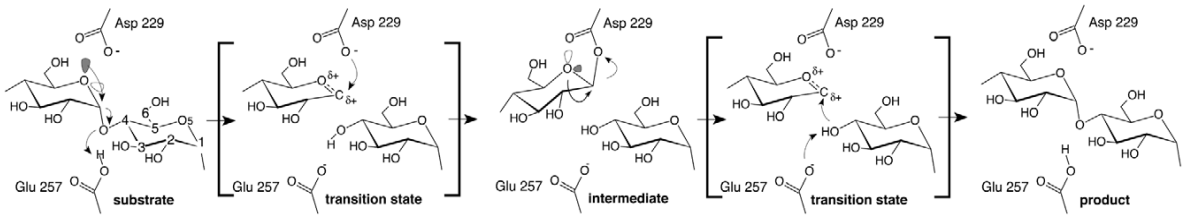

Fig. 3. Mechanism of the α-retaining α-(1-4) glycosidic bond cleavage used by the α-amylase family; Adapted from Uitdehaag *et al.* [149].

The seven most conserved amino acid residues in the α-amylase family

cluster are precisely in the center of the regions that comprises the catalytic site. Three of them are totally conserved, Asp229, Glu257, and Asp328, whereas Asp135, His140, Arg227, and His327 are almost completely conserved. The evolutionary conservation of these residues can be explained by their common substrate, starch, which contains only one basic chemical bond, the α-glycosidic bond [149].

Transglycosidases share structural and sequence similarities to retaining glycosidases, as well as mechanistic strategies. However, instead of catalyzing the hydrolysis of glycosidic linkages, they utilize a sugar rather than a water molecule to act in the final step, yielding a new glycoside linkage (transglycosylation reaction) [150]. Cyclodextrin glycosyltransferases, members of GH 13 family, use α-linked glucose polymers as substrates for the formation of cyclic oligoglucosides.

4.1. α-amylases

Amylases are among the most studied and important enzymes used in industry [148]. These starch-converting enzymes are applied in the production of maltodextrin, modified starches, or glucose and fructose syrups. α-Amylases (α-1,4-glucan-4-glucanohydrolase; E.C.3.2.1.1), which are classified into the GH 13 family, are extra-cellular enzymes that specifically catalyze the hydrolysis of α-1,4-glycosidic linkages of starch yielding low molecular weight products, such as glucose, maltose and maltotriose units; however, they do not hydrolyze β-1,6-glycosidic bonds [151, 152].

These enzymes are found throughout natural sources, including plants, animals, and microorganisms, but their commercial production has been, generally, carried out using submerged fermentation using bacteria from the genus *Bacillus,* such as *B. licheniformis*, *B. stearothermophilus*, and *B. amyloliquefaciens* [152, 153]. A large number of microbial amylases are commercially available and they have almost completely replaced chemical hydrolysis in the starch processing industries [154]. Furthermore, α-amylases have potential application in a wide number of industrial processes related to the food, fermentation, textile, paper, detergent, and pharmaceutical industries [146, 155, 156]. The properties of each α-amylase regarding thermostability, pH profile, pH stability, and Ca-dependency are important in the development of these processes.

4.1.1. Immobilization of α-amylases

α-Amylases from different sources have been immobilized onto a wide variety of organic and inorganic supports, among them, gelatin [157], gums [158, 159], magnetic nanoparticles [160, 161], silica [162], and carrier-free systems [163], increasing the enzymatic stability and adaptability to harsh conditions of reactions.

- Immobilization of α-amylases on insoluble supports

Covalent immobilization is the most used technique to attach α-amylases on solid supports. The covalent bond formed between the protein and the support usually shifts the optimal temperature of the enzyme, decreases its

conformational flexibility and protects its structure from distortion or damage by heat exchange. Rana et al. [164] immobilized α-amylase onto chitosan microspheres using glutaraldehyde as chemical agent. The optimum catalytic temperature shifted from 45 ºC to 55 ºC presenting 70 % more activity than its free form, at this temperature. The immobilized α-amylase retained 49 % of its initial activity after seven consecutive batch reuses. When compared with Amberlite MB-150 (a mixture of acidic cationic and basic anionic resin) [165], the optimum pH obtained was 8.0 for chitosan beads and 7.0 for this inorganic support, while the free enzyme showed an optimum pH of 5.5. Changes in optimum temperatures were not observed for α-amylase immobilized onto chitosan derivative, contrasting with a small increase for Amberlite. The immobilized enzyme showed a good operational stability by retaining 38 % and 58 % of its initial activity after 10 cycles for chitosan and Amberlite, respectively. When attached to DEAE-cellulose, α-amylase retained 77 % of its initial activity after 10 uses and had a change in the optimal pH, being 5.0 for soluble and 6.0 for immobilized enzyme. Immobilized and soluble enzymes showed optima activities at 70 ºC and 68 ºC, respectively [166]. On the other hand, Shukla [167] activated DEAE-cellulose with glutaraldehyde and found that *α*-amylase immobilized by covalent attachment to this support improved its optimal temperature, from 60 ºC to 70 ºC. The maxima activities of free and immobilized enzymes were observed at pH 7.0. The improved activity, at higher temperatures, in between 40 ºC and 60 ºC, was also observed by Veesar et al. [168], studying the immobilized α-amylase onto calix[4]arene activated with glutaraldehyde [169].

- CLEAs of α-amylases

The costs of immobilized enzymes should be minimized in order to increase their competitiveness for technical applications, and CLEAs have emerged as a versatile carrier-free immobilization technique. Several researchers have already reported studies on this type of immobilization applied to α-amylases. These aggregates are formed by adding a precipitant agent, such as salt or organic solvent, followed by chemical cross-linking using a bifunctional reagent, usually glutaraldehyde [11, 19, 39, 170, 171]. It was also evaluated the effect of using non-toxic and biocompatible polysaccharides such as agar, chitosan, dextran, and gum arabic as cross-linkers on α-amylase recovered activity replacing the traditional glutaraldehyde on CLEAs production [172]. The macromolecular CLEA of α-amylase using dextran showed 91 % of activity recovery, 84 % using chitosan, whereas using glutaraldehyde, it was possible to recover only 42 % of activity. The authors indicated that glutaraldehyde CLEAs had lower recovered activity owing to the compact supramolecular structure formation, resulting in serious steric hindrance effects. This may restrict the diffusion of macromolecular substrate inside the CLEA particle decreasing the catalytic activity [172]. According to Talekar et al. [173], it is expected that chemical cross-linking restricts conformational changes induced by heat and stabilizes the structure of the enzyme, increasing the stability of macromolecular cross-linked aggregates, as shown in their work.

Co-aggregation of enzymes and other proteins rich in lysine residues, such as bovine serum albumin (BSA), increase the concentration of highly reactive amino groups, facilitating the formation of the stable CLEAs [48, 174]. Torabizadeh et al. [174] introduced a two-step method for the preparation of CLEAs of a thermostable α-amylase, including calcium and sodium ions during

the enzyme aggregation. The authors selected the best ratio of enzyme:BSA, obtaining 6.2 % higher conversion than CLEAs without BSA addition. There was no significant change in the optimum pH of the enzyme activity after immobilization, but the activity and stability of produced CLEAs were significantly increased in the presence of calcium and sodium ions.

Talekar et al. [163] recovered 45 % of applied α-amylase activity in CLEAs and 100 % when magnetic CLEAs were used, this activity remaining unchanged even after 100 batch reuses. This high-recovered activity was achieved by adding amino functionalized magnetite nanoparticles into α-amylase solution, increasing the amine groups available to cross-link with glutaraldehyde. Moreover, the temperature for the highest α-amylase activity was established at 45 ºC for the free enzyme, shifting to 50 ºC for CLEAs, and to 60 ºC for magnetic CLEAs. The authors assumed that this increased thermal protection is due to the higher amount of covalent cross-linking between enzyme and amino-functionalized magnetite nanoparticles in magnetic CLEAs compared to CLEAs without addition of external amino groups. Improved thermal stability, storage stability, and reusability of magnetic CLEAs is an attractive way towards stable CLEAs preparation and could overcome the drawback of CLEAs clumping.

The enzymatic process of starch hydrolysis and sugars production involves the use of different enzymes, besides α-amylase: glucoamylase, an exo-acting enzyme that hydrolyzes α-(1,4) and α-(1,6) glycosidic bonds from the non-reducing ends, and pullulanase, which differs from amylases because it hydrolyzes pullulan, in addition to amylopectin. In the presence of high concentrations of glucose and dissolved solids, glucoamylases can also catalyze reverse condensation reaction decreasing overall glucose yield. The addition of

a pullulanase, a debranching enzyme which has also the capability to hydrolyze α-(1,6) glycosidic bonds, can be an alternative to improving the glucoamylase performance at these conditions [154, 175, 176]. In this way, Talekar et al. [177] developed a carrier free co-immobilization of these three enzymes aiming at using this combi-biocatalyst in one batch reaction. The authors reported 100 % of starch conversion with this combi-biocatalyst, compared to only 60 % and 40 % when single CLEAs mixture and free enzymes were used, respectively.

- Other approaches for α-amylase immobilization

Magnetic poly(2-hydroxyethylmethacrylate) beads carrying a dye-ligand (Cibacrom Blue) for specific proteins ligand was used for *α*-amylase immobilization by adsorption with a load of 401 ± 11 mg/g support. After adsorption, the optimal pH shifted from 7.0 to 8.0, and the pH profile of the immobilized α-amylase was much broader than that for the free enzyme, indicating a promoted protection by immobilization. This protection was also able to preserve the enzyme from temperature damage, showing a maximal catalytic activity at 10 ºC higher than the free enzyme [178]. Guo et al. [160], used magnetic Fe_3O_4 nanoparticles functionalized with 3-aminopropy-ltriethoxysilane (APTES) for the immobilization of porcine pancreatic α-amylase and reported that the enzyme exhibited higher temperature and pH resistance, in addition of better organic solvent tolerance. The authors argued that the immobilization process enhanced the rigidity and decreased thermal perturbations of the enzyme structure. Magnetic nanoparticles were coated with gum acacia and, using glutaraldehyde, they formed covalent bonds with α-amylase, enabling higher

immobilization yields (60 %) than when using unmodified magnetite nanoparticles [159].

Microspheres of gellan gum, a linear polysaccharide formed by units of glucose, glucuronic acid, and rhamnose was used to entrap α-amylase through ion tropic technique. The relative activity in the presence of amylopectin, maltodextrins, and glycogen was 73 %, 85 %, and 14 %, respectively. It was possible to inversely correlate enzyme activity with the size of the substrate [158]. Gashtasbi et al. [179], designed a new approach, using adsorption and covalent methods, to immobilize *B. licheniformis* α-amylase on the surface of *B. subtilis* spores. For the covalent method, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sulfo-N-hydroxysuccinimide (NHS) were used to activate free carboxyl groups on the spore EDC surface, followed by a condensation reaction with the amino groups of the enzyme. The optimum pH of immobilized α-amylase shifted from 5.0 to 8.0, an expected behavior because of the anionic surface of the spore. The covalently immobilized enzyme was more stable at higher temperatures, and its maximum activity changed from 60 ºC to 80 ºC. This method also allowed the enzyme to retain 64 % of its initial activity after 10 reaction cycles.

Ionic exchange and hydrophobic interactions were tested for α-amylase immobilization on gold nanorods. The immobilization promoted significant enhancement in the thermal and pH stabilities compared with the free enzyme. The immobilized enzyme showed higher activities, both in acidic and basic pH ranges, compared to the soluble form, exhibiting 33 % of activity at pH 2.0 and 52 % at pH 10, whereas the soluble enzyme was almost fully inactivated under the same conditions. Concerning temperature, maxima activities of free and immobilized α-amylase were observed at 50 ºC and 60 ºC, respectively, whereas

their irreversible thermal inactivation were determined to be 70 ºC and 80 ºC, respectively. However, the immobilized enzyme presented a thermal protection, since its half-life at 80 ºC, was three times higher than for the free form. Moreover, the immobilization allowed the retention of 45 % and 20 % of the original activities after 60 min of incubation at 70 ºC and 80 ºC, respectively. The slightly decrease of k_{cat} and increase of K_m may be explained by enzyme structure distortions associated with the immobilization [180].

4.2. Cyclodextrin glycosyltransferase

Cyclodextrin glycosyltransferases (CGTase, EC 2.4.1.19) are other important members of GH 13 family of enzymes [181, 182]. As for α-amylases, CGTases use the so-called α-retaining double displacement mechanism to react with starch substrates [183]. These enzymes can be produced by a variety of bacteria, especially of the genus *Bacillus* [184-186], having molecular weights around 75 kDa and five domains – labeled A to E. The domain A contains the catalytic $(\alpha/\beta)_8$ domain, which characterizes the members of this family [149, 187].

Besides the hydrolysis reaction, CGTases catalyze three inter- and intramolecular transglycosylation reactions: disproportionation, coupling, and cyclization, always acting on α-(1,4) glycosidic bonds. The cyclization reaction has been of great interest, since this is the unique enzyme that can produce the cyclic α-1,4-glucan with 6, 7, and 8 degrees of polymerization, named as α-, β-, and γ-cyclodextrin [188-191]. Cyclodextrins (CDs) have a hydrophobic central cavity that can incorporate various inorganic and organic compounds, forming

inclusion complexes with them [192]. Therefore, these CGTase products are widely used in the pharmaceutical, food, agricultural, and cosmetic industries [186, 193-197].

All native CGTases produce mixtures of α-, β-, and γ-CDs and the isolation and purification of a specific CD is a rather expensive and time-consuming process. According to Biwer et al. [186], solvent process purification can only isolate the CDs from the rest of the reaction mixture, but not the different CDs types from each other. Since each of them has a dimensionally distinct central cavity and different specificity for guest molecules [198-200], studies have focused on to find or engineer a CGTase that produces a specific type of CD. Additionally, it is clear the necessity for novel CGTases with improved properties related to their stability, and developments to better modulate the reaction for the production of a specific CD [149, 188].

According to Kelly et al. [201], the type of cyclodextrin produced by CGTases is dependent upon the number of glucose units that bounds at the donor substrate binding site prior to the glycosidic bond cleavage. There is an evolutionary diversification among CGTases regarding both cyclodextrin product specificity and thermal stability property. Furthermore, mutations (substitution, insertion, and/or deletion) in one or more of conserved residues at the donor subsites can directly affect the product specificity of these enzymes. Considering the importance of central tyrosine 195 in the active site of CGTase, Xie et al. [202] applied site-directed saturation mutagenesis to investigate its role on the hydrolytic and cyclization specificity of an α-CGTase. Interestingly, the authors found mutants that drastically altered the CD specificity from initial 35 % of α-CD to 34 % of β- and 38 % of γ-CD (Y195I) or even to 50 % of γ-CD (Y195R).

4.2.1. Immobilization of CGTases

Immobilization of CGTases has been developed in the attempt to reduce the costs of cyclodextrins production. Several approaches have been applied, such as adsorption [203], entrapment [204], and covalent binding [205].

Different combinations of supports and methods were studied by Sobral et al. [206] to immobilize CGTase from *B. firmus*. Adsorption and covalent immobilizations were tested using silica, chitosan, and alumina supports. The highest activity of immobilized enzyme was obtained for the covalent immobilization in chitosan. Polyethylene film functionalized with carboxylic acid groups was used as support for immobilization via covalent bonds with carbodiimide (EDC). The CGTase from *B. macerans* showed an increased production of the three types of cyclodextrins, according to the reaction time, following the order: α-CD > β-CD > γ-CD [207]. CGTase produced by *Paenibacillus macerans* was immobilized on aminated polyvinylchloride (PVC) by covalent binding with glutaraldehyde, having its thermal stability and resistance to chemical denaturation improved. Both free and immobilized enzymes had an optimum pH of 6.0, but the immobilized presented higher activities at lower pH values. The free enzyme showed optimum activities at temperature of 60 ºC, whereas for the immobilized form optimum temperature was 75 ºC [208].

The processing of starch requires the use of high temperatures to obtain its liquefaction, usually using α-amylase, thus it would be of interest to find thermostable CGTases that could be used in these processes. Norman and Jorgensen [209], isolated a thermostable CGTase from *Thermoanaerobacter* sp. ATCC53627. This is an extremely heat-stable enzyme, with optimum

temperature activities of 90 ºC to 95 ºC at pH 6.0, and, in the presence of starch, it is even stable above 100 ºC. Therefore, liquefaction and cyclization may be carried out without further enzyme addition, yielding products with high purity grade. The gene encoding this *Thermoanaerobacter* CGTase was cloned into a *Bacillus*, and this enzyme is now commercialized by Novozymes A/S as Toruzyme 3.0 L and has been used in several studies.

Tardioli et al. [210] tested the multipoint covalent immobilization of CGTase in cross-linked 6 % agarose beads activated with aldehyde groups (glyoxyl-agarose). The authors obtained increased rigidity of the CGTase (Toruzyme 3.0 L), thus a higher resistance to conformational changes caused by denaturing conditions, such as pH and temperature. Satisfactory results were obtained, as higher operational stability at 85 ºC, 30 % of the substrate conversion with only 2 h of reaction, and a two-fold increase in production rate compared to the free enzyme. Covalent attachment of Toruzyme CGTase was also carried out using Eupergit® C as support. This support contains oxirane (epoxy) groups instead of aldehyde groups, to attach the proteins. Although it was not observed any change in the optimum pH of the immobilized enzyme, it was identified a broader pH range with high catalytic activity, especially at high pH values, and the optimum temperature changed from 85 ºC to 80 ºC [205].

The immobilization on glutaraldehyde-activated chitosan spheres changed the optimal temperature of β-CGTase activity from 75 ºC to 85 ºC at pH 6.0. Free and immobilized enzymes presented optimum pH at 5.0, but the immobilized-chitosan particles presented higher operational stability, with 61 % of its initial activity still remaining after 100 batches [211]. The same optimum pH was observed when mesoporous silica functionalized using 3-

aminopropyltrimethoxysilane (APTMS) was used to immobilize the enzyme, but the best temperature for β-CD production changed from 100 ºC to 80 ºC after immobilization [212].

Although some studies have revealed good immobilization efficiencies (73 % with macroporous silica [212], 32 % with glyoxyl-agarose [210], 47 % and 25 % for ionic-exchange resins [213]), in general, it has been reported low activities recoveries of CGTases after immobilization. These activities were in the range of 5.4 % on Eupergit® C [205], 6.1 % on chitosan by covalent method [211], and 3.6 % by adsorption [203]. These reductions in specific activities after immobilization could be caused by steric hindrances, internal diffusional limitations, modifications of the enzyme conformation and/or the active site, as well as to microenvironment of the support matrix, which can differ significantly from the natural environment of the enzyme [205]. However, the possibility of reuse of the biocatalyst can overcome these drawbacks, because good operational stabilities after the immobilization have been observed. For instance, immobilized CGTases presented 40 % of its initial catalytic activity after 10 cycles of 24 h when attached to Eupergit® C [205], 60 % after 100 reuses when on chitosan [211], 85 % after 14 cycles on PVC [208], and about 60 % after 15 cycles on mesoporous silica [212].

The availability of extremely stable CGTases encouraged studies for their utilization in continuous reactors for the production of CDs [214]. For instance, Schöffer et al. [211] evaluated the operational stability of a thermostable CGTase immobilized on spheres of chitosan in a packed-bed reactor. The results showed that 100 % of the initial activity was observed even after 100 h of continuous use, indicating a promising option for its use in an industrial process. Tardioli et al.

[190], tested a fluidized-bed reactor using immobilized CGTase onto silica particles and produced, in only 4 min of residence time, the same amount of CDs normally achieved using the free enzyme after 24 h in a batch reactor. Under the reaction conditions used by the authors, it was possible to obtain a selectivity of 82 % of β-CD [215]. The use of continuous reactors has several advantages, because it simplifies the purification process of products and allows for a more rational approach of scaling-up [37].

Studies involving genetic engineering have gained more attention in the last decade for the cloning of CGTase genes. For instance, the gene coding for CGTase of *B. macerans* was fused to consecutive 10 lysine residues; its product was electrostatically immobilized on a cationic exchanger by adsorption. This new CGTase showed high thermal and operational stabilities. The operational half-life of this poly-Lys enzyme in a packed-bed reactor was 12 days at 25 ºC and pH 6.0 [216]. This modified biocatalyst also presented the advantage of enabling directional immobilization on the solid surface without blocking the active site [217].

5. The GH 70 family of enzymes

5.1. GH 70 enzymes and their characteristics

Glucansucrases, also known as glucosyltransferases, are extracellular enzymes classified as members of GH 70 family, based on four catalytically conserved sequence motifs, which are similar to those of the families GH 13 and GH 77 [218, 219]. The GH 70 family comprises dextransucrase, mutansucrase,

alternansucrase, reuteransucrase, and α-4,6-glucanotransferase, enzymes having high molecular weight, in the range of 120–200 kDa. These enzymes are produced by several lactic acid bacteria strains, such as *Leuconostoc*, *Streptococcus*, *Lactobacillus,* and *Weissella*, microorganisms that are *Generally Recognized as Safe* (GRAS) [220].

Regarding the structure of glucansucrase, its catalytic domain is predicted to be organized in a $(\alpha/\beta)_8$-barrel resembling those of GH 1 and GH 13 enzyme families, but probably circularly permuted. Sequence comparisons between the GH 13 and GH 70 families enabled the identification of only one catalytic triad composed of two aspartic acids and one glutamic acid: Asp-551, as nucleophile, Glu-589, as the acid-base catalyst, and Asp-662, as assistant in the glucosyl-enzyme formation [221]. These residues are strictly conserved for all the GH 70 family enzymes and their mutations lead to enzyme inactivation [218, 222, 223]. Furthermore, it has been reported that there are some amino acids, such as lysine, present in the catalytic domain of dextransucrase, which are responsible for anchoring the substrate and elongating the oligosaccharides in the acceptor reaction [224-226]. The modification of these amino acids is related to enzyme denaturation, probably caused by conformational changes [224, 225].

The amino acid sequence of dextransucrase includes a signal peptide, followed by a variable stretch of approximately 200 amino acids, a conserved core region of about 900 amino acids (N-terminal catalytic core domain), and a series of direct repeating units of about 400 amino acids (C-terminal glucan-binding domain) [227]. This last domain is related to the interaction with sugars during the reaction and possibly aid the anchoring of the growing polymer to the enzyme surface [228].

The glucansucrases catalyze four types of reaction: (i) polymerization, in which occurs the transfer of glucosyl moieties in presence of sucrose onto α-glucans, with the release of fructose as a by-product [229, 230]; (ii) hydrolysis, in which water is used as an acceptor substrate; (iii) acceptor reaction, when glucosyl moieties are transferred to the non-reducing end of acceptor molecules to produce oligosaccharides [218]; and (iv) disproportionation reaction, which is the transfer of the non-reducing end of an α-glucan chain to another α-glucan chain. Particularly, α-4,6-glucanotransferase only catalyzes the disproportionation reaction [231].

The products of polymerization reactions can differ in their glycosidic linkages. Each reaction product may contain a specific pattern: dextran has mainly α-1,6-bonds; mutan, mainly α-1,3-bonds; alternan has α-1,3- and α-1,6-bonds; and reuteran, mainly α-1,4-bonds [232]. Similarly, the products of acceptor reactions can vary in terms of linkages types, size, degree of branches, and spatial arrangements [228]. Maltose, glucose, fructose, D-mannose, and cellobiose have already been evaluated as acceptors for glucansucrases reactions, resulting in different oligosaccharides, such as gluco-oligosaccharides, isomalto-oligosaccharides, among others [233-238].

In this review, we will focus in a specific class of glucansucrases, the dextransucrases (E.C. 2.4.1.5). The bacteria *L. mesenteroides* NRRL B-512 F, *L. mesenteroides* B-512 FMCM, *L. mesenteroides* NRRL B-1299, and *L. citreum* KACC 91348p are among the main microorganisms that synthetize this GH 70 enzyme [239-242]. As stated above, dextransucrase catalyzes the synthesis of dextran and oligosaccharides. The dextran produced in the reaction is tightly bound to the protein, which promotes stability to the biocatalyst [218, 243, 244].

There is growing interest in the enzymatic synthesis of dextran and oligosaccharides because these products find extensive use in the food, feed, cosmetic, and pharmaceutical industries [245, 246]. Dextran have beneficial properties to human health due to antitumoral, immunomodulatory, cholesterol-lowering, and biofilm-formation-inhibiting activities [247]. As functional ingredients, these polysaccharides are versatile across a broad range of applications in the food industry as viscosifying, stabilizing, emulsifying and gelling agents [248]. Additionally, the oligosaccharides have prebiotic potential. An ingredient is classified as prebiotic when it is not digested in the stomach and it is selectively digested by *Bifidobacteria* and *Lactobacillus* in the intestine, stimulating the growth of these beneficial bacteria and, as consequence, improving the host health [249-252]. Dextransucrases have been used to convert the high sugar concentration present in beverages, like orange, mandarin, and cashew apple juices, into prebiotic oligosaccharides [237, 253, 254].

5.2. Immobilization of dextransucrase

For the industrial application of dextransucrase, either for the production of dextran or oligosaccharides, an effective immobilization technique is required to assure continuous processing and reuse of the biocatalyst. Moreover, the immobilization process can promote innumerous other advantages, as mentioned before for the other enzymes in this review.

5.2.1. Immobilization of dextransucrase by entrapment

In the literature, the most studied technique for dextransucrase

immobilization is the entrapment or encapsulation. This method was used by different groups and seems to be the most convenient, because the entrapment enables the immobilization without chemical linkages between the enzyme and the support. Compared to other strategies, the encapsulation presents higher immobilization yields, varying from 57 % to 98 % [255-259]. The enzyme-dextran complex has higher molecular weight, then the dextran-free enzyme, possibly resulting in a more suitable molecule to be retained into the gels [255].

This immobilization protocol presents some advantages and disadvantages. The major problem of this technique is the internal diffusion restriction in the beads. The high dextran content surrounding the dextransucrase probably causes a decrease in the enzymatic activity because of mass transfer phenomena limitation, as it has been reported by Berensmeier et al. [244]. As consequence, the alginate-immobilized dextransucrase can only be used for oligosaccharides production, since the high molecular weight of dextran cannot diffuse out of the beads without their rupture [260, 261]. This polymer layer is also the reason for limited operational stability of the biocatalyst and its lower productivity when it is used in continuous operation. Beyond that, the enzyme can be leaked out of the beads, as result of swelling, and their remaining enzymatic activity is considerably reduced [255, 262, 263].

Comparing dextransucrases from different strains such as from *L. mesenteroides* B-512 F and *L. mesenteroides* B-1299, it is clear that these enzymes demonstrate unique characteristics. While B-512 F dextransucrase showed recovered activity of 84 % after entrapment in calcium alginate, compared to only 57 % of the B-1299 dextransucrase [263]. The distortion caused in the beads are also different. For the immobilized dextransucrase B-1299, the

major product is the oligosaccharide with α-1,2 bonds, whereas immobilized B-512 F dextransucrase produces dextran. These singular products affect the bead conformation, since dextran remains inside the particles, whereas the prebiotic sugars are released to the medium, consequently maintaining the spherical shape of the beads [264].

Diffusion problems in encapsulation-immobilized dextransucrase may also be influenced by the content of dextran, and the size and shape of the particle. It has been observed that only 5 % of dextran is surrounding the enzyme, presumably covalently bonded, which can promote and stabilize the dextransucrase immobilization [258, 263]. Concerning the particle size, an optimal diameter of the alginate beads, which varies from 1 to 5 mm, allows the correct diffusion of substrate and products [244, 255, 258]. Concerning this aspect, Tanriseven and Doğan [235] comparing the shape of alginate fibers and beads, reported immobilization yields of 90 % and 60 %, respectively [260].

Another advantage of dextransucrase encapsulation relates to the simplification of the segregation of products, namely dextran and oligosaccharides, since dextran is limited to the bead microenvironment owing to its molecular size, whereas the smaller oligosaccharides are released to the reaction medium. Furthermore, the alginate matrix possibly acts as a protection for the biocatalyst, with the half-life of the entrapped dextransucrase, which is always reported as being higher than that of the free enzyme [244, 258, 262]. Moreover, glucose and maltose could be added as additional stabilizers [256, 265], and soluble starch as viscosity modulator, protecting the enzyme [266]. In continuous processes, this stabilization effect is even more important, especially within reaction conditions, as evaluated for the production of isomaltose and

gluco-oligosaccharides [256, 259]. For instance, Berensmeier et al. [219] compared the stability of calcium alginate immobilized and free dextransucrase by measuring their half-life at 30 ºC and reported that the immobilized enzyme had this property reduced from 23 h to 0.6 h when the dextran layer was removed by dextranase treatment. The encapsulation may also promote different product selectivity, which is a very interesting property of the system. Although this finding is difficult to explain, it has been suggested that is probably related to the nature of the support and its interaction with the enzyme [267, 268].

Stable preparations of immobilized dextransucrase may also be obtained by cross-linking the enzyme with glutaraldehyde prior to the entrapment in alginate beads and by coating these beads with chitosan films. Kubik et al. [269] found high operational stability for immobilized dextransucrase cross-linked with 10 % of glutaraldehyde. The immobilized biocatalyst could be used for 10 reaction cycles for isomalto-oligosaccharides production, reaching 60 % to 70 % of sucrose conversion, even in the last batch. When chitosan was used to coat the alginate beads aiming to promote stabilization of particles, the sucrose conversion and the isomaltose yields were slightly reduced, probably caused by diffusional limitations. Nevertheless, the chitosan, linked via electrostatic interactions with the alginate beads, avoided the leakage of encapsulated enzyme to the reaction medium [270]. These findings also demonstrate that the enzyme entrapped in alginate, without cross-linking, would leak out of the support, especially when dextranase is applied in the reaction medium.

The calcium alginate immobilized enzyme has been evaluated in fluidized and packed-bed reactors [262, 271], and depending on the reactor configuration, some variables may be studied in order to optimize the process. Packed-bed

reactor was used to continuously synthesize gluco-oligosaccharides (GOS) and it was observed the accumulation of dextran inside the beads, probably because of the high amount of initial sucrose concentration [262]. This microenvironment – forming a bead/enzyme-dextran complex – can be related to the swelling of beads. Quirasco et al. [271] demonstrated that the whole cell from *L. mesenteroides* B-1299 could be encapsulated in alginate beads in order to produce GOS in a packed-bed reactor, with immobilization yields of 93 %. These authors reported similar problems for encapsulated whole cells as reported for the purified biocatalyst.

Berensmeier et al. [219] designed a fluidized-bed reactor to operate with a high-density fluid phase of concentrated sugar solutions to produce isomalto-oligosaccharides. In order to overcome the possibility of flotation of the beads, the authors used silica flour added to alginate to increase the bead density [244]. Another important point is the weakly repressed dextran formation. It has been demonstrated that it is possible to obtain higher mechanical stability of the immobilized catalyst and higher oligosaccharides yields with the optimal sucrose/acceptor ratio in the reaction. For long-term operation, sucrose should be kept at low concentrations, whereas the acceptor concentration should be high to avoid dextran synthesis [244, 256]. Some authors reported that sucrose in high concentrations might block the reaction chain of oligosaccharides because some sugar molecules are linked to the enzyme in its allosteric site affecting the shape of the protein. Therefore, the glucose residues anchored in the dextransucrase active site cannot form the glycosidic bond [272].

Besides dextransucrase encapsulation, the co-immobilization of this enzyme with dextranase (EC 3.2.1.11) is another way to efficiently synthesize

dextrans and oligosaccharides [238, 273]. This technology allows the design of novel multi-functional biocatalysts and can display benefits owing to the synergy of the enzymes [274, 275]. However, the challenge of this kind of co-immobilization is to avoid the inactivation of dextransucrase that is promoted by dextranase. The enzyme-dextran complex can be cleaved up to 97 %. Because endogenous dextran is essential for the retention of dextransucrase activity, the enzyme stability is related to the presence of a dextran layer [244]. At the same time, the hydrolytic activity of dextranase may regulate the molecular size of the product and the availability of the acceptor, in such a way that the synthesis of products can be directed to obtain desirable characteristics, such as prebiotic effect [276].

Erhardt et al. [238] studied the immobilization of dextranase prior to co-entrapment with dextransucrase into calcium alginate. Hydroxyapatite was found to be the best support, since it was almost inert to dextransucrase and was ideal to adsorb dextranase. Their findings suggest that the co-immobilization on a solid phase prior to entrapment suppresses bead swelling owing to reduced dextran formation and slows dextransucrase inactivation. The co-immobilized biocatalyst kept only 25 % of its initial activity after 6 batches, when the ratio of dextranase/dextransucrase activities of 0.3:1 was used.

Ölçer and Tanriseven [266] developed a simple and effective co-immobilization method of dextransucrase/dextranase bearing potential for industrial-scale production of isomalto-oligosaccharides. These compounds can be produced either by acceptor reactions of dextransucrase or hydrolysis of dextran by dextranase as already reported by Goulas et al. [276]. An important aspect of this particular immobilization was the pre-immobilization of dextranase

by covalent attachment on Eupergit® C prior to alginate co-immobilization (beads, fibers, and capsules), preventing dextranase leakage and dextransucrase inactivation. The best immobilization yield was 71 % in alginate capsules. The enzymes retained their activities during 20 repeated batch reactions and for a month when stored at 4 ºC [266]. Goulas et al. [273, 276] investigated the production of isomalto-oligosaccharides using the free form of both enzymes, in batch and in a continuous recycling ultrafiltration membrane reactor. The authors reported similar yields of sucrose conversion by using free dextransucrase or its immobilized preparations. However, when the authors co-immobilized the dextransucrase and dextranase, the rate of formation and the size of acceptor products were regulated, in contrast with the use of absolute concentrations, leading to polymers with more persistent prebiotic effect.

Another interesting material for the entrapment of biocatalysts is the hydrogel formed by polyvinyl alcohol (PVA). PVA is highly elastic, stable, and suitable as entrapper to immobilize dextransucrase. Commercially available PVA-particles (LentiKats®) have lens-shaped form, a diameter in between 3 and 5 mm, and a thickness of 300 to 400 mm, and they have been used to immobilize dextransucrases. Dextransucrase from *L. mesenteroides* B-1299 encapsulated in LentiKats® had similar recovery of activity (approximately 55 %) when compared with entrapment in calcium alginate gels. In addition, the conversion to α-1,2-linear and branched oligosaccharides using LentiKats®-dextransucrase was higher than that obtained for alginate-dextransucrase, probably because of the reduction of diffusional limitations derived from its lenticular shape. Other important parameter is the protein leaching. This problem was reduced from 18 % to 4 % by pre-treating dextransucrase with glutaraldehyde. However, this

complex glutaraldehyde-enzyme possibly affected some amino acids in the catalytic site, as the yields and the specific activity of this preparation were lower than expected [268].

5.2.2. Immobilization of dextransucrase by covalent immobilization

Several groups have reported the covalent immobilization of dextransucrase. In this case, the carriers must display high density of reactive groups for attachment of the enzyme to the support. The enzyme was covalently immobilized in Bio-Gel P-2 [277], polyacrylamide gel, cellulose acetate membranes, polysulfone hollow fiber [278, 279], and akylamine porous silica [280]. The covalent binding of dextransucrase to porous silica activated with α-aminopropyl and glutaraldehyde has also been described [265, 281]. Most of these studies reported low immobilization yields, low specific activities and poor operational stability. These findings indicate that some of the reactive groups present at the catalytic domain, such as lysine, could react with the aldehyde and epoxy groups of the covalent immobilization matrices. Moreover, the dextran associated with the enzyme covers the reactive groups on its surface, which possibly affects the covalent immobilization of dextransucrase [224, 261, 282].

Epoxy supports have been demonstrated to be suitable supports for enzyme immobilization, bearing industrial potential [283, 284]. These epoxy-activated supports were able to chemically react with different nucleophile groups placed on the protein surface: lysine, histidine, cysteine, tyrosine, among others [285]. Gómez de Segura et al. [263] immobilized the dextransucrase on epoxy activated acrylic polymers with different textural properties (Eupergit® C and

Eupergit® C 250L). In order to promote the accessibility of reactive groups of the enzyme surface to the epoxide centers of the support, easing the covalent coupling, the native dextransucrase, which may contain up to 80 g glucose/g protein [286], was treated with dextranase to assure the removal of dextran layer. These findings suggest that Eupergit® C 250L works better to bind enzyme molecules inside its macroporous matrix, since has higher volume and higher diameter of pores compared to Eupergit® C, which explains its higher specific activity (up to 710 U/g). The maximum activity recovered was 22 %, with an immobilization yields of 72 % using Eupergit® C 250L and the immobilized enzyme kept more than 40 % of its initial activity over 2 days at 30 ºC and pH 5.4. Unlike alginate beads, the authors reported that dextransucrase did not diffuse into swelled matrices [263].

Hashem et al. [287] evaluated the covalent immobilization of dextransucrase from *Enterococcus faecalis* Esawy by Fe^{3+}-cross-linked alginate/carboxymethyl cellulose beads modified with polyethylenimine and glutaraldehyde. The immobilization yields reached 94.35 %. Moreover, the immobilization process improved the thermal and pH stability of the enzyme to great extent, probably caused by covalent attachment that protects against protein conformational changes [41, 285]. Reusability tests proved that the enzyme retained 60 % of its initial activity after 15 batch reactions [287].

Alcalde et al. [258] investigated the immobilization of dextransucrase by covalent attachment on activated silica (silica X030). The removal of the dextran covering the enzyme surface was essential to promote the bonding and, indeed, while the dextran-free enzyme showed immobilization yields of 13 %, the native dextransucrase presented yields of only 0.6 %. These low immobilization yields

might be related to the participation of a lysine residue in the catalytic domain of dextransucrase. The interaction of this Lys with the support probably changes the protein conformation, reducing its catalytic activity [224, 288, 289]. The residual activity of the biocatalyst immobilized on the silica decreased from 58 % to 17 % after 48 h of reaction, fact that did not occur when the dextransucrase was immobilized in alginate beads [258].

5.2.3. Immobilization of dextransucrase by adsorption

There are few works on dextransucrase immobilization using the methodology of adsorption. Kaboli and Reilly [280] tried to attach the dextransucrase from *L. mesenteroides* B-512 F by anion exchange to DEAE-cellulose, DEAE-Sephadex A-25 and A-50, and by cation exchange SP-Sephadex C-25 and C-50, without satisfactory results. Hydroxyapatite [290], Sephadex G200 [291], and phenoxyacetyl cellulose [292] have also been studied as carriers to adsorb the biocatalyst, all of them without success.

5.2.4. New approaches in dextransucrase immobilization

Some research groups have been using different protein engineering tools to enhance several dextransucrase parameters, such as the catalytic activity and type of reaction products [223, 228, 293]. These strategies, combined with immobilization techniques, have provided good results. Parlak et al. [261] developed a bioengineering study, in which a novel dextransucrase was fused to a glutathione-S-transferase (GST), to facilitate the covalent immobilization on Eupergit® C 250L. The modification provided additional 21 lysines, 18 aspartic

acids, 16 glutamic acids and 4 cysteines. The results showed the importance of the fusion protein because the immobilization yields and recovered activity were 100 % and 83.3 %, respectively. Comparatively, under the optima conditions, the immobilization of truncated dextransucrase without GST resulted in 100 % of immobilization yields, but only 3 % of recovered activity. Furthermore, the immobilized enzyme showed no decrease in activity for 15 batch reactions and retained its initial activity at 4 ºC storage for 35 days.

The challenge remains about the glucansucrase structure and its surface reactive groups to improve the immobilization parameters. Until now, there are only five three-dimensional structures known of GH 70 glucansucrases: glucansucrase GTF180-ΔN from *L. reuteri* (PDB: 3KLK; [294]), glucansucrase from *S. mutans* (PDB: 3AIE; [295]), dextransucrase DSR-E from *L. mesenteroides* NRRL B-1299 (PDB: 3TTQ; [296]), glucansucrase GTFA from *L. reuteri* 121 (PDB: 4AMC; [297]), and dextransucrase DSR-E from *L. mesenteroides* NRRL B-1299 (PDB: 4TVD; [298]). Comparatively, the Protein Data Bank (www.rcsb.org) presents more than 200 three-dimensional structures of members of GH 1 and GH 13 families. This lack of information about the crystal structures of glucansucrases makes difficult to predict which bonds will be involved in the immobilization process.

Further developments in elucidating aspects of the domains of glucansucrases must also be addressed. The glucan-binding domain, found at C-terminal, is a highly conserved region. Some authors discovered that this domain plays a major role in polymer elongation, since it is a sucrose and/or polymer-binding site [224, 299] and its truncation can result in an enzyme much less efficient in catalyzing high molecular weight polymers [228]. On the other

hand, this domain does not seem to be involved in linkage specificity [228], and it has been demonstrated that the truncation of dextransucrase in this region, associated with the fusion to other protein, preserves the catalytic activity and enables the immobilization with satisfactory results [261]. Therefore, it is important to find which domains could interact with supports, and to evaluate if these bonds are responsible for activity losses or enzyme inactivation.

Kaboli and Reilly [280] stated in 1980 that “dextransucrase is an extraordinary difficult enzyme to immobilize”. In fact, it has been demonstrated that glucansucrases present some shortcomings. To the best of our knowledge, there is no work with glucansucrases immobilized via adsorption or covalent binding applied in continuous reactors. Therefore, there are still many aspects to be investigated about their immobilization and their behavior under continuous operation applied to synthesize different desirable products. Further research is in need to develop a stable, versatile, and robust immobilized biocatalyst based on glucansucrases.

6. Future perspectives

We discussed the main protocols used to immobilize the enzymes from GH 1, GH 13, and GH 70 families of enzymes, with special focus on β-glucosidases, α-amylases, cyclodextrin glycosyltransferase, and dextransucrase. Immobilization is not only a strategy to turn the soluble enzymes into heterogeneous catalysts, but it is also a way to improve and/or change their activities, stabilities or specificities. In general, developments in material science and molecular biology are the main tools to obtain new support matrices and to

improve enzyme properties for immobilization.

In the case of the GH 1 family, although carriers and immobilization methods have been intensively investigated, there are still few studies using techniques as co-immobilization on solid supports, or the preparation of CLEAs, or even combi-CLEAs, using the β-glucosidase in combination with other enzymes. These studies are very interesting because the co-immobilization of several enzymes can be applied in sequential biocatalytic processes, thus reducing costs for industrial applications. In addition, recent progress on the field of the structural characteristics of β-glucosidases from GH 1 family, have been providing the starting point to improve and orientate the immobilization by site-directed mutagenesis on enzyme surface following the immobilization on solid supports. This may increase the stability and could be useful to obtain mutants with more advantageous characteristics, such as lower product inhibition, and higher activities and/or specificities.

Concerning the GH 13 family, although CGTases are well known for their use in the production of CDs, many additional applications have been explored based on the other reactions catalyzed and a huge industrial demand for these enzymes has emerged over the last decades. Efforts have been made to improve these enzymes to better suit industrial applications by immobilization techniques. Recent researches are being directed to improve the enzyme performance using the heterologous expression and molecular engineering. Site-directed mutagenesis has been applied extensively, enhancing properties such as substrate conversion, product specificity, stability, and specific activities. However, further developments are still necessary in order to biological models suitable for the heterologous expression aiming at improving the quantity and

quality of these enzymes and to enable their use in different areas.

Glucansucrases from GH 70 family were first immobilized more than 40 years ago. However, a high-recovered activity of immobilized biocatalyst for industrial applications remains as a challenge. So far, best technologies for the immobilization of glucansucrases are mainly based on entrapment. Nevertheless, as for β-glucosidases, CLEAs appear as a new perspective to be used with glucansucrases. Finally, further investigations on protein engineering combined with immobilization techniques may be useful to identify amino acids that can be modified in order to enhance the immobilization parameters of glucansucrases.

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Author contributions

N.G.G researched about GH 70 family, J.N.S. researched about GH 13 family, D.A. researched about GH 1 family. P.H., M.A.Z.A. and R.C.R are responsible for all research and revise the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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CAPÍTULO III – DEXTRANSUCRASE IMMOBILIZED ON ACTIVATED- CHITOSAN PARTICLES AS A NOVEL BIOCATALYST

O Capítulo III, intitulado *Dextransucrase immobilized on activated-chitosan particles as a novel biocatalyst*, está apresentado na forma de artigo, publicado no periódico *Journal of Molecular Catalysis B: Enzymatic* (DOI: http://dx.doi.org/10.1016/j.molcatb.2016.12.007).

O trabalho versa sobre a imobilização covalente de dextransucrase de *L. mesenteroides* B-512 F em esferas de quitosana funcionalizadas com glutaraldeído. Nesse estudo, o efeito da carga de proteína na imobilização foi avaliado. A fim de caracterizar o novo biocatalisador, pH e temperatura ótimos, estabilidade ao armazenamento, estabilidades térmica e operacional foram determinados.

Dextransucrase immobilized on activated-chitosan particles as a novel biocatalyst

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ABSTRACT

Dextransucrase from *Leuconostoc mesenteroides* B-512 F was covalently immobilized on glutaraldehyde-activated chitosan particles. The best initial protein loading (400 mg/g of dried support) showed 197 U/g of catalytic activity. The optimal reaction pH and temperature of this new biocatalyst were determined to be 4.5 and 20 ºC, respectively. Regarding the thermal stability, the immobilization enhanced enzyme protection against high temperatures, whereas glucose and maltose acted as stabilizers. The biocatalyst was stable under storage at 5 ºC for a month. The biocatalyst presented good operational stability, retaining up to 40 % of its initial activity after ten batch cycles of reaction to obtain oligosaccharides. These results suggest the use of the immobilized dextransucrase on chitosan particles as a promising novel biocatalyst to produce dextran and oligosaccharides.

Keywords: Dextransucrase; enzymatic covalent immobilization; chitosan; dextran; oligosaccharides.

1. Introduction

Glucansucrases, belonging to the glycoside-hydrolase GH 70 family of enzymes, are extracellular enzymes, recognized as GRAS for food applications, and are produced by lactic acid bacteria, especially of the genera *Leuconostoc*, *Streptococcus*, *Lactobacillus,* and *Weissella* [1]. Among the glucansucrases, dextransucrase (sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferases, EC 2.4.1.5) has become the most intensively studied enzyme, especially the one from *Leuconostoc mesenteroides* strain B-512 F, because of the characteristics of dextran and oligosaccharides obtained by this enzyme [2-4]. This enzyme catalyzes mainly polymerization and acceptor reactions. Polymerization comprises the transfer of glucosyl moieties, in presence of sucrose, onto α-glucans [5, 6], whereas in the acceptor reaction, the glucosyl moieties are transferred to the non-reducing end of acceptor molecules producing oligosaccharides [7].

There is a growing interest in the enzymatic synthesis of dextran and oligosaccharides because these products are so far difficult to be obtained via chemical synthesis. These molecules have an extensive use in the food, feed, cosmetic, and pharmaceutical industries [8-10]. Dextran can act as functional ingredients as stabilizing, emulsifying, and gelling agents [11]. Concerning oligosaccharides, many of them hold prebiotic potentials, being selectively digested by *Bifidobacteria* and *Lactobacillus* in the intestine of humans and animals, stimulating the growth of these beneficial bacteria and, as a consequence, improving the host health [12-15].

In order to assure continuous processing and reuse of the biocatalyst in industrial applications of dextransucrase, an effective enzyme immobilization

technique would be required. Reports on the literature show that the most studied immobilization technique for dextransucrase is the process of enzyme encapsulation. Comparing this technique with other immobilization systems, such as covalent binding and adsorption, the encapsulation presents higher immobilization yields, which varies from 57 % to 98 % [16-20]. However, this method presents some limitations, especially concerning substrates and products diffusion problems, swelling of particles, protein leakage to the medium, as well as low operational stability under continuous operation [16, 21-23].

Another important technique for enzyme immobilization is the covalent attachment using glutaraldehyde, which is the most used approach to immobilize many enzymes [24]. The linkage of enzyme and activated-support takes place between the most reactive amino group exposed on the enzyme structure, which usually is the terminal amino group [24, 25]. The covalent attachment methodology is simple to be carried out and efficient, the glutaraldehyde-protein bonds formed are stable, promoting good stabilization factors. This approach can be used in very different reaction conditions [24, 26, 27].

Several supports have already been evaluated to covalently bind dextransucrase, among them the Bio-Gel P-2 [28], polyacrylamide gel, cellulose acetate membranes, polysulfone hollow fibers [29, 30], akylamine porous silica [31], and porous silica activated with α-aminopropyl and glutaraldehyde [32, 33]. Most of these studies reported low immobilization yields, low specific activities and poor operational stabilities. The main problems associated with these types of immobilizations might be associated with the dextran layer linked to the enzyme that covers the reactive groups on its surface blocking the interaction with the carrier [4, 34, 35]. Some authors studied the rupture of the complex dextransucrase-dextran in

order to make available the amino acids on the enzyme surface, thus allowing the immobilization via covalent binding [19, 23].

Chitosan, a cationic polymer of β-1,4 glycosidic linkages, is obtained from chitin, a major natural structural component of the invertebrate's exoskeleton and fungi cell wall [36]. This polymer has been successfully applied for enzyme immobilization to be used in feed and food process, because of its non-toxicity, high protein affinity, and biocompatibility and biodegradability properties [37-39]. Moreover, the enzyme can be strongly linked to the chitosan surface [40], and the major part of the immobilized enzyme is located on the external surface, which can effectively reduce mass-transfer limitations [41]. Several studies from our group reported the increase in thermal and operational stabilities of different enzymes when immobilized on glutaraldehyde-activated chitosan particles [27, 42, 43]. Additionally, lysozyme, stem bromelain, and lipases are other examples of enzymes that are used in food applications, which were immobilized on chitosan-based supports [44, 45]. To the best of our knowledge, there are no reports regarding the use of activated chitosan as support for the immobilization of dextransucrase.

Considering all these aspects, the objective of this work was to study the covalent immobilization of dextransucrase on chitosan particles. The immobilization procedure followed two steps: first, the hydrolysis of the dextran-layer around dextransucrase molecules was carried out using dextranase; then, the immobilization of the enzyme proceeded on glutaraldehyde-activated chitosan particles. Optimal pH and temperature, the thermal and storage stabilities, and the operational stability of the immobilized biocatalyst in repeated reaction batches of oligosaccharides formation were also tested.

2. Experimental

2.1 Materials

Chitosan from shrimp shells (> 75 % deacetylated) was purchased from Sigma-Aldrich (USA). Dextranase Plus L (Novozymes A/S) were kindly provided by LNF Latino Americana (Bento Gonçalves, Brazil). Glutaraldehyde (50 % mass fraction solution) was bought from Dinâmica Ltda. (Brazil). Sucrose, glucose, and maltose (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade, purchased from readily available commercial sources.

2.2 Microorganism, culture medium and enzyme production

A strain of *L. mesenteroides* B-512 F obtained from Fundação Tropical de Pesquisas e Tecnologia André Tosello (Campinas, SP, Brazil) was used for dextransucrase production. The strain was preserved as frozen samples in MRS (de Man, Rogosa, and Sharpe) medium and 20 % (volume fraction) of glycerol.

The culture medium used for microbial growth and enzyme production was composed of (in g/L): sucrose, 50; yeast extract, 20; $MgSO_4 \cdot 7H_2O$, 0.20; $MnSO_4 \cdot 2H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.02; NaCl, 0.01; K_2HPO_4, 20, pH adjusted to 6.7 with phosphoric acid 10 % (volume fraction) as proposed by Rabelo et al. [46].

Batch cultivations of *L. mesenteroides* B-512 F were carried out in stirred tank bioreactors (Biostat B model, B. Braun Biotech International, Germany) to produce the

enzyme. Pre-inoculum was prepared by transferring one isolated colony of *L. mesenteroides* B-512 F to 500 mL conical flasks containing 100 mL of the culture medium and incubated in an orbital shaker at 180 rpm for 16 h at 30 ºC. From this culture, a volume of 200 mL with a cell concentration corresponding to OD_{600} = 1.0 was added to the bioreactor, which was filled with 1.8 L of culture medium. Culture conditions were based on the work of Rabelo et al. [46], who defined the best temperature for bacterial growth and enzyme production as 30 ºC. Under these conditions, the dextransucrase production was 7.5-fold higher than that reported by Goyal et al. [47]. Other cultivation parameters were controlled pH at 6.7, agitation speed of 500 rpm, and air flow of 0.5 L air/min for 6 h. This pH value was kept by the addition of a solution containing sucrose (300 g/L) and NaOH (120 g/L), to optimize the enzyme production, following procedures described by Rabelo et al. [46]. At the end of feeding, culture proceeded for another 2 h to allow the pH to reach 5.2, the optimal pH to preserve enzyme activity. At this point, cells were harvested by centrifugation at 12,000 × *g* for 15 min at 4 ºC to recover the supernatant. The enzyme was partially purified from the culture broth by the precipitation using polyethylene glycol (PEG 1500 – 50 % mass fraction), according to Paul et al. [48] with some modifications. The samples were centrifuged at 4,500 × *g* for 15 min at 4 ºC. The salts and media components were not removed. After the centrifugation, the denser (bottom) phase contained dextran along with approximately 90 % of dextransucrase activity, whereas the upper phase contains glucose, fructose, and polyethylene glycol (PEG), according to reports by Paul et al. [48]. The enzymatic extract prepared in this way was described as being free of levansucrase activity. The supernatant containing the concentrated enzyme (enzymatic extract) was stored at -20 ºC until further

analyses. The obtained enzymatic preparation, containing 49 U/mL (specific activity of 7.9 U/mg protein), was used for the immobilization protocol.

2.3 Synthesis of chitosan spheres for immobilization

The chitosan particles were prepared by dripping the chitosan solution into the alkaline coagulation solution, as described by Klein et al. [27]. The prepared particles had spherical shape with a diameter of approximately 2 mm and pore sizes varying from 20 to 200 Å, and a dry weight of 0.343 ± 0.008 mg per sphere. The activation was carried out incubating the chitosan particles with 5 % (v/v) of glutaraldehyde solution at 25 ºC for 3 h, as reported by Lorenzoni et al. [42].

2.4 Dextransucrase immobilization

Because dextran is a polymer, and its removal by dialysis is a complex process, it was chosen the treatment using dextranase prior to the immobilization. Therefore, before the immobilization, the dextran layer surrounding the enzyme molecule was removed using Dextranase Plus L [23]. The dextranase was added to the solution containing dextransucrase and the reaction proceeded for 8 h at 5 ºC. After this treatment, the specific activity of dextran-free dextransucrase was 7.9 U/mg. The immobilization was carried out incubating the activated-chitosan particles with the enzyme solution, properly diluted in activity buffer (50 mM sodium acetate, pH 5.2) to obtain different protein concentrations (25, 50, 100, 200, and 400 mg protein/g support), at 5 ºC under gentle shaking for 16 h.

After immobilization, the biocatalyst was washed with 1.0 M NaCl solution and ethylene glycol (30 %, volume fraction) to eliminate the non-covalently bonded enzyme. The equations of immobilization yield (IY), immobilization efficiency (IE), and recovered activity (RA) were calculated as described by Sheldon and van Pelt [49] and are shown below as equations 1, 2, and 3. Soluble protein was determined by the Lowry method using bovine serum albumin (BSA) as protein standard [50].

$$Immobilization\ yield\ (\%) = \left[\frac{Immobilized\ activity}{Starting\ activity}\right] \times 100 \quad (1)$$

$$Efficiency\ (\%) = \left[\frac{Observed\ activity}{Immobilized\ activity}\right] \times 100 \quad (2)$$

$$Activity\ recovery\ (\%) = \left[\frac{Observed\ activity}{Starting\ activity}\right] \times 100 \quad (3)$$

2.5 Activities of free and immobilized dextransucrases

To measure the free dextransucrase activity, a volume of 100 µL of the enzymatic extract was mixed with 900 µL of 20 mM sodium acetate, pH 5.2, which contained 292 mM sucrose and 0.45 mM $CaCl_2$. This preparation was then incubated at 30 ºC for 1 h [51]. The activity of immobilized biocatalyst was measured by incubating the chitosan particles in 2 mL of the same substrate solution, at 30 ºC for 1 h, under agitation. The fructose released was measured using the 3,5-dinitrosalicylic acid method [52]. One unit of dextransucrase activity was expressed as the amount of enzyme that releases 1 µmol of fructose per minute under these conditions. All enzymatic activities measurements were carried out comparing against the control (reaction medium without the addition of sucrose).

2.6 Optimal pH and temperature for enzyme activity

The effect of temperature on the activity of free and immobilized enzymes was measured at different values (5-60 ºC), using 50 mM sodium acetate buffer, pH 5.2. The effect of pH was investigated using different buffers that were varied from 3 to 8 (it was used acetate buffer for pH from 3 to 5, and phosphate buffer for pH from 5.5 to 8), at 30 ºC. The enzymatic activity was measured as described above. The relative activity was calculated as the ratio between the activity at each temperature or pH and the maximum obtained.

2.7 Biocatalyst thermal stability

The thermal stability of the soluble and immobilized enzymes was performed by incubating the biocatalyst in a sodium acetate buffer (50 mM, pH 5.2), at 30 ºC, 40 ºC, and 50 ºC. Periodically, samples were withdrawn and the dextransucrase activity was measured. The thermal inactivation was described by a first order reaction (Equation 4):

$$\frac{A}{Ao} = \exp(-kt) \quad (4)$$

Where *A* is the enzyme activity at time t, *Ao* the initial enzyme activity, *t* is the reaction time, and *k* is the inactivation rate constant at the studied temperature. The half-life ($t_{1/2}$) was calculated from the values of *k* (Equation 5):

$$t_{1/2} = \frac{\ln(2)}{k} \quad (5)$$

2.8 Storage and operational stabilities

Storage stability of biocatalysts was carried out by storing free and immobilized enzymes at 5 ºC for 30 days. The activity of both biocatalysts was periodically measured as described in Section 2.6.

The operational stability for oligosaccharides production, using a total of 0.3 U of immobilized enzyme, was performed by repeated batch reactions at 30 ºC with 100 mM solution of sucrose and 600 mM solution of maltose, both diluted in 50 mM sodium acetate buffer pH 5.2, at 30 ºC for 3 h. At the end of each batch, the immobilized enzyme was separated from the reaction medium by a simple filtration, it was washed using 50 mM sodium acetate buffer, pH 5.2 to remove possible residues of substrate or product, and reused in a new fresh reaction. The enzymatic activity measured in the first cycle was considered as 100 % for the calculation of the remaining activity after each successive batch. The concentrations of substrates and products were determined by the HPLC analysis.

2.9 HPLC analyses of sugars in reaction medium during operational stability tests

The samples obtained in the operational stability test were analyzed using a HPLC system (Shimadzu, Tokyo, Japan) equipped with refractor index using Shodex NH2 P50-4E (4.6 mm × 250 mm) column. Acetonitrile:ultra-pure water (75:25) was used as eluting solvent at a flow rate of 1.0 mL/min. The column temperature was kept constant at 30 ºC. The samples were diluted and filtrated (0.22 μm), before the injection. Glucose, fructose, sucrose, and maltose were used as external standards.

3. Results and Discussion

3.1 Dextransucrase immobilization

Initially, it was tested the immobilization of the native dextransucrase on chitosan particles that were functionalized with glutaraldehyde. Immobilization yields were lower than 1 % because the dextran layer surrounding the enzyme blocks the access of reactive groups in the enzyme surface to access the glutaraldehyde on the support surface. Thus, dextran hydrolysis was performed to solve this problem by pre-treating dextransucrase enzymatic extract with dextranase. Low immobilization yields were also observed by other authors for native dextransucrase using activated silica X030 (aminopropyl trimethoxysilane/glutaraldehyde) [19], Eupergit® C, and Eupergit® C 250L [23] as supports, and the immobilization was only possible after the hydrolysis of dextran layer by a fungal dextranase [23]. The immobilization of dextransucrase after the removal of dextran was carried out at pH 5.2 and 4 ºC to avoid protein inactivation during this step [19].

The protein loading for dextran-free dextransucrase immobilization was tested and the immobilization parameters are shown in Table 1. In contrast with observations for encapsulated enzymes, in which the amount of protein is limited by the gel-pore size [22], it was possible to load from 25 up to 400 mg of protein/g of dried support during the immobilization of dextransucrase using chitosan particles, as the final activity obtained for the immobilized biocatalyst was high. Immobilization yields obtained in this work were similar to those reported in the literature for the immobilized dextransucrase by encapsulation in Ca-alginate beads, reported as the most successful technique [17, 19, 20, 22].

Table 1. Effect of protein loading on dextransucrase immobilization (*)

Protein loading (mg/g of dried support)	Immobilization yield (%)	Immobilization efficiency (%)	Recovered activity (%)	Activity of immobilized biocatalyst (U/g of dried support)
25	76.89 ± 1.31	11.05 ± 1.34	8.48 ± 0.58	14.99 ± 0.11
50	84.68 ± 0.47	10.15 ± 1.29	8.52 ± 0.72	25.99 ± 0.20
100	90.79 ± 0.24	8.12 ± 0.56	8.11 ± 0.38	43.64 ± 0.08
200	95.75 ± 0.41	6.05 ± 0.99	11.58 ± 0.33	85.94 ± 0.72
400	98.20 ± 0.15	8.19 ± 0.08	8.05 ± 0.72	197.27 ± 0.97

(*) Values represent the mean of duplicates.

Kaboli and Reilly [31] reported the covalent immobilization of dextransucrase on alkylamine porous silica, recovering only 10 % of activity, producing an immobilized biocatalyst with only 0.51 U/g [31]. Monsan and López [32] and Monsan et al. [33] used glutaraldehyde as crosslinking agent on aminated silica and Spherosil to immobilize dextransucrase, obtaining less than 2 % of recovered activity in both supports [32, 33]. This may be explained by the formation of a complex glutaraldehyde-enzyme that can affect some important amino acids involved in the catalysis, by steric hindrance for example, which was also observed for the encapsulation of dextransucrase in LentiKats® when using the cross-linker [53].

The highest activity of covalently immobilized dextransucrase on glutaraldehyde-activated chitosan particles was 197 U/g of dried support (1.8 U/mg of

specific activity). The immobilization time was 16 h, which is much shorter than the 72 h used for the immobilization of dextransucrase on the commercial epoxy-supports, Eupergit® C and Eupergit® C 250L [23]. The high activity of the immobilized dextransucrase in this work represents an improvement compared to other covalent immobilization approaches, such as using epoxy supports and glutaraldehyde-activated silica [4, 19, 23].

The main difference among these protocols of immobilization is related to the distinct properties of each solid support, such as pore and particle sizes, and surface area. The pore diameter is important in allowing high amounts of enzyme molecules to bind in the support. In the case of chitosan particles, silica X030, Eupergit® C, and Eupergit® C 250L, they show pore diameters varying from 20 to 3,000 Å [19, 23, 27]. There is an expected relationship between recovered activity and pore diameter, since the dextransucrase is an enzyme with high molecular weight, thus it can block the diffusion of molecules into swelled matrices. Nevertheless, the interaction of support and enzyme is also essential for a high recovered activity in the immobilization process.

Concerning the amount of total protein loading offered to the immobilization reaction, in the case of this study, the immobilized biocatalyst with initial protein loading of 400 mg of protein/g of dried support was chosen for further experiments, because it allowed for the highest activity of the final biocatalyst.

3.2 Optimal pH and temperature of enzyme activity

Fig. 1 shows that the optimum activity for native, dextran-free and immobilized

enzyme were 5.0, 5.5, and 4.5, respectively. The immobilized biocatalyst presented higher activity in a broader range of pH than the free forms, similarly as reported by some authors [4, 20, 54, 55]. It was observed a catalytic activity of approximately 30 % for pH 7, and 20 % for pH 8. Similarly, at the lower end of pH values (3 and 3.5), the immobilized dextransucrase also presented higher activity compared to the free enzymes. Some authors have evaluated the application of dextransucrases in fruit juices that would work as acceptors for oligosaccharides *in situ* production because of their high concentrations of sugars [56, 57]. Therefore, it would be highly desirable to obtain an immobilized dextransucrase that would work at low pH values, which it is characteristic of these beverages.

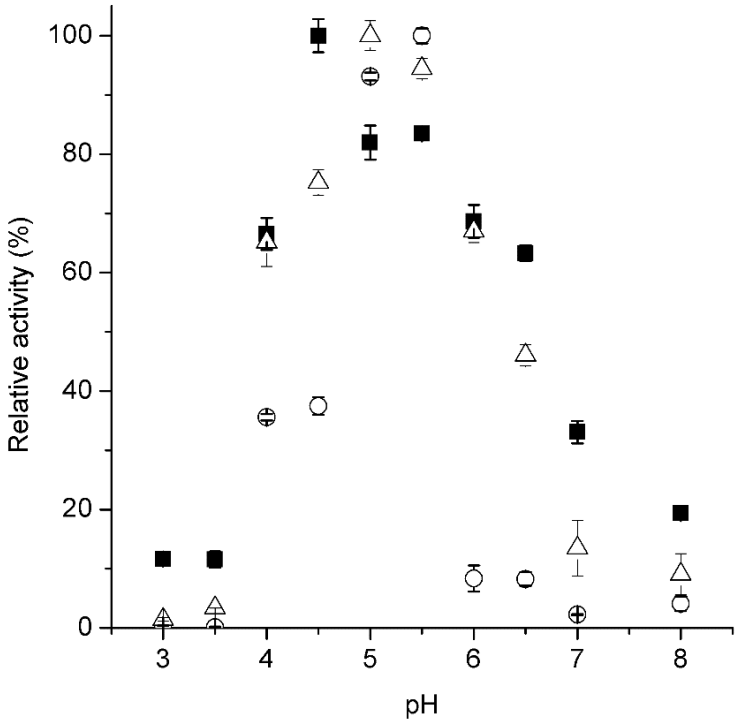

Fig. 1. *L. mesenteroides* B-512 F dextransucrase activity as function of pH: (■) enzyme covalently immobilized on glutaraldehyde-activated chitosan particles; (○) dextran-free soluble enzyme; (△) native soluble enzyme. Tests were carried out at 30

ºC. The specific activities of native enzyme, dextran-free soluble enzyme, and immobilized dextransucrase were 10.1 U/mg, 7.9 U/mg, and 1.8 U/mg, respectively. Values represent the mean of duplicates.

The temperature activity profile of soluble forms and covalently immobilized dextransucrase is presented in Fig. 2. The optimal temperature for native and immobilized enzyme was 20 ºC, whereas for the dextran-free soluble enzyme was 30 ºC. The differences of enzymatic activities at 20 ºC and 30 ºC are small. Possibly, it is likely that the removal of the dextran layer could change the enzyme conformation and increase the reaction rate with the temperature. Additionally, the immobilized biocatalyst showed more than 50 % of the maximal activity from 5 ºC to 40 ºC. In fact, the residual activity of the immobilized biocatalyst for most of the evaluated range of temperatures was higher compared to the residual activity of the soluble forms. Some authors have reported similar behaviors using other immobilized preparations, confirming the positive effects of the immobilization process [20, 23, 55].

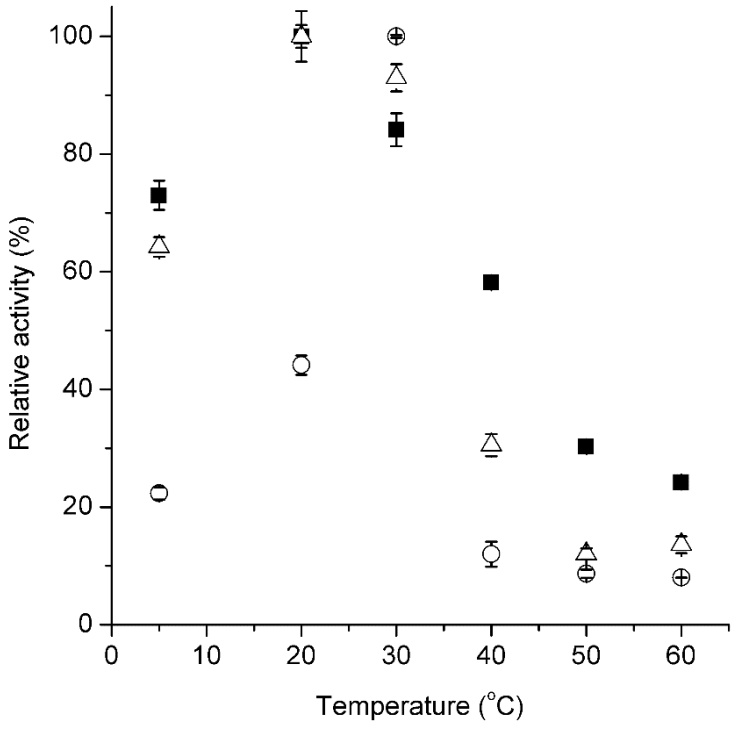

Fig. 2. *L. mesenteroides* B-512 F dextransucrase activity as function of temperature: (■) enzyme covalently immobilized on glutaraldehyde-activated chitosan particles; (○) dextran-free soluble enzyme; (△) native soluble enzyme. Tests were performed in 50 mM sodium acetate buffer, pH 5.2. The specific activities of native enzyme, dextran-free soluble enzyme, and immobilized dextransucrase were 10.1 U/mg, 7.9 U/mg, and 1.8 U/mg, respectively. Values represent the mean of duplicates.

3.3 Thermal stability

A typical phenomenon associated with the immobilization of enzymes is the protection conferred against thermal denaturation [25]. Additionally, for dextransucrases, the dextran-layer surrounding the enzyme promotes stability [19, 23]. As results shown in Table 2 demonstrate, for the free forms, the half-life of native enzyme is, approximately, 4-fold higher than the dextran-free dextransucrase. This improvement on the thermal stability, related to the polymer involving the enzyme, was not observed at 50 ºC, probably explained by the almost complete inactivation of the enzyme molecule, for both forms of free enzymes, at this high temperature.

Table 2. Thermal inactivation constant (k), half-life ($t_{1/2}$), and stabilization factor of soluble and immobilized dextransucrases from *L. mesenteroides* B-512 F

T (ºC)	Biocatalyst		k (1/min)	$t_{1/2}$ (min)	Stabilization factor*
40	Soluble	Native	0.077	11.20	-
		Dextran-free	0.239	2.89	-

	Immobilized	Buffer	0.148	4.92	1.70
		Glucose	0.017	31.91	11.0
		Maltose	0.011	54.29	18.8
50	Soluble	Native	2.510	0.38	-
		Dextran-free	3.306	0.35	-
	Immobilized	Buffer	0.523	1.47	4.2
		Glucose	0.243	2.18	6.2
		Maltose	0.222	3.46	9.9

* The stabilization factor was calculated in comparison with the half-life of immobilized enzyme and its soluble form, the dextran-free soluble dextransucrase.

Since the hydrolysis of dextran-layer is needed for covalent immobilization, the protection promoted by this process is even more important. The results presented in Table 2 show that the enzyme stability was enhanced by the immobilization on chitosan, when tested at 40 ºC and 50 ºC. No significant loss of activity was observed at 30 ºC in 24 h for the immobilized biocatalyst. The stabilities of the immobilized biocatalyst were approximately 2 and 4 times higher at 40 ºC and 50 ºC, respectively, in comparison with the native enzyme. This observed stabilization effect might be a result of the immobilization, which promotes a rigidified three-dimensional form of the enzyme [25]. In comparison, immobilization of dextransucrase on activated silica X030 and Eupergit® C 250L (truncated form fused to GST) kept only around 18 % and 15 % of its initial activity, at 30 ºC, respectively [4, 19]. The thermal protection promoted by the immobilization was also observed for the encapsulated enzyme, since the alginate matrices promoted stabilization at different temperatures and the half-life of the

entrapped dextransucrase was always higher, compared to the free enzyme, as reported in another works [17, 21, 22].

Several enzyme stabilizers, such as Tween 80, glycerol, PEG-8000, dextran, sugars, and glutaraldehyde, have been studied to enhance the stability of dextransucrase [17, 58]. The dextransucrase covalently immobilized on glutaraldehyde-activated chitosan particles, in the presence of maltose and glucose, retained its initial activity at 30 ºC for 24 h (data not shown). Therefore, these sugars seem to stabilize the immobilized biocatalyst, producing stabilization factors of 18.8 at 40 ºC and 9.9 at 50 ºC (Table 2). The presence of glucose (2,523 mM) was also able to increase 10-fold the thermal stability at 40 ºC for dextransucrase immobilized on Ca-alginate beads [17]. Maltose, which is a strong acceptor for dextransucrase [59], possibly protects the active site, preventing damages to the enzyme due to harsh reaction conditions.

3.4 Storage and operational stabilities

Fig. 3 shows the results obtained for the storage stability of immobilized dextransucrase on chitosan particles. The enzyme showed to be stable at least for one month at 5 ºC, which is similar to findings reported by Kothari et al. [20], Gómez de Segura et al. [23] and Ölçer and Tanriseven [60]. In contrast, the catalytic activity of dextran-free soluble dextransucrase was reduced to 70 % at the same storage

interval.

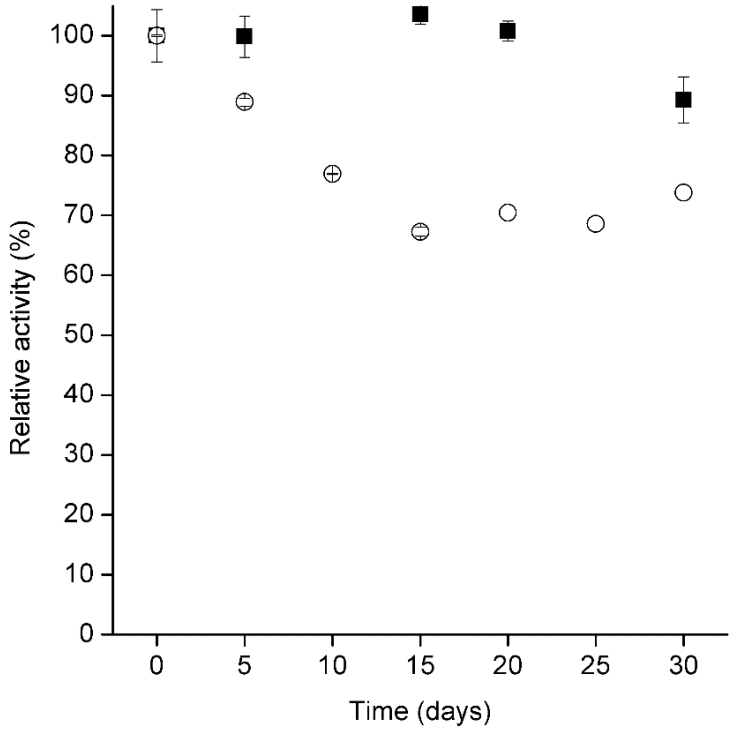

Fig. 3. *L. mesenteroides* B-512 F dextransucrase storage stability tests: (■) enzyme covalently immobilized on glutaraldehyde-activated chitosan particles incubated in 50 mM sodium acetate buffer containing 0.05 g/L $CaCl_2$, at 5 ºC; (○) dextran-free soluble enzyme incubated at 5 ºC.

In the industrial application of dextransucrases for the production of dextran and prebiotic oligosaccharides, the reusability of the biocatalyst is of special importance. Therefore, in order to evaluate the operational stability of immobilized dextransucrase on chitosan particles, it was tested a reaction using a sucrose:maltose ratio of 1 to 6 as proposed by Alcalde et al. [19], in order to shift the reaction direction to the production of oligosaccharides [61, 62]. Although it was not possible to quantify and identify the produced oligosaccharides in this work, it was observed the sucrose and

maltose consumption and the absence of glucose in the reaction medium, indirectly confirming the synthesis of oligosaccharides. Fig. 4 presents the relative activity, in terms of fructose released, for the repeated batches, where the immobilized biocatalyst retained 40 % of its initial activity after 10 cycles.

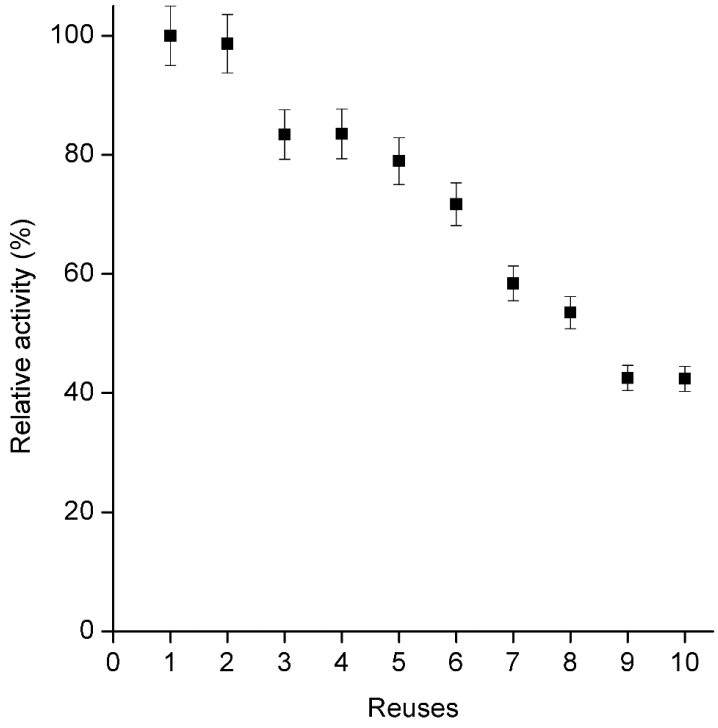

Fig. 4. *L. mesenteroides* B-512 F dextransucrase operational stability tests: (■) covalently immobilized on glutaraldehyde-activated chitosan particles. Reaction conditions were 100 mM sucrose, 600 mM maltose at 30 ºC; each cycle was carried out for 3 h.

The immobilized dextransucrase on chitosan particles seems to be more feasible for industrial applications than Ca-alginate beads, which are the most studied immobilized biocatalysts so far used for oligosaccharides production. The inherent characteristics of Ca-alginate beads cause diffusion restrictions, particle swelling,

protein leakage, and even the collapse of the support, all factors probably involved in the low operational stability of this system [21-23]. Even when the suppression of bead swelling promoted by dextranase action, as in the co-immobilization of dextransucrase encapsulated with dextranase adsorbed on hydroxyapatite, the operational stability of the biocatalyst, regulated by the ratio of both enzymes, was only 25 % of the initial activity after the 6th batch [63].

The good mechanical properties of chitosan have also been demonstrated for other enzymes, as for instance, favoring the high operational stability for β-fructofuranosidase and β-galactosidase, allowing their use for at least 50 batches without significant losses of activity [27, 42].

4. Conclusion

It was described for the first time the covalent immobilization of dextransucrase on glutaraldehyde-activated chitosan particles. The obtained biocatalyst showed interesting properties in terms of activity and thermal stability. The immobilized biocatalyst activity was 197 U/g of dried support, which is one of the highest activities reported in the literature. After hydrolyzing the dextran-layer that surrounded the enzyme molecule the biocatalyst became unstable at 30 ºC or even at 5 ºC, whereas the immobilized preparation presented good stability at both temperatures. In the presence of maltose, the immobilized dextransucrase increased its thermal stability by 19 and 10 times at 40 ºC and 50 ºC, respectively, compared to dextran-free soluble enzyme. More important, the immobilized biocatalyst showed good operational stability, which is very interesting for its possible industrial application. Based on these results, we intend to test this novel dextransucrase preparation to produce dextran

and prebiotic oligosaccharides, using packed-bed and fluidized reactors in continuous system operations. Furthermore, a full study on the characterization and optimization of oligosaccharides production will also be pursued.

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CAPÍTULO IV – PREPARATION AND CHARACTERIZATION OF CROSS-LINKED ENZYME AGGREGATES OF DEXTRANSUCRASE FROM *LEUCONOSTOC MESENTEROIDES* B-512 F

O Capítulo IV, intitulado *Preparation and characterization of cross-linked enzyme aggregates of dextransucrase from Leuconostoc mesenteroides B-512 F*, está apresentado na forma de artigo, publicado no periódico *Process Biochemistry*.

O trabalho aborda a produção e a caracterização de agregados enzimáticos entrecruzados (CLEAs) de dextransucrase de *L. mesenteroides* B-512 F. Nesse estudo, diferentes tempos de tratamento com a dextranase foram avaliados. Além disso, no preparo dos CLEAs, os agentes precipitantes etanol, isopropanol, butanol, e acetona, diferentes tempos de reação e de centrifugação, e diferentes concentrações de glutaraldeído, foram investigados. A fim de caracterizar o novo biocatalisador, pH e temperatura ótimos, e estabilidade operacional, nas reações de síntese de dextrana e oligossacarídeos, foram determinados.

Preparation and characterization of cross-linked enzyme aggregates of dextransucrase from *Leuconostoc mesenteroides* B-512 F

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ABSTRACT

We describe the development of cross-linked enzyme aggregates (CLEA) of dextransucrase of *Leuconostoc mesenteroides* B-512 F. Treatments of enzyme preparation using dextranase were evaluated varying incubation time to remove the dextran layer involving the enzyme molecule, turning it suitable for immobilization. Results showed 21 days of treatment as the best outcome. Subsequently, we tested different water-miscible organic solvents as precipitants, the reaction and centrifugation times, and the concentration of cross-linker agent (glutaraldehyde) in the preparation of CLEA. Optimal conditions were: the use of isopropanol as solvent, 30 min of centrifugation time, 3 h of cross-linking time, and 100 mM of glutaraldehyde. A central composite design was carried out to optimize conditions to obtain the highest enzymatic activity, testing the pH (3.0-7.0) and temperature (20 ºC-60 ºC). Results showed that dextransucrase CLEA operate at optimal pH of 3.0 and temperature of 60 ºC. The operational stability of the immobilized biocatalyst showed up to 30 % of residual activity after 10 cycles of reuse, in a solution of 100 mM of sucrose and 600 mM of maltose. The preparation of dextransucrase CLEA is described for the first time and results suggest that this novel immobilized biocatalyst has potential in many industrial applications.

Keywords: Dextransucrase; cross-linked enzyme aggregates (CLEA); dextran layer; glutaraldehyde; oligosaccharides.

1. Introduction

Dextransucrase (E.C. 2.4.1.5) is a glucansucrase, member of GH 70 enzyme family. Using sucrose as substrate, this enzyme catalyzes transglycosylation forming polymers of glucose (dextrans) [1, 2]. In the acceptor reaction, oligosaccharides are synthesized from an acceptor molecule such as maltose, glucose, or cellobiose, which are glycosylated in the reaction, releasing fructose as a by-product in all cases [3-6].

There is a growing commercial interest in this enzyme because the produced compounds (dextran and oligosaccharides) have a broad range of applications in food, pharmaceutical, and cosmetic industries [7]. For example, the polysaccharides are used as viscosifying, stabilizing, emulsifying, and gelling agents, whereas some specific oligosaccharides are considered prebiotics [8, 9]. These compounds, also called as nutritional ingredients, are not digested in the stomach, being selectively digested by *Bifidobacteria* and *Lactobacillus* in the intestine, stimulating the growth of probiotic bacteria in the host [8, 10].

Enzyme immobilization is a well-studied and important technique to produce robust catalysts for industrial applications. Concerning dextransucrase, there is scarcity of immobilization protocols because native dextransucrase has its surface covered by a dextran layer, which blocks the access of the reactive groups of the enzyme to those in the immobilization support [11, 12]. As a consequence, the most investigated immobilization method for dextransucrase is the enzyme encapsulation, which is based only on the physical restraining of the molecule, the dextran layer not being a problem under this condition [13-15]. On the other hand, it has been shown that the removal of the dextran layer is essential to allow the immobilization via covalent binding [16]. The use of dextranase, which removes dextran residues from

the surface of dextransucrase, eases the access of the reactive groups on the enzyme surface, consequently allowing the interaction between enzyme and carrier, or even enzyme-to-enzyme [12, 16, 17]. In these cases, different supports have been tested, such as porous silica activated with γ-aminopropyl methoxysilane/glutaraldehyde [17], epoxy-activated acrylic polymers [12], and chitosan functionalized with glutaraldehyde [16].

Alternatively, another immobilization methodology is based on the preparation of cross-linked enzyme aggregates (CLEA). CLEA is a carrier-free immobilization technique, which has been shown to produce biocatalysts possessing interesting properties, such as improved storage and operational stabilities, high enzyme loading, easy recovery and recycling, although the aggregation may alter some enzymatic properties [18-20]. Generally, glutaraldehyde is used as a cross-linker because of its low cost and its ability to form covalent bonds with several enzymes [21]. Another important characteristic of CLEA, being a combination of precipitation and immobilization, is that it does not require highly purified enzymes for the preparation of cross-linked enzyme crystals, thus being of good prospect in industrial applications [20]. These immobilized particles are usually employed in fluidized bed and membrane slurry reactors, which are very cost-effective systems for continuous biotransformations on an industrial scale [22]. Notwithstanding these facts, protocols for the preparation of CLEA of dextransucrase from *L. mesenteroides* are scarce in the literature.

Based on these considerations, we set to investigate the possible preparation of CLEAs of dextransucrase and study some of the properties of this new biocatalyst. Different times of dextranase treatment were evaluated in order to remove dextran layers from around the enzyme molecule and to obtain the best suitable enzyme

molecule to immobilize. Different precipitant solvents, reaction and centrifugation times, and concentration of the cross-linker agent glutaraldehyde were also tested. Finally, the optimal pH and temperature were characterized for CLEAs activities, as well as their operational stability in repeated batch cycles during the production of dextran and oligosaccharides.

2. Material and methods

2.1 Materials

Dextranase Plus L (Novozymes A/S) was kindly provided by LNF Latino Americana (Bento Gonçalves, Brazil). Glutaraldehyde (50 % mass fraction solution) was bought from Dinâmica Ltda. (Brazil). Sucrose, glucose, and maltose (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade, purchased from readily available commercial sources.

2.2 Microorganism, culture medium and dextransucrase production

Leuconostoc mesenteroides B-512 F strain was obtained from Fundação Tropical de Pesquisas e Tecnologia André Tosello (Campinas, SP, Brazil), and was used for dextransucrase production. The strain was preserved as frozen samples in MRS (de Man, Rogosa, and Sharpe) medium and 20 % (volume fraction) of glycerol. The culture medium used for microbial growth and enzyme production was based on work described by Rabelo et al. [23]. At the end of cultivation, the dextransucrase was

partially purified from the culture broth by precipitation using polyethylene glycol (PEG 1500 – 50 % mass fraction). The samples were centrifuged at 4 500 × *g* for 15 min at 4 ºC. The salts and media components were not removed. After the centrifugation, the denser phase contained dextran along with approximately 90 % of dextransucrase activity, whereas the upper phase contained glucose, fructose, and polyethylene glycol (PEG), according to Paul et al. [24]. The enzymatic extract was stored at -20 ºC until further analyses.

2.3 Dextranase treatment

The treatment using dextranase was carried out prior to the CLEA preparation. A volume of 2 mL dextransucrase was diluted in 2 mL of 50 mM sodium acetate buffer (pH 5.2) containing 0.45 mM $CaCl_2$ and 20 µL of Dextranase Plus L (100 U g^{-1}) (Novozymes A/S, Denmark) was added. The reaction proceeded for up to 31 days at 5 ºC.

2.4 Determination of dextransucrase activity

A volume of 100 µL of the enzyme solution (soluble dextranase-treated enzyme or CLEA preparation) was mixed with 900 µL of 20 mM sodium acetate, pH 5.2 containing 292 mM sucrose and 0.45 mM $CaCl_2$. This preparation was incubated at 30 ºC for 1 h [25]. The released fructose was measured using the 3,5-dinitrosalicylic acid method [26]. One enzymatic unit (U) of dextransucrase activity was expressed as the amount of enzyme that releases 1 µmol of fructose per minute under these conditions.

2.5 CLEA preparation

For preparation of dextransucrase CLEA, the enzyme, after dextranase treatment described in 2.3, was used without any further purification. CLEA were prepared mixing 0.1 mL of enzyme solution (6 mg mL^{-1} of protein), 0.9 mL of organic solvent and glutaraldehyde for up to 24 h, at 5 ºC. The obtained CLEA was recovered by centrifugation, washed twice using sodium acetate buffer (50 mM, pH 5.2) to assure the elimination of all free enzyme and residual glutaraldehyde. The kind of precipitant agent, the concentration of glutaraldehyde, the reaction and centrifugation times varied for each experiment as described in the following sections.

Immobilization yield and activity recovery of the CLEA were calculated as described by Sheldon and van Pelt [19] and is shown in equations 1 and 2:

$$Immobilization\ yield\ (\%) = \left[\frac{Immobilized\ activity}{Starting\ activity}\right] \times 100 \quad (1)$$

$$Activity\ recovery\ (\%) = \left[\frac{Observed\ activity}{Starting\ activity}\right] \times 100 \quad (2)$$

2.5.1 Screening of precipitant agents

Four organic solvents (ethanol, isopropanol, butanol, and acetone) were evaluated for the preparation of CLEA. For this, 0.1 mL of the treated dextransucrase (6 mg mL^{-1} of protein) was added to 0.9 mL of the organic solvent, using 100 mM of glutaraldehyde as cross-linker. Then, the mixture was homogenized in a roller-mixer (model KJMR-II, Global Trade, USA) for up to 24 h, at 5 ºC. The obtained CLEA was recovered by centrifugation (15 000 × *g*, 5 min). The supernatant was removed and

the CLEA was washed twice with sodium acetate buffer (50 mM, pH 5.2) to assure the elimination of all free enzyme and residual glutaraldehyde.

2.5.2 Reaction and centrifugation times

Three different reaction times (3, 6, and 24 h) were tested for CLEA production. For this experiment, 0.1 mL (6 mg mL^{-1} of protein) of the dextranase-treated dextransucrase was added to 0.9 mL of isopropanol, using 100 mM of glutaraldehyde. The immobilization was carried out in a roller-mixer at 5 ºC and after the required time, the solution was centrifuged (15 000 × *g*, 5 min).

In order to evaluate the effect of centrifugation time, the separation between the CLEA and the solvent was carried out using 15 000 × *g* for 5 min, 15 min or 30 min in the centrifuge (model 2K15, B. Braun). This experiment was performed as described above, using 100 mM of glutaraldehyde and 3 h of reaction time.

2.5.3 Cross-linker concentration

Different concentrations of glutaraldehyde were evaluated for the CLEA preparation. The cross-linker concentration varied from 10 mM to 400 mM. As described in Section 2.5, 0.1 mL of the dextranase-treated dextransucrase (6 mg mL^{-1} of protein) was added to 0.9 mL of isopropanol. The reaction was carried out in the roller-mixer at 5 ºC for 3 h. The CLEA were centrifuged at 15 000 × *g* for 30 min.

2.6 Optimal pH and temperature

A central composite design (CCD) with two variables at five levels was carried out in order to obtain the optimal conditions for enzymatic activity. Soluble dextranase-treated dextransucrase and CLEA were analyzed and compared. The pH ranged from 3.0 to 7.0 (sodium acetate buffer 50 mM for pH 3.0, 3.6 and 5, and sodium phosphate buffer 50 mM for pH 6.4 and 7), whereas the temperature ranged from 20 ºC to 60 ºC. The relative activity was calculated as the ratio between the activity at each temperature or pH and the maximum activity obtained. The sets of experiments for each enzyme are shown in Table 1.

Table 1. Experimental design and results of optimal pH and temperature for enzymatic activities of soluble treated dextransucrase and CLEAs preparation

Run	X_1 Temperature (ºC)	X_2 pH	Soluble enzyme*		CLEA	
			Relative activity (%)	Enzymatic activity (U)	Relative activity (%)	Enzymatic activity (U)
1	-1 (25.8)	-1 (3.6)	13.86	0.108	30.39	0.045
2	-1 (25.8)	1 (6.4)	15.23	0.118	21.14	0.031
3	1 (52.4)	-1 (3.6)	22.49	0.175	72.26	0.107
4	1 (52.4)	1 (6.4)	18.48	0.144	37.19	0.055
5	-1.41 (20)	0 (5.0)	100.0	0.777	34.24	0.051
6	1.41 (60)	0 (5.0)	27.70	0.215	100.0	0.149

7	0 (40)	-1.41 (3.0)	16.61	0.129	69.05	0.103
8	0 (40)	1.41 (7.0)	14.03	0.109	22.30	0.033
9	0 (40)	0 (5.0)	41.07	0.319	54.28	0.081
10	0 (40)	0 (5.0)	42.91	0.333	48.88	0.073
11	0 (40)	0 (5.0)	41.27	0.321	52.99	0.079

* The soluble enzyme is the dextran-free dextransucrase from *L. mesenteroides* B-512 F.

2.7 Operational stability of CLEA

The operational stability of CLEA was carried by repeated batch reactions at 30 ºC for 3 h, using 0.1 U of immobilized enzyme in 1 mL of reaction medium containing (A) 100 mM of sucrose or (B) 100 mM of sucrose and 600 mM of maltose, both diluted in 50 mM sodium acetate buffer. At the end of each batch, the immobilized enzyme was separated from the reaction medium by centrifugation (15 000 × *g* for 30 min), washed using 50 mM sodium acetate buffer (pH 5.2) to remove possible residues of substrate or product, and reused in a new fresh reaction. The enzymatic activity measured in the first cycle was considered as 100 % for the calculation of the remaining activity after each successive batch. The concentrations of substrates and products were determined by the HPLC.

2.8 HPLC-RID and LC-MS analyses

Chromatographic analyses were performed using a HPLC system (Shimadzu, Tokyo, Japan) equipped with refractor index (RID-20) and a mass spectrometer with an Q-TOF analyzer and electrospray ionization (ESI) source (Bruker Daltonics, model micrOTOF-QIII, Bremen, Germany). The compounds were separated using a Shodex NH2 P50-4E (4.6 mm × 250 mm) column. Acetonitrile:ultra-pure water (75:25) was used as eluting solvent at a flow rate of 1.0 mL min^{-1} and the column temperature was kept constant at 30 ºC. The samples were diluted and filtrated with cellulose acetate membrane of 0.22 µm (Millipore, Massachusetts, USA) and 20 µL was directly injected into the chromatographic system. Glucose, fructose, sucrose, and maltose were used as external standards.

The mass spectra were acquired with a scan range from m/z 100 to 1000. The MS parameters were set as follows: ESI source in positive ion modes; capillary voltage, 3000 V (positive); end plate offset, −500 V; dry gas (N_2) temperature, 310 ºC; flow rate, 8 L min^{-1}; nebulizer gas, 2 bar. The sugars were identified on the basis of the following information: elution order and retention time, MS spectra features compared to standards analyzed under the same conditions and data available in the literature.

3. Results and discussion

3.1 Dextranase treatment

The production of native dextransucrase from *L. mesenteroides* B-512 F is induced by sucrose, and consequently dextran is formed. This polymer has been reported to be covalently attached to the enzyme [27]. Because the dextran surrounds

the biocatalyst, it is important to remove this physical barrier making the reactive groups of the enzyme surface more accessible to binding with the activated support sites, or to allow the enzyme-to-enzyme molecule link via cross-linker agent, as for CLEA preparation. The results concerning the dextranase treatment is presented in Fig. 1., in which the optimal treatment time under the conditions of this work was 21 days. There was an almost 4-fold increase of the recovered activity of CLEA from the first day of treatment to the 21st day of experiment, suggesting that more reactive groups were exposed to link with glutaraldehyde. On the other hand, it was also observed a decrease in the recovered activity after this optimal time. This fact may be related to the stability promoted by the polymer as already described in the literature [12, 17].

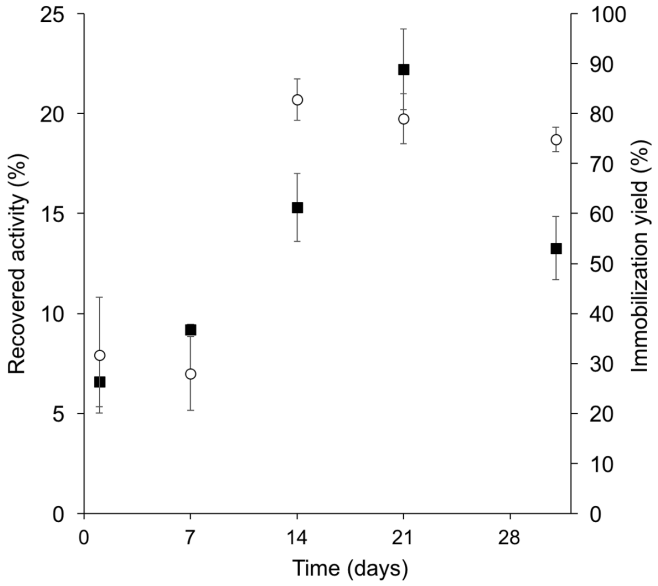

Fig. 1. Effect of different dextranase treatment time in *L. mesenteroides* B-512 F dextransucrase solution; (○) immobilization yield; (■) recovered activity; The measurements were performed in triplicates. CLEA preparation conditions: glutaraldehyde concentration: 100 mM; centrifugation: 15 000 × *g* for 30 min; reaction

time: 3 h; precipitant agent: isopropanol.

For the immobilization of *L. mesenteroides* B-512 F dextransucrase on epoxy supports, it was essential the removal of dextran layer surrounding the enzyme, since the recovered activity in the biocatalyst increased from 0.6 % to 13 % after the dextranase treatment [17]. Similarly, the dextran removal allowed the dextransucrase immobilization on activated-chitosan beads, reaching recovered activities of 8 % [16].

3.2 Screening of precipitant agents and reaction time

The precipitant agent plays an important role in activity recovery of CLEA. It is desirable, after the precipitation, that the enzyme should keep its supramolecular structure, which will be cross-linked to form the aggregate [28]. Therefore, it is important to analyze the activity recovery after the cross-linking step, as proposed in our work. In addition, it is also important to investigate the best length of time for the contact between enzyme molecules, precipitant and cross-linker agents, in order to obtain the highest recovered activity of the immobilized biocatalyst [19]. The effects of the water-miscible organic solvents and the reaction times are presented in Table 2. Among the water-miscible organic solvents, isopropanol showed the highest recovered activity, followed by acetone, butanol, and ethanol. The use of acetone as precipitant agent was only satisfactory for the dextransucrase CLEA preparation in 3 h of reaction time, whereas for ethanol in all times recovered activities were below 5 %. Possibly these two organic solvents caused partial denaturation of dextransucrase. Another explanation may reside in the change of the enzyme flexibility and its

conformation, or deep infiltration of the solvents within the enzyme molecule, which might have hampered the tridimensional structure of the biocatalyst [20, 28].

Table. 2. Effect of organic solvents and cross-linking time in the preparation of CLEA of *L. mesenteroides* B-512 F dextransucrase

Immobilization yield (%)				
Time (h)	Ethanol	Isopropanol	Butanol	Acetone
3	11.92 ± 1.27	28.49 ± 1.77	28.82 ± 1.34	47.07 ± 3.41
6	42.21 ± 2.12	34.80 ± 1.32	60.63 ± 2.54	33.10 ± 2.70
24	38.90 ± 1.13	41.76 ± 5.31	52.52 ± 2.79	41.76 ± 3.85
Recovered activity (%)				
Time (h)	Ethanol	Isopropanol	Butanol	Acetone
3	3.12 ± 2.87	17.00 ± 0.67	7.43 ± 1.41	7.77 ± 1.45
6	1.43 ± 1.25	6.20 ± 1.05	9.69 ± 2.32	1.37 ± 1.12
24	1.19 ± 1.33	1.73 ± 0.89	1.77 ± 1.09	0.00 ± 0.02

The best precipitant is particular for one enzyme, or even for the same enzyme from different sources because of different biochemical and structural properties of

proteins. For example, isopropanol was reported to produce the best precipitation of laccase [29], whereas ethanol was chosen as the optimal precipitant agent for combi-CLEA of pectinases and cellulases [18]. In the preparation of CLEA of β-mannanase, using ethanol, acetone, and isopropanol, results showed that an increase in the size of alcohol aliphatic chain corresponded in higher retention of the enzymatic activity, as well as in increased hydrophobicity, producing the best results [30].

Reaction time is another important parameter to study when preparing CLEA. An optimal reaction time may imply a compromise between efficient cross-linking and enzyme stability. A very short reaction time may result in inadequate cross-linking. On the other hand, prolonged cross-linking time can restrict the enzyme flexibility, abolishing enzyme activity caused by more intensive bindings [20, 28, 31]. Results in Table 2 showed that the best reaction time for the preparation of *L. mesenteroides* B-512 F dextransucrase CLEA is 3 h. At 6 h, there was an increase in the yields of immobilization using any of the studied solvents. However, reaction times longer than 3 h led to reduced recovered activities. For acetone in particular, it was not observed the formation of CLEA of dextransucrase after 24 h of cross-linking. Similar behavior was found for the preparation β-mannanase CLEA, in which the recovered activity of the immobilized biocatalyst was reduced by 40 % when the crosslinking time was prolonged from 16 h to 24 h [30].

3.3 Centrifugation time

During enzyme immobilization, the most common unit operations used for the separation of the immobilized biocatalyst are filtration or centrifugation. In particular for CLEA, because of the particle size, centrifugation is extensively applied, becoming

a crucial step to recover the insoluble protein cross-linked particles and remove the precipitant agent in the CLEA preparation, but also to remove possible residues of substrate or product in the reusability tests [20, 28].

The immobilization yields were 59.3 %, 59.0 %, and 69.0 %, whereas the recovered activities were 14.4 %, 15.8 %, and 17.2 % obtained for 5, 15, and 30 minutes as centrifugation time, respectively. As can be seen, an increase in time led to higher recovered activity of CLEA of dextransucrase from *L. mesenteroides* B-512 F. This parameter was not extensively studied for CLEA preparation in the literature, nevertheless it is demonstrated the importance in our work, since it was shown an improvement of about 20 % when the centrifugation time was increased from 5 to 30 min. The lower recovered activity obtained under 5 min treatment may result from insufficient time to precipitate small CLEA particles during the centrifugation step, as similarly reported for oxidation reactions using laccase CLEA [29]. Comparatively, in the preparation of CLEA, several centrifugation conditions were reported, such as 10 000 $\times$ *g* for 10 min for phenylalanine ammonia lyase [32], 10 000 $\times$ *g* for 15 min for cellulase [33], and 3 000 $\times$ *g* for 10 min for papain and neutrase [34].

3.4 Cross-linker concentration

The number of free amino groups on the protein surface varies from enzyme to enzyme, so it is important to determine the optimal cross-linker concentration for each biocatalyst. As reported in the literature, the recovered activity of CLEA reaches a maximum value proportionally to increasing concentrations of cross-linker, up to a point. The most common chemical used as cross-linker is glutaraldehyde because this chemical is inexpensive, easily handled, and it is capable to bind to different groups

on the enzymes surfaces, mainly involving primary amino groups (from terminal amino and Lys), although it can eventually react with other groups, such as thiol (from Cys), phenol (from Tyr), and imidazole (from His) [21].

Results in Fig. 2 show the optimal concentration of 100 mM glutaraldehyde as cross-linker for the production of *L. mesenteroides* B-512 F dextransucrase CLEA. The low recovery activity obtained using 10 mM and 25 mM of glutaraldehyde may be explained by the insufficient linkages in the aggregate, which resulted in an unstable CLEA, which loses its activity on the washing step. On the other hand, 200 mM and 400 mM of glutaraldehyde had possibly promoted a rigidification of the biocatalyst, and this non-flexibility in the enzyme structure led to internal mass transfer limitations, as reported for the preparation of CLEA of hydroxylnitrile lyase and catalase [35, 36]. Concerning optimal glutaraldehyde concentration, our results are similar to those reported for the production of CLEA of cutinase [37], lipase [38], and laccase [29]. Lower concentrations lead to unstable and insufficient linkages in CLEA, whereas higher concentrations limit the conformational structure of the enzyme, thus diminishing the enzymatic activity. In addition, this cross-linker agent is a small reactive molecule that could penetrate the internal structure of the protein and react with amino residues that are crucial for enzyme catalytic activity [29].

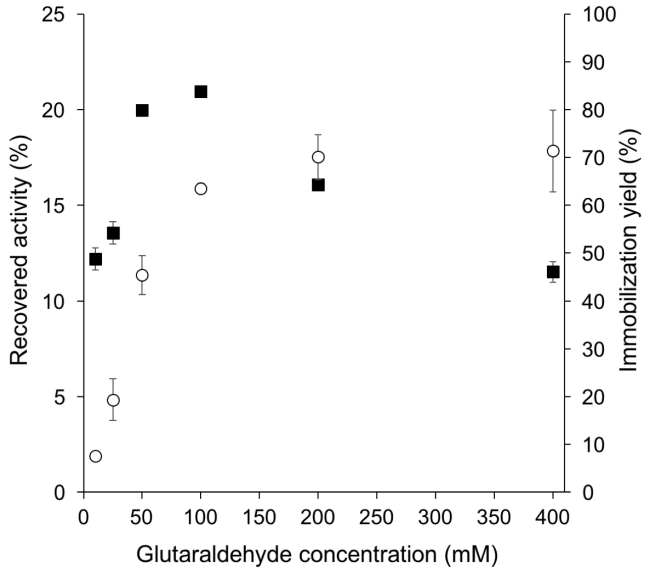

Fig. 2. Effect of cross-linker concentration in CLEA of *L. mesenteroides* B-512 F dextransucrase; (○) immobilization yield; (■) recovered activity; The measurements were performed in triplicates. CLEA preparation conditions: centrifugation: 15 000 × *g* for 30 min; reaction time: 3 h; precipitant agent: isopropanol.

3.5 Determination of optimal pH and temperature of CLEA activities

The dextransucrase from *L. mesenteroides* B-512 F has optimal activity around pH 5.4 and temperature of 30 ºC. If immobilized, however, these characteristics may change [39]. In Fig. 3 are presented the contour plots for optimal pH and temperature activities of the CLEA of dextransucrase, for which the optimum conditions of preparation were isopropanol as solvent, 30 min of centrifugation time, 3 h of crosslinking time, and 100 mM of glutaraldehyde concentration.

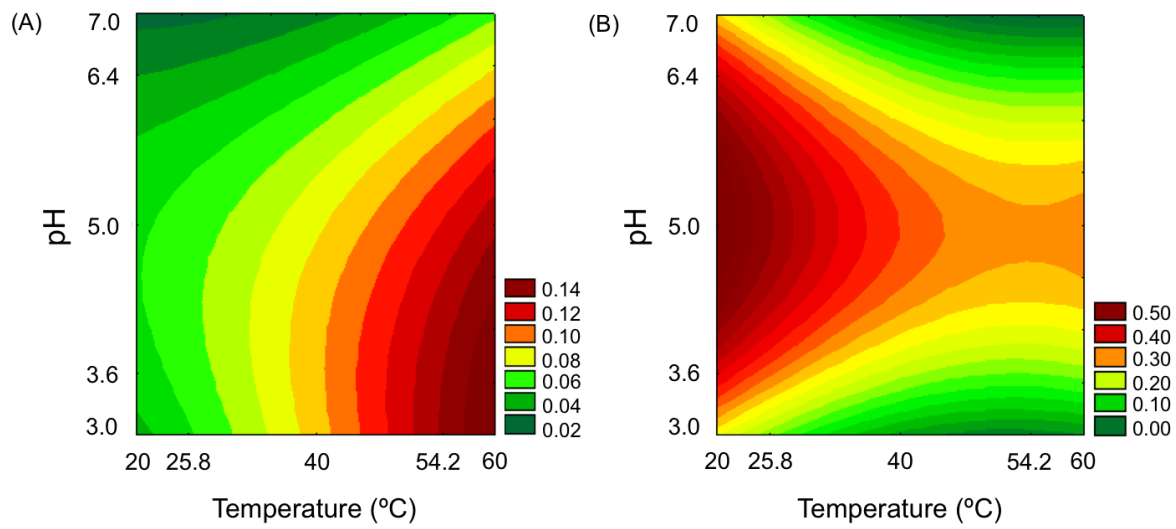

Fig. 3. Contour plots for optimal pH and temperature for enzymatic activity (U). (A) *L. mesenteroides* B-512 F dextransucrase CLEA (B) Soluble dextranase-treated dextransucrase from *L. mesenteroides* B-512 F.

In our work, the activity of dextransucrase CLEA was higher than the soluble dextranase-treated enzyme in the range of 40 ºC to 60 ºC for most of the studied pH range. This indicates the stabilization caused by immobilization, leading to a shift in the optimal temperature of the immobilized biocatalyst (Fig. 3A). Usually, the linkages promoted by immobilization increase the stability of 3D protein structure, protecting the enzyme against denaturation agents such as temperature, pH, organic solvents. Concerning the pH, the maximal activity of dextransucrase CLEA was found under acidic conditions, which could be interesting in replacement of soluble form of dextransucrase in the production of oligosaccharides in fruit juices with low pH, as observed for cashew apple, orange, and mandarin [3, 40, 41]. Moreover, the immobilized enzyme was active in a broader pH range than the soluble enzyme. Although the CLEA presented higher activity at high temperatures, even at low

temperatures the activity in acidic pH (pH 3-3.5) was similar to the activity under the usual optimal pH reported in the literature (pH 5.2).

On the other hand, the impact of the pH and temperature in the enzymatic activity of soluble dextranase-treated dextransucrase is very different, as is shown in Fig. 3B. The soluble enzyme presented relative activity of approximately 50 %, compared to the highest activity, in all the range of studied pH. Moreover, at temperatures higher than 30 ºC, the enzymatic activity was drastically reduced, specially under acidic and neutral conditions. This behavior of soluble dextranase-treated dextransucrase under harsh conditions was already described in other reports, as a consequence of the removal of the polymer layer, causing the instability of the biocatalyst [12, 17]. Additionally, if we consider the enzyme activity (U), the soluble enzyme presents higher activity than the CLEAs. However, it is important to note that the initial activity of both preparations is different. CLEA activity is lower because it was used the same protein content, and the recovered activity after immobilization was 22 %, as shown in Fig. 2.

Comparing to other studies of CLEAs systems, our preparation presented similar behavior regarding the effect of temperature in the enzyme activity. Goetze et al. [42] demonstrated that the combi-CLEA of pectinases exhibited higher optimal temperature than the soluble enzyme, using bovine serum albumin and feather meal as proteic feeders. In another work, Tükel et al. [36] showed that CLEA of catalase had higher relative activities in the range of 10 ºC-35 ºC, compared to the soluble enzyme, reaching maximum activity at 35 °C, whereas the optimal temperature for the soluble enzyme was 25 ºC.

3.6 Operational stability of CLEA

The reusability of a biocatalyst is an important characteristic to be investigated, especially for industrial applications, and dextransucrases can be used to produce dextran and prebiotic oligosaccharides, thus we tested the operational stability of CLEA for this purpose. Results presented in Fig. 4A show that the residual activity after 10 cycles was approximately 40 %, when using only sucrose as substrate. It can be seen a significant loss of activity until the fourth cycle, then, the rate of inactivation stabilized. However, when maltose was added to the medium, the relative activity was 30 % after 10 cycles (Fig. 4B). A possible explanation to the higher operational stability found in the medium containing only sucrose is related to the stability promoted by the produced dextran. The polymer may protect the CLEA during the repeated cycles of reaction. On the other hand, the rapid decrease in the enzymatic activity of the dextransucrase CLEA observed after the first cycle (Fig. 4B) could be explained by mechanical losses, due to possible small particles of CLEAs that are unable to precipitate under used conditions, as already described in the recyclability tests of laccase CLEA [29].

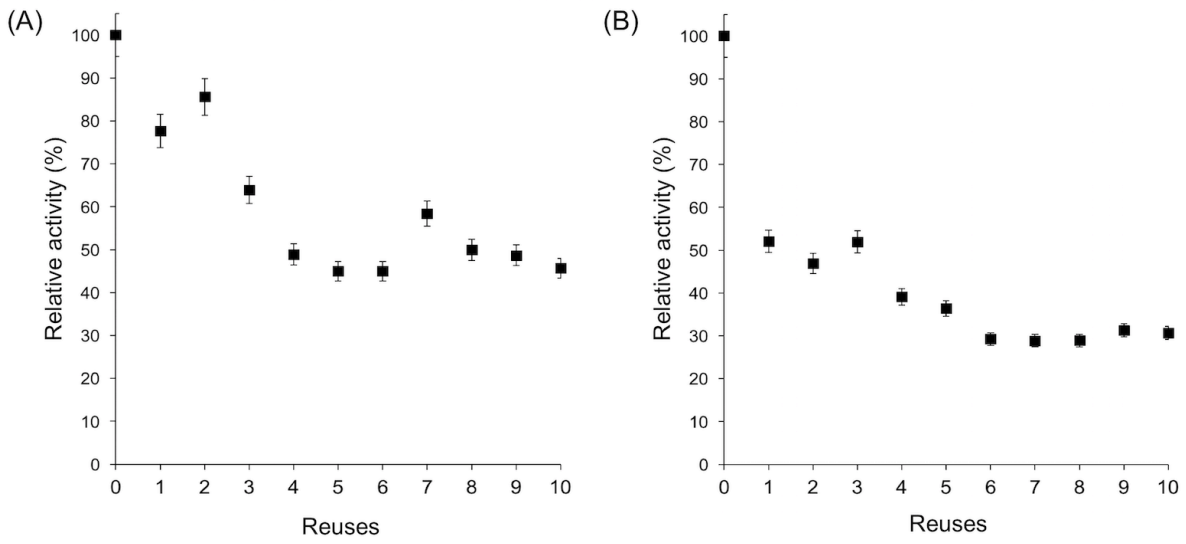

Fig. 4. Operational stability tests with dextransucrase CLEA for dextran and oligosaccharides using the substrates (A) 100 mM sucrose and (B) 100 mM sucrose + 600 mM maltose. Reaction conditions were: temperature: 30 ºC; reaction time for each cycle: 3 h.

The operational stability of combi-CLEA of pectinases for pectin hydrolysis presented approximately 20 % of its initial activity after 10 cycles [42]. Results of recycling experiments demonstrated that the laccase CLEA could be reused several times without appreciable loss of activity, showing that this immobilized biocatalyst could be used in the oxidation of primary alcohols to the corresponding aldehydes [29]. Similarly, the remaining activity of catalase CLEA was around 50 % of its initial activity at the end of the batches [36].

The results of HPLC-MS analysis identified only one trisaccharide under the evaluated reaction conditions and using only sucrose as substrate. This compound may be a glucooligosaccharide, a dextran precursor, found in low concentrations. On the other hand, two different trisaccharides were obtained using sucrose and maltose as substrates (Fig. 5), probably isomaltotriose (three glucose units linked by α-1,6 glycosidic bonds), and panose (one unit of maltose linked by α-1,6 glycosidic bond to one unit of glucose), as already described in the literature [6, 17].

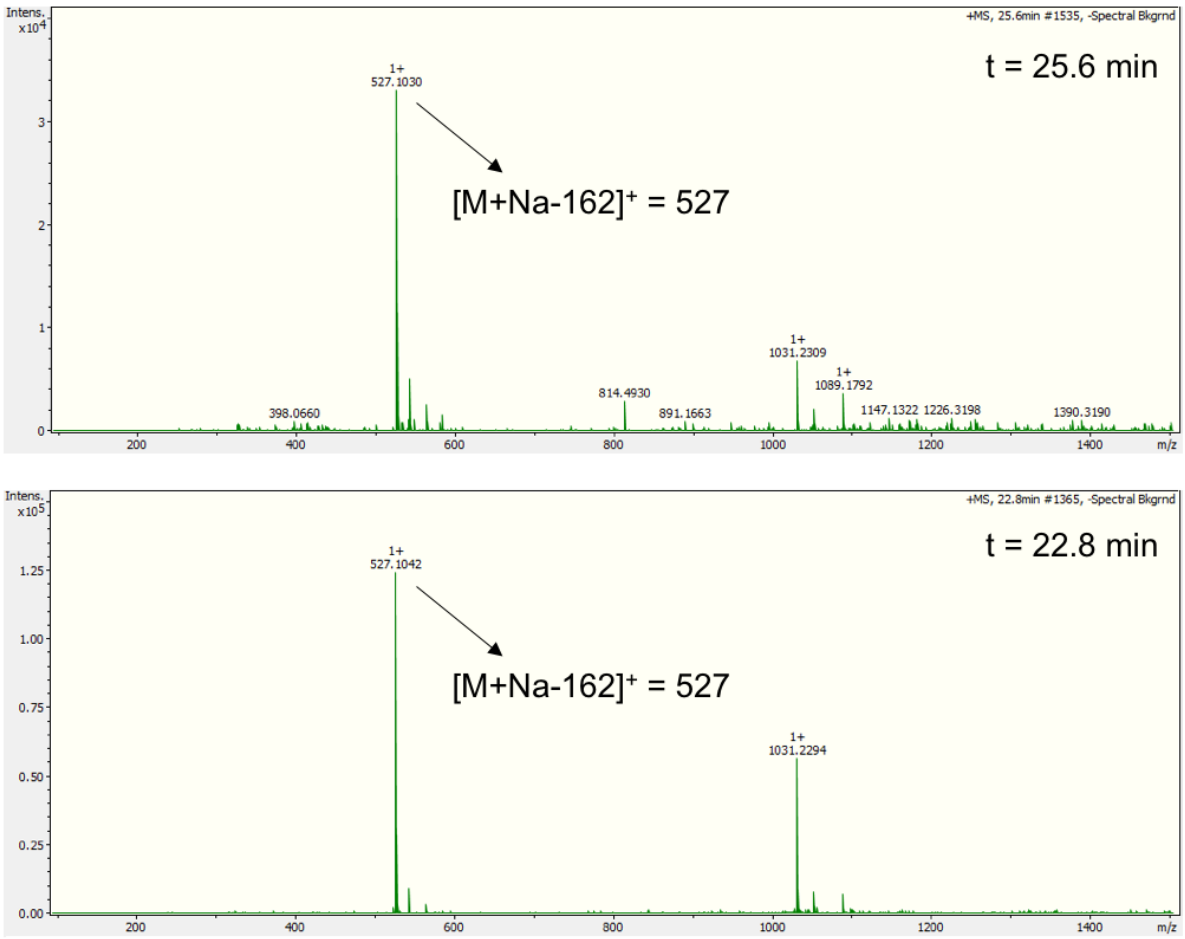

Fig. 5. Identification of trisaccharides (MM = 527 g moL^{-1}) produced by *L. mesenteroides* B-512 F dextransucrase CLEA in the reaction containing 100 mM of sucrose and 600 mM of maltose.

4. Conclusion

Dextransucrase CLEA were prepared for the first time to the best of our knowledge. It was also demonstrated that the treatment using dextranase is essential for making the reactive group on the enzyme surface more accessible to binding with the cross-linker agent. The best conditions for the preparation of dextransucrase CLEA were obtained using isopropanol as water-miscible precipitant solvent, centrifugation

during 30 min, cross-linking reaction of 3 h, and glutaraldehyde concentration of 100 mM. The immobilization led to a shift in the optimal pH and temperature of dextransucrase CLEA. The immobilized biocatalyst presented higher activities under harsh conditions, probably to a more favorable conformation that promoted stability to the enzyme. The immobilized biocatalyst kept up to 40 % and 30 % of residual activity after 10 cycles using only sucrose, and sucrose and maltose, as substrates, respectively. Our results open the possibility to further researches in the field, such as the use of magnetic nanoparticles to aid the separation of CLEA at the end of the reaction, the application of the CLEA in continuous processes in fluidized bed reactors, and the possible scaling up to pilot and industrial scales.

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CAPÍTULO V – HOMOLOGY MODELING AND PROTEIN ENGINEERING OF TWO TRUNCATED FORMS OF GLUCANSUCRASES: DSR-S VARDEL Δ4N AND ASR C-APY DEL

O Capítulo V, intitulado *Homology modeling and protein engineering of two truncated forms of glucansucrases: DSR-S vardel Δ4N and ASR C-APY del*, está apresentado na forma de artigo, a ser submetido ao periódico *Biotechnology and Bioengineering*. O material suplementar referente a esse trabalho encontra-se no Anexo I.

O trabalho versa sobre o estudo da modelagem das estruturas tridimensionais e da mutagênese sítio-dirigida das glucansucrases DSR-S vardel Δ4N and ASR C-APY del. A fim de verificar a qualidade dos modelos preditos, os diagramas de Ramachandran, e os valores 3D-1D QMEANS, ProSA foram avaliados. Além disso, está apresentada a discussão sobre a seleção dos aminoácidos alvos, bem como a influência da substituição dos resíduos Ser por resíduos Cys na atividade enzimática de cada mutante.

Homology modeling and protein engineering of two truncated forms of glucansucrases: DSR-S vardel Δ4N and ASR C-APY del

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ABSTRACT

In this study, the three-dimensional structure of the truncated enzymes dextransucrase DSR-S vardel Δ4N and alternansucrase ASR C-APY del were modeled by using Swiss-model and their quality was evaluated. The accuracy of the predicted structure was checked using Ramachandran plot and the analysis showed that 87.2 % and 81.7 % of total residues were confined to the core region for DSR-S vardel Δ4N and ASR C-APY del models, respectively. The values of QMEANS and ProSA z-score were 0.66 and -9.6 for dextransucrase model, whereas the results obtained were 0.66 and -10.6 for altenansucrase three-dimensional structure. The compatibility of an atomic model (3D) with amino acid sequence (1D) for the DSR-S vardel Δ4N and ASR C-APY del models were observed as 91.76 % and 77.8 % using Verify 3D at SAVES server. The DSR-S vardel Δ4N variants presented enzymatic activities varying from 0.0 to 83.5 U mL^{-1}. The enzyme activities for the ASR C-APY del variants ranged from 26.4 to 70.3 U mL^{-1}. The mutation of Ser 326 in DSR-S vardel Δ4N negatively influenced the enzymatic activity, probably caused by changes in the conformational structure. These findings may help to construct a theoretical approach about the structure of these truncated glucansucrases, which can be useful in immobilization studies.

Keywords: Glucansucrases; molecular modeling; model evaluation; site-directed mutagenesis.

1. Introduction

Glucansucrases (GS) (EC. 2.4.1.) are transglucosidases, classified into GH 70 family from CAZy (http://www.cazy.org), which synthesize high molecular weight α-glucans and oligosaccharides from sucrose (Ebert *et al.* 1968; Buchholz and Monsan 2003). Depending on the enzyme specificity, these products show several types of linkages and polymerization degrees (Robyt and Walseth 1978; Robyt 1995). These enzymes are produced by lactic acid bacteria of the genera *Leuconostoc*, *Streptococcus*, *Lactobacillus* and *Weissella*. Because of their ability to produce carbohydrates of diverse sizes, structures, and physicochemical properties, GS are attractive tools for glycodiversification (Remaud-Simeon *et al.* 2000). The polymers produced by GS have been described as thickening, stabilizing, and gelling agents, whereas the oligosaccharides have been shown to present prebiotic effects (Koepsell *et al.* 1953, Monsan *et al.* 2000).

GS were first immobilized more than 40 years ago. The most studied GS for immobilization is dextransucrase, being encapsulation widely employed (Reischwitz *et al.* 1995; Alcalde *et al.* 1999; Berensmeier *et al.* 2006). Particularly for native dextransucrase, i.e, from *L. mesenteroides* B-512 F, the enzyme production is induced by sucrose, and dextran is, consequently, formed around the enzyme surface, blocking the access of the reactive groups (Jeanes *et al.* 1954; Alcalde *et al.* 1999; Gómez de Segura *et al.* 2004). To overcome this problem, the heterologous expression in *E. coli*, for example, is an alternative to produce GS without concomitant polymer production, due to the absence of sucrose in the medium (Moulis *et al.* 2006).

Taking advantage of this heterologous expression of GS in *E. coli*, several studies were carried out using site-directed mutagenesis to enhance properties such

as product specificity, stability, and specific activities, as well as to understand the mechanisms of polymerization catalyzed by GS (Joucla *et al.* 2006; Moulis *et al.* 2006; Irague *et al.* 2013). In addition, protein engineering has been useful to improve enzyme immobilization, by orientating the immobilization by a specific side of the protein or by enhancing the multipoint covalent attachment (Hernandez and Fernandez-Lafuente 2011). The first could be achieved by introduction of a Cys on the enzyme surface, whereas the second approach can be obtained by the addition of several Lys residues on the enzyme surface (Batista-Viera *et al.* 1991; Hernandez and Fernandez-Lafuente 2011). Researchers can, in both cases, choose the best area of the protein surface to perform the site-directed mutagenesis. However, one of the requisites to apply this method is the knowledge of the enzyme structure or its analogue.

To date, 497 enzymes are described as a member of GH 70 family. Nevertheless, 66 enzymes are biochemically characterized and only seven GS have their three-dimensional structures available. These structures were obtained by crystallization of recombinant truncated forms of glucansucrase GTF180-ΔN from *L. reuteri* (PDB code: 3KLK (Vujičić-Žagar *et al.* 2010)), glucansucrase from *S. mutans* (PDB code: 3AIE (Ito *et al.* 2011)), dextransucrase DSR-E from *L. mesenteroides* NRRL B-1299 (PDB code: 3TTQ (Brison *et al.* 2012)), glucansucrase GTFA from *L. reuteri* 121 (PDB code: 4AMC (Pijning *et al.* 2012)), dextransucrase DSR-E from *L. mesenteroides* NRRL B-1299 (PDB code: 4TVD (Brison *et al.* 2016)), α-4,6-glucanotransferase GTFB from *L. reuteri* 121 (PDB code: 5JBD (Bai *et al.* 2017)), and dextransucrase DSR-M from *L. mesenteroides* B-1299 (PDB code: 5NGY (Claverie *et al.* 2017)).

Homology modeling techniques have been developed to build three-dimensional models of a protein (target) from its amino-acid sequence on the basis of

an alignment with a similar protein with known structure. This is of a great interest since the number of known protein sequences is much larger than the number of experimentally solved protein structures. Furthermore, homology models of a protein under investigation can provide a valuable tool for the interpretation of sequence variation and the design of mutagenesis experiment to elucidate biological and structural features (Ali 1995; Peitsch 2002).

Based on these aspects, we proposed a 3D-model for dextransucrase DSR-S vardel Δ4N and alternansucrase ASR C-APY del. In addition, the models were evaluated about their stereochemical quality and their overall structural geometry. Site-directed mutagenesis was used to introduce Cys residues in different positions on the surface of DSR-S vardel Δ4N and ASR C-APY del. The selected amino acids were chosen considering their position on the GS surface, their involvement in secondary structures and their relative solvent accessible surface area. All new single mutants were investigated according to their enzymatic activities.

2. Material and methods

2.1 DSR-S vardel Δ4N and ASR C-APY del homology modeling

2.1.1 Template structure selection

The enzyme sequences of DSR-S vardel Δ4N (Moulis *et al.* 2006) and ASR C-APY del (Joucla *et al.* 2006) were matched against those found in Protein Data Bank

(PDB). The search for the closest structural homologue templates to model the protein was achieved by using BLASTP program (https://blast.ncbi.nlm.nih.gov). According to the RCSB Protein Data Bank, the X-ray crystal structure of DSR-S vardel Δ4N and ASR C-APY del are not available. It was selected the structures with highest identity score as the most suitable templates. The multiple sequence alignment of target and template protein sequences were performed using dynamics programming based Clustalω, a general purpose multiple sequence alignment program for DNA and proteins (Larkin *et al.* 2007).

2.1.2 Three-dimensional molecular modeling

GS homology for comparative protein structure modeling was performed with the help of SWISS-MODEL workspace, which was an integrated web-based modeling expert system (Arnold *et al.* 2006; Biasini *et al.* 2014). On the basis of a sequence alignment between the DSR-S vardel Δ4N or ASR C-APY del and the suitable template structure, a three-dimensional model for each target protein was generated. SWISS-MODEL workspace derived the restraints automatically from related known structure (template) present in the database. All 3D-structures images were prepared through PyMol Molecular Graphic System, version 2.0 (http://www.pymol.org/).

2.1.3 Model evaluation

The constructed models of DSR-S vardel Δ4N and ASR C-APY del were verified by using Procheck and Verify 3D programs available in the Structural Analysis and Verification Server (SAVES) (http://services.nihserver.mbi.ucla.edu/SAVES). The

stereochemical quality and the overall structural geometry of GS models were investigated by the Procheck program (Laskowski *et al.* 1993) and by the Verify 3D program (Bowie *et al.* 1991; Lüthy *et al.* 1992). Further, the global quality of the generated models was also assessed by checking QMEAN and ProSA (Weiderstein and Sippl 2007; Benkert *et al.* 2008).

2.2 Target amino acids for site-directed mutagenesis

The target amino acids for site-directed mutagenesis were investigated concerning their internal/external exposition and relative solvent accessible surface area (SASA) calculated by Get Area software (Fraczkiewicz and Braun 1998). They were also evaluated about their conservation compared to the characterized sequences of GH 70 family.

The multiple sequence alignment of target and characterized protein sequences were performed by Clustalω (Larkin *et al.* 2007) and this alignment was submitted in WebLogo software (http://weblogo.berkeley.edu/) to have a visual representation of each mutated amino acid.

2.3 Mutagenesis studies

All single mutants were constructed by site-directed mutagenesis using DSR-S vardel Δ4N and ASR C-APY del as templates. The primers are described in Table S1 and S2. PCR amplifications were carried out using Phusion® High-Fidelity DNA polymerase for 20 cycles (98 ºC, 10 s; 55 ºC, 30 s; 72 ºC, 2 min). The parental plasmid

template was digested with DpnI and PCR products were purified using a GenElute PCR Clean Up kit (Sigma-Aldrich, St. Louis, USA), following manufacturer recommendations. *E. coli* TOP10 (Invitrogen) cells were transformed with the plasmid and the resulting clones were selected on LB agar plates supplemented with 100 µg mL^{-1} of ampicillin at 37 ºC for 24 h. Plasmids were extracted with Qiaprep® Spin Miniprep kits (Qiagen), verified by restriction analyses, and the genes of interest sequenced (GATC Biotech, France). DpnI restriction enzyme and Phusion® High-Fidelity DNA Polymerase were purchased from New England Biolabs (Beverly, MA, USA). Oligonucleotides were synthetized by Eurogentec (Liège, Belgium).

2.4 Production of parental and mutant enzymes

E. coli BL21 AI (Invitrogen) cells carrying the parental plasmid (DSR-S vardel Δ4N-His or ASR C-APY del) or the recombinant plasmids encoding ASR or DSR single mutants were grown in flasks containing ZYM5052 medium supplemented with ampicillin (100 µg mL^{-1}) and 0.01 % arabinose (weight fraction), and incubated at 21 ºC for 26 h under agitation (150 rpm) (Studier 2005). Cells were centrifuged (4 500 × *g*, 15 min, 4 ºC) and the pellets were resuspended to a final $OD_{600\ nm}$ of 80 in 50 mM sodium acetate buffer (pH 5.2) containing 0.05 g L^{-1} of $CaCl_2$, before being sonicated and centrifuged (15 000 × *g*, 60 min, 4 ºC) to remove cell debris. Supernatants (cell free extracts) were harvested for glucansucrase activity measurements.

2.5 Glucansucrase activity

One unit of glucansucrase activity was expressed as the amount of enzyme

that releases 1 µmol of fructose per minute at 30 ºC in 20 mM sodium acetate, pH 5.2 containing 292 mM sucrose and 0.45 mM $CaCl_2$. The fructose released was measured using the 3,5-dinitrosalicylic acid method (Miller 1959).

2.6 Electrophoresis analysis

The mutant enzymes were verified by 8 % SDS (weight fraction) polyacrylamide gel (Laemmli 1970) with Bio-Rad Mini-PROTEAN® Tetra System (California, USA). Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

3. Results and discussion

3.1 DSR-S vardel Δ4N and ASR C-APY del models

The enzymes dextransucrase DSR-S vardel Δ4N (Moulis *et al.* 2006) and alternansucrase ASR C-APY del (Joucla *et al.* 2006) are truncated mutants of the enzymes DSR-S from *L. mesenteroides* NRRL B-512 F and ASR from *L. mesenteroides* NRRL B-1355, respectively. Previous studies showed that DSR-S vardel Δ4N produces isomaltooligosaccharides and high molecular weight dextran, whereas ASR C-APY del synthesizes a myriad of branching oligosaccharides with various α-1,3 and/or α-1,6 links, using sucrose and maltose as substrates (Moulis *et al.* 2006). Molecular modeling investigations were never carried out before for GS aiming the development of new mutated enzymes to orientate the immobilization, although the dextransucrase from *L. mesenteroides* B-512 FMC was fused with

glutathione S-transferase to improve the activity recovery after the covalent immobilization in epoxy supports (Parlak *et al.* 2013).

In order to perform the homology modeling, the sequences of DSR-S vardel Δ4N or ASR C-APY del were compared by protein-protein BLAST to those of the Protein Data Bank (PDB) database. The template protein for each GS was selected by the highest sequence identity. For DSR-S vardel Δ4N, the template showing the highest homology with the sequence identity of 57 % was the glucansucrase from *S. mutans* (PDB code: 3AIB_A), whereas the model of ASR C-APY del was constructed using the structure of the glucansucrase GTFA from *L. reuteri* 121 (PDB code: 4AMC_A), which was 51 % identical. The percentage sequence identity between target and template is a good predictor of the accuracy of a model, being 30 % of identity the lower limit for comparative modeling (Bordoli *et al.* 2009). For comparison, the crystallographic structure of N-terminally truncated glucansucrase GTF180-ΔN (PDB code: 3KLK) was used in the molecular modeling of dextransucrase from *L. mesenteroides* 0326 (Wang *et al.* 2017) showing that each enzyme has its own best template, according to the protein sequence.

Afterwards, both sequences of target and template proteins were aligned using MUSCLE, and only the part of the sequence with the highest homology was used to construct the 3D-model. For example, DSR-S vardel Δ4N was a truncated form of DSR-S. The native protein has 1527 amino acids, while DSR-S vardel Δ4N was truncated from Thr 152 to Ser 1450. For model construction, the sequence used was from Asp 168 to Asp 1007. The alternansucrase ASR C-APY del was the truncated form from Met 1 to Gly 1425 of the 2057 amino acids of native enzyme. The 3D-model was constructed using the sequence from Arg 363 to Gly 1425.

The constructed models with the help of online server SWISS-MODEL workspace are presented in Fig. 1. In order to evaluate the model qualities, Ramachandran plots were drawn using a Procheck program for DSR-S vardel Δ4N and ASR C-APY del models (Fig. 2). To DSR-S vardel Δ4N three-dimensional structure, the model had good quality, because 87.2 % of total residues were confined to the core region (red area), 12.4 % in additional allowed region (yellow area), remaining 0.3 % residues in generously allowed region and only one residue in the disallowed region (white area) (Fig. 2A). These values confirm that the protein back bone dihedral angles phi (φ) and psi (ψ) occupy reasonably accurate positions in the model. Furthermore, compared to the template, the plot statistic results are very similar, as can be seen in Table 1.

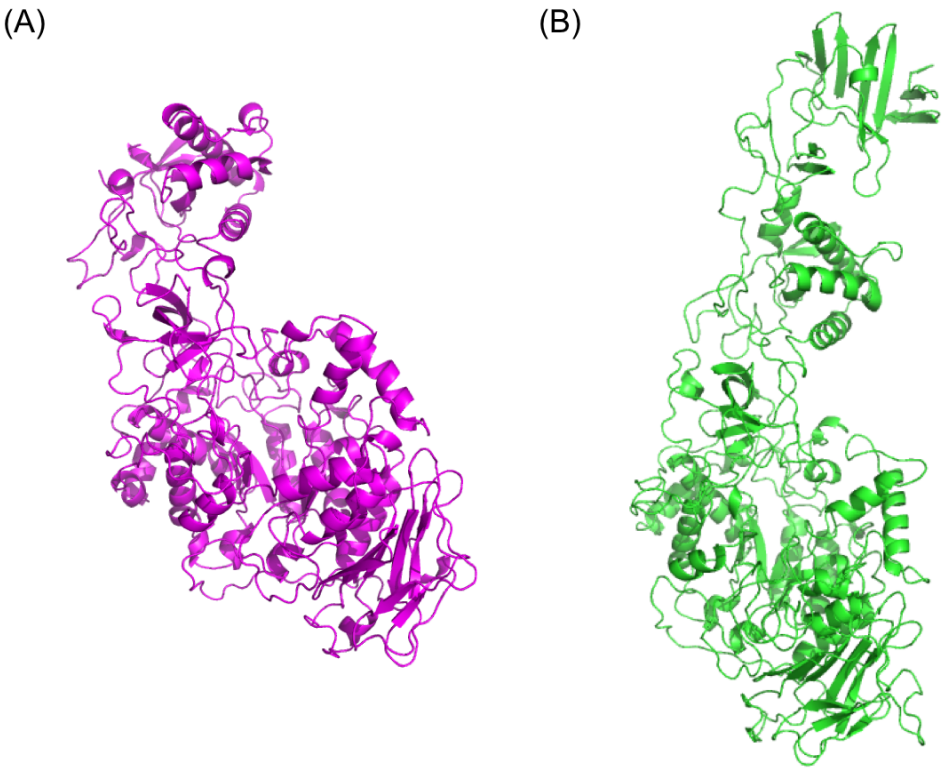

Fig. 1. 3D-structures of (A) DSR-S vardel Δ4N and (B) ASR C-APY del, modeled by

SWISS-MODEL online server. The images were prepared using PyMol Molecular Graphic System, version 2.0 (http://www.pymol.org/).

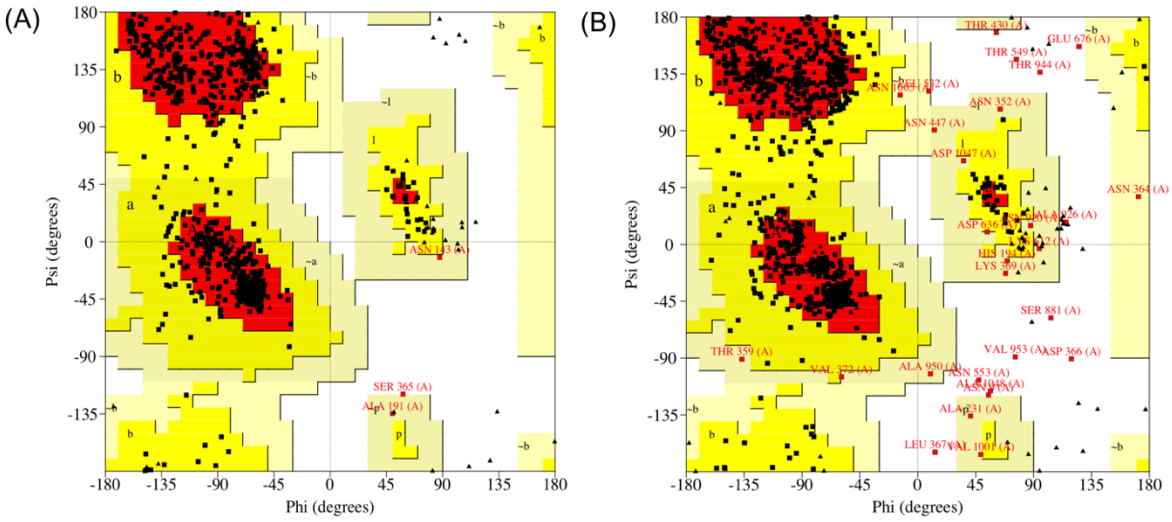

Fig. 2. Ramachandran plot of modeled GS (A) DSR-S vardel ∆4N and (B) ASR C-APY del, obtained by Procheck validation package.

Other parameters used to evaluate the quality of the model are generated by Verify 3D, QMEANS, and ProSA. The program Verify 3D determines the compatibility of an atomic model (3D) with its own amino acid sequence (1D) assigning a structural class based on its location, environment (α, β, loop, polar, non-polar) and comparison of results with excellent database structures (Bowie *et al.* 1991; Lüthy *et al.* 1992). QMEANS is a combined scoring function consisting of three statistical potential terms and two additional terms describing the agreement of the predicted and observed secondary structure and solvent accessibility, respectively. A good model is represented by a QMEANS score near to 1, in the range of 0-1 (Benkert *et al.* 2008). Protein statistical analysis (ProSA) provides the z-score, a parameter that indicates

overall model quality. A negative score shows a good model, while a positive value would show errors.

The DSR-S vardel Δ4N model revealed an interesting result, because 91.76 % of the amino acids presented a 3D-1D score ≥ 0.2 and the QMEANS observed value was 0.69. The z-score of the DSR-S vardel Δ4N model was −9.6 compared with the z-score of the template 3AIB_A, which was −10.65.

Table 1. Plot statistics of model and template by Procheck

	Most favored region	Additional allowed region	Generally allowed region	Disallowed region
DSR-S vardel Δ4N				
Model	87.2 %	12.4 %	0.3 %	0.1 %
Template	87.1 %	12.5 %	0.4 %	0.0 %
ASR C-APY del				
Model	81.7 %	15.3 %	1.7 %	1.3 %
Template	85.0 %	13.6 %	1.0 %	0.4 %

The results of Ramachandran plot (Fig. 2B), Verify 3D, QMEANS, and ProSA for ASR C-APY del structure confirm that the generated model is reliable and of good quality. The value for Verify 3D was 77.8 %, whereas for QMEANS was 0.6. The z-score of the ASR C-APY model was -10.6, compared to -12.68 obtained for the

4AMC_A structure. As presented in Table 1, the model also showed an analogous statistic result verified by Procheck.

In the study of homology modeling for cellulase from *Acinetobacter* sp., Selvam *et. al* (2017) found 81.1 %, 14.9 %, 2.5 %, and 1.5 % of the residues within the most favorable region, additional allowed region, generally allowed region, and disallowed region, respectively. The authors reported the overall quality factor and compatibility of an atomic model with amino acid sequence for the model was observed as 78.21 % for Verify 3D at SAVES server. The model of xanthine oxidoreductase from *Arthrobacter* sp. XL26 produced a QMEANS score within the reliability (0-1), 0.71, whereas the overall stereochemical quality of model was assessed by Procheck, and the Ramachandran plot revealed 94.1 % of residues in most favorable region, 4.9 % in allowed region, 1.1 % in additional and disallowed region, showing that the majority of the amino acids are in a phi-psi distribution consistent with right handed α-helix (Bodade *et al.* 2010).

3.2 Selection of amino acid targets

In our work, the use of site-directed mutagenesis was addressed to control the orientation of the protein on the support surface during the immobilization and the simplest and most versatile way is possibly to introduce a unique Cys residue into the enzyme surface (Hernandez and Fernandez-Lafuente 2011). Particularly, both GS enzymes DSR-S vardel Δ4N and ASR C-APY del do not present Cys residues in their primary amino acid sequences, indicating the importance of our hypothesis. The choice for the mutation of Ser residues by Cys residues was established because of the similarity between these amino acids, such as polarity and side chain (Zimmerman

et al. 1968). Furthermore, this selection also aimed at finding Ser residues close to lysine-rich regions, since Lys residues can interact with glyoxyl or epoxy groups to promote multipoint covalent immobilization. Thus, the use of an heterofunctional support containing a disulfide group and glyoxyl or epoxy groups surrounding it, allows the site-directed immobilization/rigidification, orientating the immobilization by thiol-disulfide exchange by Cys, and the rigidification of the immobilization by additional covalent bonds by Lys, as already showed in the literature (Godoy *et al.* 2011; Przybysz *et al.* 2013). Moreover, the Ser residues were selected according to their position on the GS surface, their involvement in secondary structures (α-helix and β-sheet) and their relative SASA.

It is well-established that the conformational stability of the protein is mainly due to the involvement of several covalent and non-covalent bonds presented in α-helices and β-sheets. The perturbation of these forces may result in losses of biological activity caused by changes in the three-dimensional structure (Kumar *et al.* 2011). Other important consideration is solvent accessible surface area that determines the accessibility of an amino acid side-chain on the surface of a protein that can be accessed by solvent. In order to obtain a relative solvent accessible surface area, a normalization is used considering the maximum value observed for the amino acid. This value represents a metric, identifying buried or exposed residues (Lee and Richards 1971).

As demonstrated in Table 2, the number of Ser residues evaluated by PyMol software was very distinct for each GS. The quantity of Ser residues on the surface of ASR C-APY del was higher than those of DSR-S vardel Δ4N. Furthermore, approximately 50 % of the Ser residues located on the surface of DSR-S vardel Δ4N are involved in secondary structures, being a limitation factor in the choice of target

amino acids, since these residues may play a key role in the conformational enzyme structure.

Table 2. Quantity and location of Ser residues present in DSR-S vardel Δ4N and ASR C-APY del structures

	Quantity of Ser residues	
	DSR-S vardel Δ4N	ASR C-APY del
Total	65	71
Located on the surface	18	30
Located on the surface and not involved in α-helix or β-sheet secondary structures	10	25

All Ser residues located on the surface and not involved in α-helix or β-sheet structures of DSR-S vardel Δ4N were chosen to be mutated, however only 14 Ser residues of those not involved in secondary structures and located on the surface of ASR C-APY del were selected, because of their distinct position. Fig. 3 shows that the selected Ser residues to be substituted by a Cys residue of both GS are located on the distal side of the protein surface away from the active site. Przybysz *et al.* (2013) investigated the immobilization of a thermostable endo-β-1,3-glucanase on disulfide-epoxy support. The authors constructed an enzyme variant with an accessible cysteine located far away from the catalytic site. Their results demonstrated that the long distance of target Cys from the active site was crucial to guarantee a high

conservation of the enzyme activity. On the other hand, Simons *et al.* (2013) verified the negative effect caused by mutated amino acids close to catalytic site in the site-directed immobilization of glucose-6-phosphate dehydrogenase, which led to losses in the enzymatic activity.

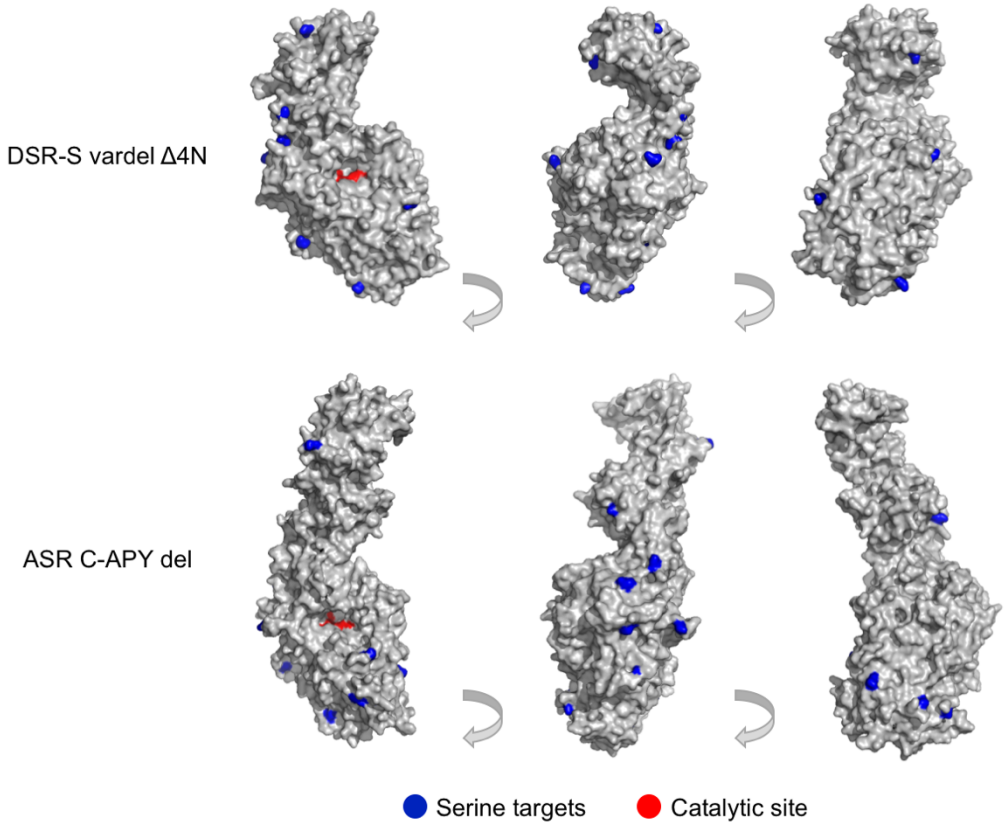

Fig. 3. Target Ser residues in three-dimensional structures of DSR-S vardel Δ4N and ASR C-APY del; The target Ser residues are colored in blue; The catalytic sites of DSR-S vardel Δ4N – D400, E438, D511 – and ASR C-APY del – D635, E673, D767 – are colored in red.

Table 3 shows the relative SASA of selected Ser residues for DSR-S vardel Δ4N and ASR C-APY del. The maximum value of SASA proposed by Tien *et al.* (2013) for serine, 161 Å, was used to calculate the relative SASA. The highest SASAs

identified for the Ser residues found in the sequence of DSR-S vardel Δ4N and ASR C-APY del were 116.07 Å and 126.46 Å, respectively.

Table 3. Relative solvent accessible surface area of the exposed serine on the surfaces of DSR-S vardel Δ4N and ASR C-APY del

Relative solvent accessible surface area (%)			
DSR-S vardel Δ4N		ASR C-APY del	
Ser 170	78.5	Ser 415	58.8
Ser 278	22.4	Ser 563	48.6
Ser 318	11.5	Ser 579	69.8
Ser 326	15.8	Ser 713	50.1
Ser 474	41.7	Ser 882	40.8
Ser 641	56.0	Ser 883	4.9
Ser 646	50.6	Ser 907	25.7
Ser 759	41.7	Ser 964	60.7
Ser 788	42.4	Ser 971	43.3
Ser 954	63.0	Ser 1027	42.5
		Ser 1042	37.2
		Ser 1235	45.1
		Ser 1251	58.3
		Ser 1313	68.3

3.2 Effect of the site-directed mutagenesis in GS activities

The truncated variants were successfully produced as demonstrated by the colloidal blue stained SDS-PAGE (Fig. 4). As already described in the literature, the majority of the enzymatic activities of DSR-S vardel Δ4N and ASR C-APY del were found in the soluble extract (Joucla *et al.* 2006; Moulis *et al.* 2006).

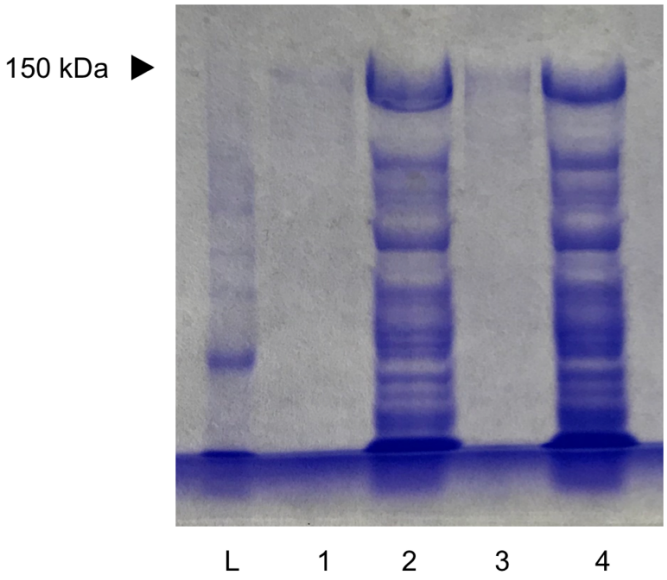

Fig 4. SDS-PAGE of two GS variants. Loaded samples are purified DSR_S170C (lane 1), soluble extract of DSR_S170C (lane 2), purified ASR_S415C (lane 3), soluble extract of ASR_S415C (lane 4). Arrow indicates the molecular weight marker.

The enzyme activities of all single mutants of DSR-S vardel Δ4N and ASR C-APY del are presented in Table 4. The highest enzymatic activities were found in mutants DSR_S954C and ASR_S579C, whereas the mutants DSR_S326C and

ASR_S713C showed the lowest enzymatic activities. These differences may be related with changes in the accessibility of modified sites, as well as with effects on dynamics and flexibility of the enzyme caused by the substitution of amino acids via site-directed mutagenesis (Li *et al.* 2018; Zhang *et al.* 2018). An increased activity of β-glucuronidase variants was observed after the mutation, revealing the importance of the amino acid presented in the parental sequence on the enzyme structure and function (Zhang *et al.* 2018).

Table 4. Enzyme activities of single mutants of DSR-S vardel Δ4N and ASR C-APY del

Enzyme activity (U mL^{-1})			
DSR-S vardel Δ4N: 25.0 ± 8.8		ASR C-APY del: 35.2 ± 5.8	
DSR_S170C	48.1 ± 8.0	ASR_S415C	55.7 ± 2.7
DSR_S278C	14.0 ± 2.0	ASR_S563C	55.0 ± 4.3
DSR_S318C	50.9 ± 19.8	ASR_S579C	70.32 ± 10.0
DSR_S326C	0.0 ± 0.01	ASR_S713C	26.4 ± 11.0
DSR_S474C	18.2 ± 8.4	ASR_S882C	40.8 ± 9.7
DSR_S641C	38.1 ± 14.2	ASR_S883C	37.5 ± 10.0
DSR_S646C	42.1 ± 9.1	ASR_S907C	39.0 ± 7.3
DSR_S759C	47.3 ± 13.6	ASR_S964C	36.5 ± 2.9
DSR_S788C	27.8 ± 6.8	ASR_S971C	43.5 ± 3.6

DSR_S954C	83.49 ± 11.8	ASR_S1027C	36.5 ± 4.9
		ASR_S1042C	36.9 ± 2.9
		ASR_S1235C	43.3 ± 13.2
		ASR_S1251C	41.4 ± 1.2
		ASR_S1313C	32.4 ± 1.0

On the other hand, the site-directed mutagenesis may affect the protein in a negative way. A possible explanation for the complete loss of activity in the variant DSR_S326C is its conservation in the sequence. The comparison obtained by Clustalω with the characterized enzymes of GH 70 family indicated that this particular Ser residue is well-conserved (Fig. 5). Additionally, this amino acid may have impacted on the enzyme structure, since serine is known to form hydrogen linkages, reinforcing the protein structure stability (Li *et al.* 2018). All other mutants did not present conserved Ser residues as can been seen in Fig. S1 and S2.

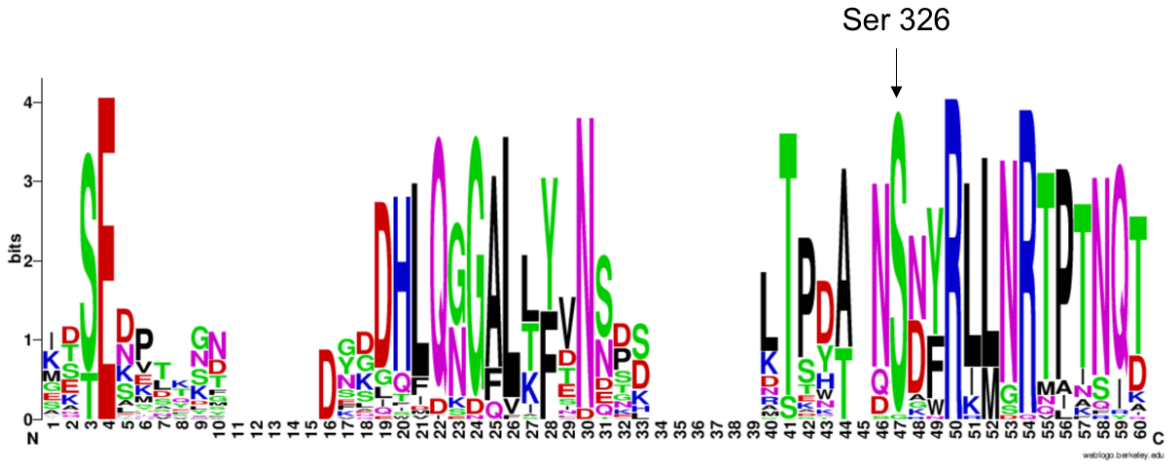

Fig. 5. Primary structural alignment of all GH 70 family enzymes corresponding to the

interval from E449 to T490 of DSR-S from *L. mesenteroides* B-512 F (GenBank accession number: I09598). Ser 447 in the sequence of DSR-S from *L. mesenteroides* B-512 F corresponds to Ser 326 of DSR-S vardel ∆4N. The overall height of the stack indicates the sequence conservation at that position, while the height of the symbols within the stack indicates the relative frequency of each amino acid at that position. Amino acids are colored according to their chemical properties: polar amino acids are green; neutral, pink; basic, blue; acidic, red; and hydrophobic amino acids are black.

Kang *et al.* (2011) investigated the mutagenesis of well conserved amino acids in GS and their results revealed that the substitution of these amino acids caused drastic losses in the variant enzyme activity, reaching only 8 % of residual activity after the mutation. Concerning the insertion of Cys residues, Simons *et al.* (2013) investigated the influence on catalytic activity of glucose-6-phosphate dehydrogenase from *L. mesenteroides* by the introduction of cysteine at different positions on the site-directed immobilization. The authors found that the mutation of an aspartic acid by a cysteine did not lead to losses in the enzymatic activity. In contrast, the substitution of a leucine indicated a loss of 30 % compared to the non-mutated fusion enzyme upon site-directed mutagenesis (Simons *et al.* 2013). The effect of Cys mutation was also evaluated for the lipase from *Geobacillus thermocatelunatus*. The variants showed a higher degree of variation of their properties relative to their corresponding native enzymes. This suggests that the parental enzyme is prone to being disturbed by amino acid substitutions, due to its complex catalytic mechanism (Godoy *et al.* 2011).

4. Conclusion

The three-dimensional models of DSR-S vardel ∆4N and ASR C-APY del were constructed and evaluated, presenting good quality. The site-directed mutagenesis did not promote significant losses in the variant enzyme activities. Only one mutant (DSR_S326C) had shown no dextransucrase activity, possibly explained by its conservation and this role in the protein structure stability. Overall, this work may provide a good perspective about the structure of these truncated dextransucrase and alternansucrase that could be used in immobilization studies on laboratory-scale and commercially-available supports. Further studies about site-directed immobilization are being conducted by our group to verify the effect of protein orientation in the activity recovery and synthesis of oligosaccharides by the constructed mutants.

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CAPÍTULO VI – CONSIDERAÇÕES FINAIS

Esse trabalho foi desenvolvido visando o aprimoramento da imobilização de glucansucrases. Para tal, uma extensa revisão sobre as diferentes técnicas de imobilização da dextransucrase, glucansucrase mais amplamente referida na literatura, foi desenvolvida. Inúmeras discussões foram levantadas em relação às vantagens e desvantagens de cada método, bem como a influência de alguns aspectos relevantes na imobilização, tais como origem da enzima, formato do suporte, presença ou ausência de aditivos no biocatalisador.

Na segunda parte do trabalho, por sua vez, os resultados referentes à imobilização covalente da dextransucrase de *L. mesenteroides* B-512 F em esferas de quitosana funcionalizadas com glutaraldeído foram apresentados. O novo biocatalisador demonstrou ser mais estável termicamente do que sua forma livre, bem como ser estável operacionalmente, o que é de extrema importância para sua utilização em escala industrial.

A seguir, demonstrou-se a produção e a caracterização de agregados enzimáticos entrecruzados (CLEAs) de dextransucrase de *L. mesenteroides* B-512 F. Nesse estudo, ficou clara a importância do tratamento com dextranase para a exposição dos grupos reativos dos aminoácidos localizados na superfície da enzima, para que esses pudessem reagir com o agente bifuncional glutaraldeído. Assim como a dextransucrase imobilizada em esferas de quitosana funcionalizadas com glutaraldeído, os CLEAs de dextransucrase exibiram boa estabilidade operacional na síntese de oligossacarídeos, sendo ambos imobilizados caracterizados como promissores.

Por fim, estudos sobre a modelagem das estruturas tridimensionais e a mutagênese sítio-dirigida das glucansucrases DSR-S vardel Δ4N and ASR C-APY del foram apresentados. Os modelos preditos demonstraram boa qualidade e a mutagênese sítio-dirigida não promoveu perdas significativas na atividade enzimática dos mutantes. Somente o mutante DSR_S326C, mostrou-se inativo, possivelmente explicado pelo papel da Ser 326 na estabilidade conformacional da proteína.

Os resultados obtidos nesse trabalho sugerem que a imobilização da dextransucrase foi satisfatória e que cada técnica utilizada possibilitou diferentes características ao imobilizado. Como perspectivas, sugere-se a investigação das novas formas imobilizadas em reatores enzimáticos. Também indica-se estudos de imobilização covalente multipontual em suportes heterofuncionais dos novos mutantes da dextransucrase DSR-S vardel Δ4N e da alternansucrase ASR C-APY del.

ANEXO I

Table S1. Primers of site-directed mutagenesis of dextransucrase DSR-S vardel Δ4N.

Mutant enzyme	Primer 5'-3' (forward)
DSR_S170C	f: CACGGATTATTGCGAACATAATGC
	r: GCATTATGTTCGCAATAATCCGTG
DSR_S278C	f: GTGCGCAACAATGTACAGAGTGG
	r: CCACTCTGTACATTGTTGCGCAC
DSR_S318C	f: CTTATGTCAACTGTCCACTGACAC
	r: GTGTCAGTGGACAGTTGACATAAG
DSR_S326C	f: CCTGACGCTAATTGTAACTTTAGACTAC
	r: GTAGTCTAAAGTTACAATTAGCGTCAGG
DSR_S474C	f: CTCTAACAAAATCATGTGACATACGAGG
	r: CCTCGTATGTCACATGATTTTGTTAGAG
DSR_S641C	f: GCCATGACAGCTTGTGACACTGGAAC
	r: GTTCCAGTGTCACAAGCTGTCATGGC
DSR_S646C	f: GACACTGGAACATGTGAGACGCGTAC
	r: GTACGCGTCTCACATGTTCCAGTGTC
DSR_S759C	f: CAACAAACACGTGTGATAAAGTGTTC
	r: GAACACTTTATCACACGTGTTTGTTG
DSR_S788C	f: GCATTTGCTACAGACTGCAGTGAATATAC
	r: GTATATTCACTGCAGTCTGTAGCAAATGC
DSR_S954C	f: CCAATTGATGGCTGTGTAAAGATTACTG
	r: CAGTAATCTTTACACAGCCATCAATTGG

Table S2. Primers of site-directed mutagenesis of alternansucrase ASR C-APY del

Mutant enzyme	Primer 5'-3'
ASR_S415C	f: GCCGCAAAGTCTTATGATAAATTAGTTTTGAAAATGTTGATGG
	r: CCATCAACATTTTCAAAACTACATTTATCATAAGACTTTGCGGC
ASR_S563C	f: CGCCGAATACTGATTGTGGAAATAACAGAAAACTAGG
	r: CCTAGTTTTCTGTTATTTCCACAATCAGTATTCGGCG
ASR_S579C	f: CAGTTGTATCTTTGCAACCATCG
	r: CGATGGTTGCAAAGATACAACTG
ASR_S713C	f: GCCAACAACAGGTGTAACATGTGG
	r: CCACATGTTACACCTGTTGTTGGC
ASR_S882C	f: GGCTACCGATTGTTCTGGAAAAGACC
	r: GGTCTTTTCCAGAACAATCGGTAGCC
ASR_S883C	f: GGCTACCGATAGTTGTGGAAAAGACC
	r: GGCTACCGATAGTTGTGGAAAAGACC
ASR_S907C	f: GGAATTATGACATGTGATCAAACCAC
	r: GTGGTTTGATCACATGTCATAATTCC
ASR_S964C	f: GCCTTAGTATTATCAAATGACTGTGGAATTGATG
	r: CATCAATTCCACAGTCATTTGATAATACTAAGGC
ASR_S971C	f: GTTTATGATTGTGATGATAAAGCACC
	r: GGTGCTTTATCATCACAATCATAAAC
ASR_S1027C	f: CCAGTTGGAGCTTGTGATTCACAAG
	r: CTTGTGAATCACAAGCTCCAACTGG

ASR_S1042C	f: GAGTCATCATCATGTAATGATGGTTCTG
	r: CAGAACCATCATTACATGATGATGACTC
ASR_S1235C	f: GAAGAGTACCCATGCTTATTCAAACAG
	r: CTGTTTGAATAAGCATGGGTACTCTTC
ASR_S1251C	f: GCCAATTGATGCTTGTACAAAAATTAAGC
	r: GCTTAATTTTTGTACAAGCATCAATTGGC
ASR_S1313C	f: GTGATGCCTGCGGTGTAAAATATTAC
	r: GTAATATTTTACACCGCAGGCATCAC

Fig. S1. Primary structural alignment of all GH 70 family enzymes indicating the selected Ser residues for the substitution in the sequence of dextransucrase DSR-S vardel Δ4N. The overall height of the stack indicates the sequence conservation at that position, while the height of the symbols within the stack indicates the relative frequency of each amino acid at that position. Amino acids are colored according to their chemical properties: polar amino acids are green; neutral, pink; basic, blue; acidic, red; and hydrophobic amino acids are black.

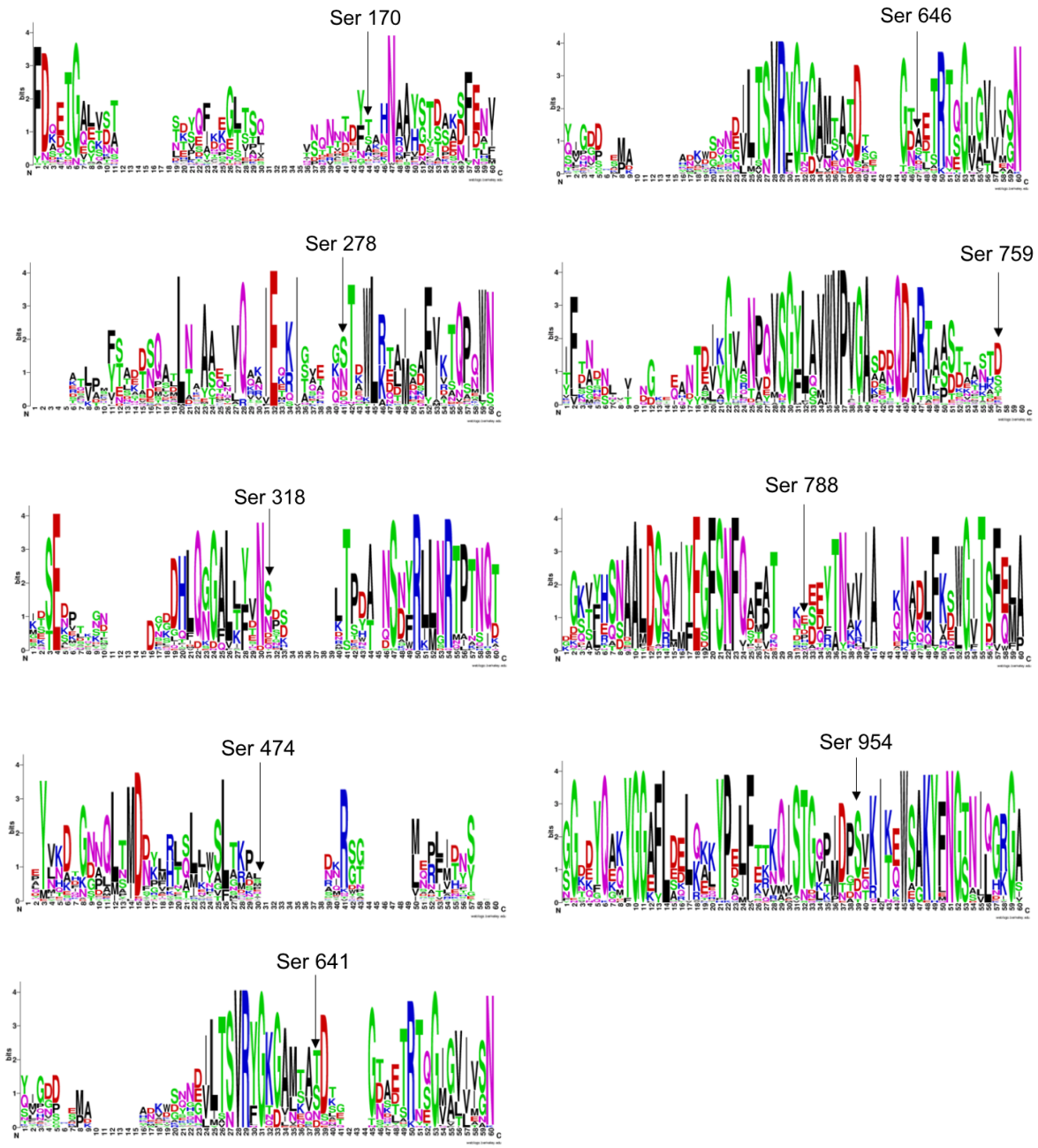
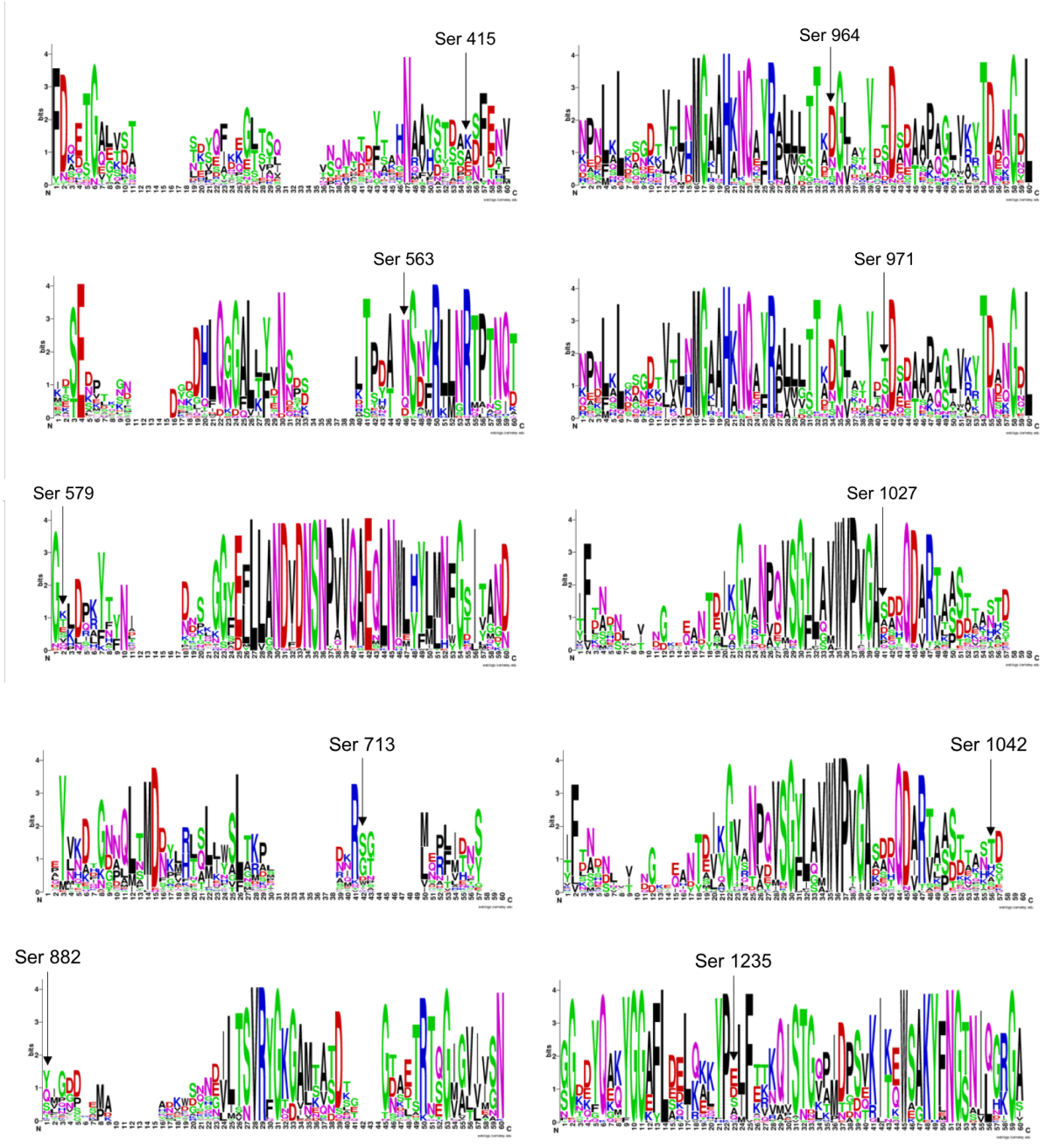
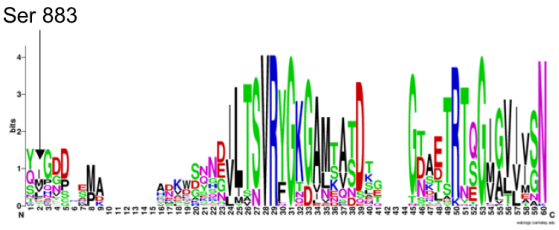


Fig. S2. Primary structural alignment of all GH 70 family enzymes indicating the selected Ser residues for the substitution in the sequence of alternansucrase ASR C-APY del. The overall height of the stack indicates the sequence conservation at that position, while the height of the symbols within the stack indicates the relative frequency of each amino acid at that position. Amino acids are colored according to their chemical properties: polar amino acids are green; neutral, pink; basic, blue; acidic, red; and hydrophobic amino acids are black.

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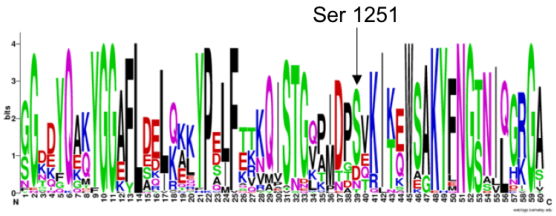
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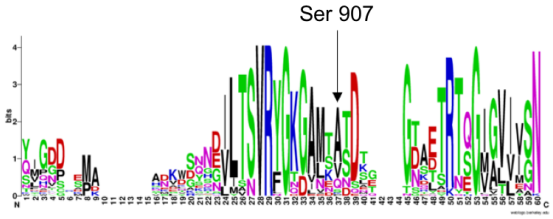
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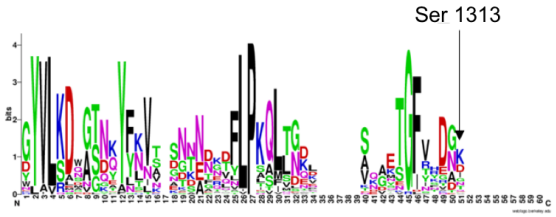
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