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PRODUÇÃO DE FRUTO-OLIGOSSACARÍDEOS E AÇÚCAR INVERTIDO UTILIZANDO ENZIMAS IMOBILIZADAS

Porto Alegre
Março de 2014

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Dissertação apresentada ao Curso de Pós Graduação em Ciência e Tecnologia de Alimentos como um dos requisitos para obtenção do grau de mestre em Ciência e Tecnologia de Alimentos.

Orientador: Plinho Francisco Hertz

Coorientador: Rafael Costa Rodrigues

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Sumário

Lista de Figuras

Resumo:

Fruto-oligossacarídeos (FOS) são fibras prebióticas com poder adoçante considerável, sendo um produto de alto valor para a indústria de alimentos. Açúcar invertido é o produto da hidrólise da sacarose possuindo maior poder adoçante, menor susceptibilidade à cristalização e maior higroscopicidade com relação à sacarose, sendo de grande interesse industrial. Ambos produtos podem ser produzidos por reações enzimáticas, utilizando β-frutosiltransferase e β-frutofuranosidase respectivamente, no entanto processos enzimáticos costumam ser caros devido ao alto custo e baixa estabilidade de enzimas. Esses fatores podem ser contornados com a imobilização da enzima, permitindo a reutilização e por vezes aumentando a estabilidade. No presente trabalho a enzima β-frutosiltransferase proveniente de um extrato comercial de *Aspergillus aculeatus* (Viscozyme L) foi parcialmente purificada, com resina de troca iônica, imobilizada covalentemente em esferas de quitosana e utilizada na produção de FOS. O processo de purificação aumentou a atividade específica em 6 vezes. A estabilidade do biocatalisador imobilizado foi avaliada em 50 bateladas para produção de FOS, foi observado cerca de 55 % de rendimento em cada batelada, sem perda de atividade detectada após as utilizações. Após esse experimento foi testada a utilização das esferas em reatores contínuos com leito fixo e fluidizado, com rendimentos de 59 % e 54 % respectivamente. A produção de açúcar invertido foi feita utilizando a enzima Maxinvert L (β-frutofuranosidase de *Saccharomyces cerevisiae*) que foi imobilizada, da mesma forma, em esferas de quitosana e sua utilização foi testada em reatores de leito fixo e fluidizado com rendimentos de 98 % e 94 % respectivamente. Os reatores de leito fixo possuem potencial para estudos envolvendo aplicações industriais tanto para produção de FOS quanto para produção de Açúcar Invertido.

Palavras Chave: Imobilização de enzimas, Reatores enzimáticos, Fruto-oligossacarídeos, Açúcar invertido, Quitosana.

Abstract:

Fructooligosaccharides (FOS) are prebiotic fibre with sweetening power, being a high-value product for the food industry. Invert sugar is the product of sucrose hydrolysis; it has a higher sweetening power, it is less susceptible to crystallization and has a higher hygroscopicity than regular sugar. Finding many uses in food industry processes. Both products can be obtained by enzymatic reactions using β-fructosyltransferase and β-fructofuranosidase, respectively. However, enzymatic processes are often costly because of high enzymatic cost and lack of operational stability. These drawbacks can be overcome by immobilization of enzyme, enabling reuses and usually increasing its stability. In the present work, β-fructofuranosidase from a commercial preparation from *Aspergillus aculeatus* (Viscozyme L) was partially purified, covalently immobilized on chitosan spheres and used for FOS production. Partial purification resulted in a 6-fold increase in specific activity. Operational stability of biocatalyst was evaluated along 50 batches, resulting in around 55 % yield on each batch and no loss of activity after batches. The immobilized biocatalyst was also used for FOS production in packed bed and fluidized bed reactors with yields of 59 % and 54 % respectively. Invert sugar production was carried out using Maxinvert L (β-fructofuranosidase from *Saccharomyces cerevisiae*) immobilized, by the same method, on chitosan spheres. Its application on packed bed and fluidized bed reactors was evaluated resulting in yields of 98 % and 94 % respectively. The packed bed reactors presented potential for further studies aiming industrial applications for FOS and Invert Sugar production.

Keywords: Enzyme immobilization, Enzymatic reactors, Fructooligosaccharides, Invert sugar, Chitosan.

1. Introdução:

O desenvolvimento de produtos de valor agregado obtidos por transformações químicas tem contribuído significativamente para o aumento da qualidade de vida ao longo de séculos e atingiu um alto padrão. No entanto muitas reações químicas estequiométricas utilizadas atualmente, embora produtivas, ainda devem ser substituídas por reações catalisadas (Noyori, 2009). Os catalisadores empregados atualmente na indústria, podem ser inorgânicos, organometálicos, orgânicos e biocatalisadores. Os biocatalisadores, ou enzimas, destacam-se por possuírem diversas qualidades, não são tóxicos, são obtidos de fontes renováveis, são facilmente biodegradáveis, possuem alta especificidade e processos enzimáticos são seguros e consomem pouca energia (Illanes, 2008; Wohlgemuth, 2010).

Embora possuam muitas vantagens, o uso de enzimas em processos industriais possui algumas inconveniências. Enzimas em geral são matérias primas caras que quando utilizadas na sua forma livre elevam substancialmente o custo do processo. Além disso, como são de origem biológica, as enzimas foram naturalmente sofrendo adaptações para operar em condições fisiológicas que diferem de condições de uso industriais, tornando-as frágeis (Illanes, 2008).

Ultimamente, novas tecnologias possibilitaram o uso de enzimas em uma série de aplicações industriais, principalmente na produção de medicamentos, vitaminas e aromas (Bornscheuer *et al.*, 2012). Entre estas tecnologias está a imobilização de enzimas, permitindo que a enzima seja reutilizada várias vezes, eventualmente em processos contínuos, e ainda pode aumentar a estabilidade da enzima (Garcia-Galan *et al.*, 2011).

Entre outras técnicas, a imobilização covalente em suporte insolúvel é de grande interesse, devido ao fato de ser irreversível e usualmente gerar biocatalisadores estáveis. A quitosana foi utilizada como suporte por ser barata, atóxica, possuir alta resistência a degradação química e poder ser sintetizada em várias formas diferentes (Ravi Kumar, 2000; Krajewska, 2004). Como possui grupos amino pode ser facilmente funcionalizada com glutaraldeído, tornando-se suscetível a formação de ligações com o amino terminal de proteínas.

No presente trabalho, a enzima invertase foi o foco da pesquisa. Esta enzima, utilizada na indústria de alimentos, é capaz de hidrolisar a sacarose produzindo glicose e frutose, como demonstrado na Figura 1a. Trata-se de uma das mais simples carboidrases comerciais e é muito utilizada em função disso (Kotwal e Shankar, 2009). Sob condições específicas, altas concentrações de sacarose, esta enzima é capaz de produzir também fruto-oligossacarídeos (FOS), sendo nesse caso chamada preferencialmente de β-frutosiltransferase como mostrado

na Figura 1b. Os fruto-oligossacarídeos são oligossacarídeos formados por unidades de frutosil, eles não são metabolizados pelo organismo humano e são considerados prebióticos, pois promovem o crescimento de probióticos (Passos e Park, 2003). Atualmente não há nenhuma invertase imobilizada disponível comercialmente, e as invertases comercializadas na forma livre não possuem atividade de β-frutosiltransferase para produção de FOS.

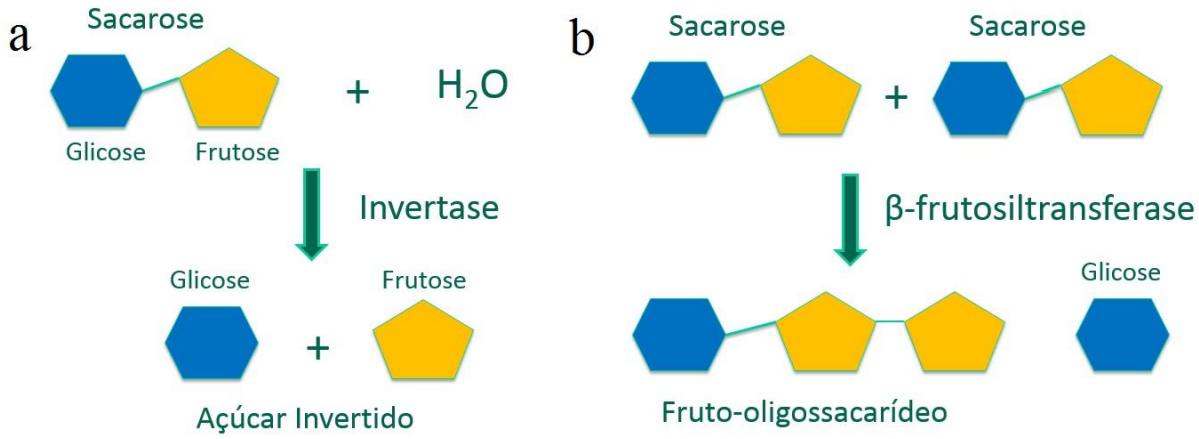

Figura 1 Mecanismo de ação: a Invertase. b β-frutosiltransferase.

1.1 *Objetivos*

Considerando isto, o objetivo geral deste trabalho foi produzir fruto-oligossacarídeos e açúcar invertido utilizando enzimas imobilizadas.

Os objetivos específicos foram: obter uma enzima com alta atividade específica para produção de fruto-oligossacarídeos; produzir um biocatalisador estável para produção de fruto-oligossacarídeos e outro para produção de açúcar invertido; avaliar a melhor forma de utilizar os biocatalisadores obtidos para produção contínua de fruto-oligossacarídeos e açúcar invertido.

2. Revisão:

Nas seções seguintes serão apresentadas revisões sobre imobilização de enzimas, reatores enzimáticos, fruto-oligossacarídeos e açúcar invertido os quais foram o alvo de estudo desta dissertação.

2.1 *Imobilização de Enzimas*

Existem muitos métodos de imobilização diferentes e várias formas de classifica-los (Katzbauer, Narodoslawsky e Moser, 1995). Neste trabalho serão classificados em três categorias de métodos diferentes para imobilização de enzimas, ligação cruzada, confinamento e ligação em um suporte, ilustradas na Figura 2 (Sheldon e Van Pelt, 2013). Cada método possui vantagens e desvantagens que serão descritas a seguir:

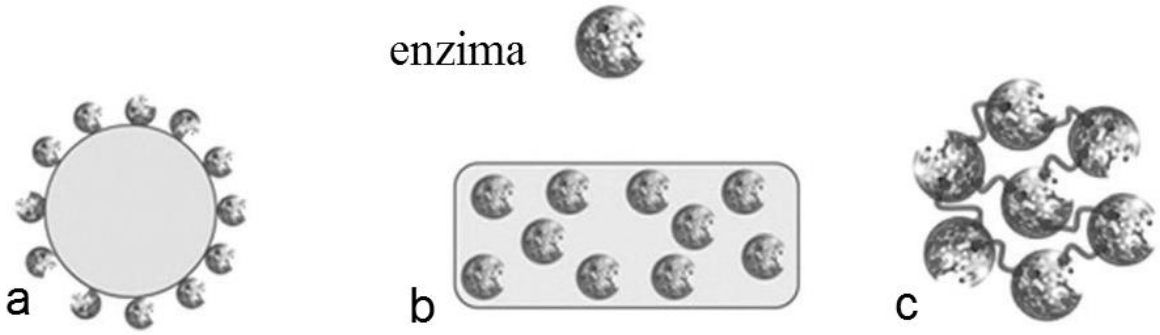

Figura 2 Métodos de imobilização de enzimas: a Ligação ao Suporte. b Confinamento. c Ligação Cruzada, adaptado de Sheldon e Van Pelt (2013).

A ligação cruzada, mostrada na Figura 2c é uma categoria de métodos de imobilização que não necessitam de suporte. Uma vantagem primária desta categoria é o fato de a atividade não ser "dissolvida" por uma massa não catalítica, ou seja, o suporte (Cao, Langen e Sheldon, 2003). O primeiro método utilizado foi o de formação de agregados de enzimas insolúveis por agentes de ligações cruzadas, como o glutaraldeído. Apesar de ainda possuir parte da atividade catalítica, este tipo de agregado costuma perder boa parte de sua atividade inicial. Isto somado a sua baixa estabilidade fizeram com que este método fosse pouco explorado (Sheldon e Van Pelt, 2013).

Para superar o problema de baixa estabilidade foram desenvolvidos os cristais de enzimas agregadas. Tais cristais são robustos, com tamanho de partícula controlável e apresentam uma estabilidade mais alta que suas respectivas enzimas na forma livre. Entretanto, a necessidade de obtenção de uma enzima pura e a dificuldade de cristalização, somados ao desenvolvimento de uma nova tecnologia, fizeram com que o uso comercial destes compostos fossem abandonados (Cao, Langen e Sheldon, 2003; Sheldon e Van Pelt, 2013).

A nova tecnologia utilizada para imobilizar enzimas sem suporte consiste em formar agregados de enzimas por precipitação, ligações cruzadas são realizadas subsequentemente de forma que os precipitados permaneçam insolúveis. Esse tipo de agregado é denominado CLEA (Cross-Linked Enzyme Aggregate) (Cao, Van Rantwijk e Sheldon, 2000). CLEAs possuem propriedades parecidas com cristais de agregados enzimáticos porém não necessitam do mesmo grau de pureza podendo inclusive serem preparados a partir de sobrenadantes de fermentações. Isto fez com que os CLEAs fossem utilizados comercialmente para diversas finalidades (Sheldon e Van Pelt, 2013). Embora o desenvolvimento de enzimas imobilizadas sem suporte possua diversas vantagens, alguns fatores como o emprego em reatores, e condições de preparo trabalhosas, fazem com que estes agregados dificilmente sejam a primeira opção entre os engenheiros de bioprocessos (Cao, 2006a).

O confinamento, encapsulamento ou engaiolamento, mostrado na Figura 2b, é definido pelo fato de o suporte ser fabricado (solidificado, polimerizado ou sintetizado) em contato com a enzima, de forma que ela fique inserida no suporte ou envolvida por ele (Sheldon e Van Pelt, 2013). O confinamento é um dos tipos de métodos mais simples de imobilização de enzimas, porém, em geral a enzima se desprende facilmente do suporte. Além disso, a matriz pode dificultar a difusão de substrato e dificilmente a estabilidade da enzima é aumentada (Cao, 2006a).

A ligação da enzima em um suporte, mostrada na Figura 2a, pode ser feita por adsorção ou ligação covalente. A adsorção da enzima em um suporte pode ser não específica (van der Waals, ligação de hidrogênio e interação hidrofílica), bioespecífica, hidrofóbica e iônica. Já a ligação covalente é feita ligando covalentemente um resíduo de aminoácido ou o amino terminal da enzima a um grupo funcional do suporte. Basicamente qualquer suporte pode ser utilizado para imobilizar enzimas por adsorção, ao passo que para ligação covalente ele deve possuir grupos funcionais específicos e em muitos casos uma etapa de ativação do suporte precisa ser realizada (Cao, 2006b; c; Garcia-Galan *et al.*, 2011).

Métodos de imobilização covalente costumam ser mais complexos que os de adsorção, porém resultam em ligações mais estáveis e irreversíveis (Garcia-Galan *et al.*, 2011), diferentemente de enzimas imobilizadas por adsorção que costumam se desprender do suporte ao longo de processos industriais. No entanto, uma vez que a ligação é irreversível, enzimas ligadas covalentemente quando inativadas tornam o suporte inútil (Sheldon e Van Pelt, 2013). Este fato pode ser facilmente contornado utilizando um suporte barato. Também é importante ressaltar que a imobilização covalente muitas vezes aumenta a estabilidade térmica da enzima (Garcia-Galan *et al.*, 2011; Lorenzoni *et al.*, 2014).

Dentre os tipos de ligação covalente podemos citar ligação peptídica, ligação dissulfeto, diazotização, alquilação/acilação, entre outros. Como mencionado anteriormente os grupos funcionais do suporte podem se ligar tanto aos resíduos de aminoácidos como no amino ou carbono terminal. Ligações envolvendo apenas as extremidades das enzimas dificilmente envolvem o sítio ativo da enzima, portanto a perda de atividade costuma ser menor devido a imobilização, no entanto, neste caso apenas uma ligação por monômero é permitida e o efeito de estabilização da enzima é menor. As ligações envolvendo resíduos de aminoácidos permitem que sejam feitas várias ligações por monômero, ou seja, ligações multipontuais. Estas ligações estabilizam a estrutura tridimensional da enzima resultando em biocatalisadores ainda mais estáveis, porém caso um aminoácido próximo ao sítio ativo seja envolvido na ligação ocorrerá perda de atividade (Fessner *et al.*, 1999; Mateo *et al.*, 2000; Cao, 2006b).

Agentes de ligação são necessários quando o suporte não possui grupos funcionais disponíveis para formar ligações covalentes diretamente com a enzima. Genipina, carbodiimida e glutaraldeído são exemplos de agentes de ligação, dentre estes o mais versátil é o glutaraldeído (Chiou e Wu, 2004; Migneault *et al.*, 2004). O Glutaraldeído é um dialdeído com uma cadeia linear com 5 carbonos sendo altamente reativo, fazendo ligações com grupos amino em meios pouco ácidos e alcalinos (Okuda *et al.*, 1991). Em pHs próximos a neutralidade há uma tendência a ligação com o amino terminal, já em pHs mais alcalinos o glutaraldeído pode fazer ligações também com resíduos de lisina. Ligações com lisina são interessantes do ponto de vista de imobilização, já que resíduos deste aminoácido usualmente estão localizados na superfície das proteínas, fora de sítios ativos devido a polaridade do grupo amino (Migneault *et al.*, 2004). O glutaraldeído também pode ser utilizado como agente de ligação entre proteínas sem o uso de suportes, que serão descritos mais adiante.

2.1.1 Uso de Quitosana como Suporte de Imobilização de Enzimas

A quitosana é um poliaminosacarídeo natural, sendo comercialmente obtido através da desacetilação da quitina, processo que a torna solúvel em meios ácidos. A quitina é um dos recursos naturais orgânicos mais abundantes na natureza, é o constituinte principal de cascas de crustáceos, exoesqueletos de insetos e da parede celular de fungos. É estimado que aproximadamente 10 bilhões de toneladas de quitina sejam sintetizadas e degradadas a cada ano (Krajewska, 2004; Vaaje-Kolstad *et al.*, 2013). Em função disso o custo da quitosana é desprezível comparado com o custo de enzimas, além disso, ela possui grupos funcionais

(amino) com alta afinidade à proteínas, não é tóxica e possui alta resistência à degradação química (Ravi Kumar, 2000; Krajewska, 2004).

Os primeiros trabalhos envolvendo imobilização de enzimas em suportes de quitosana foram publicados na década de 70. Muzzareli *et al.* (1976) testaram a adsorção de quimotripsina em quitosana sem utilizar agente de ligação. Kasumi *et al.* (1977) imobilizaram glicose isomerase, urease, glicoamilase, tripsina e glicose oxidase em quitosana utilizando carbodiimida como agente de ligação. Recentemente, Seo *et al.* (2012) imobilizaram quitinases em quitosana utilizando glutaraldeído como agente de ligação.

Ultimamente, vários trabalhos foram desenvolvidos pelo nosso grupo de pesquisa envolvendo imobilização de enzimas em quitosana, também utilizando o glutaraldeído como agente de ligação, possibilitando a obtenção de biocatalisadores estáveis. Valerio *et al.* (2012) utilizaram nanopartículas de quitosana para imobilização de invertase e produção de açúcar invertido. Klein *et al.* (2012) avaliaram o efeito do tamanho do suporte de quitosana na imobilização da enzima β-galactosidase, e verificaram que apesar de possibilitarem uma maior carga de enzima, as nanopartículas são de manuseio mais difícil e não proporcionam melhoria significativa na estabilidade da enzima. Em relação a estabilidade, Schöffer *et al.* (2013) alcançaram resultados promissores, uma vez que, testando a utilização de esferas de quitosana para produção de β-ciclodextrinas em reator de leito fixo, observaram que mesmo após 100 horas de uso contínuo, a atividade se manteve em 100 %. Klein *et al.* (2013) produziram galacto-oligossacarídeos utilizando esferas de quitosana em reator de leito fixo, alcançando alta produtividade e aumento da estabilidade do biocatalisador.

Neste trabalho também foi utilizado o método de imobilização covalente em esferas de quitosana pré ativadas com glutaraldeído.

2.2 ***Reatores Enzimáticos***

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

A Figura 3 mostra diferentes tipos de reatores enzimáticos. Estes tipos de reatores serão descritos a seguir:

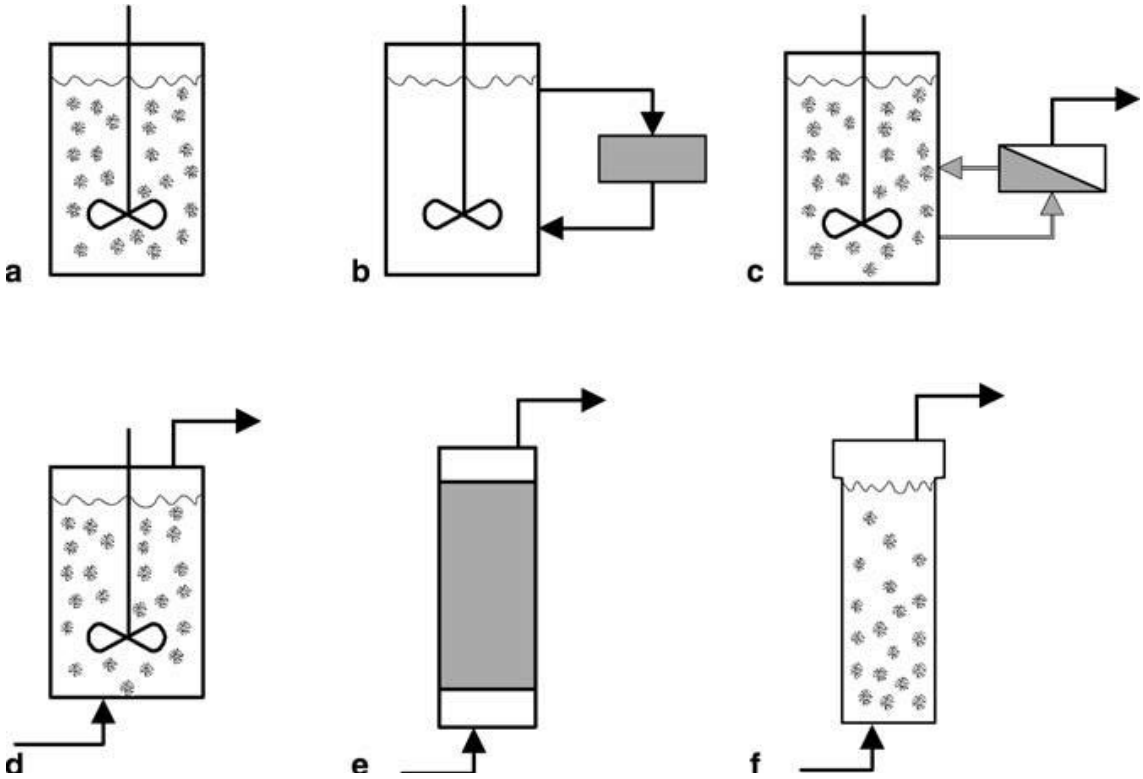

Figura 3 Diferentes tipos de reatores enzimáticos: a Batelada. b Batelada com Recirculação. c Batelada com Ultrafiltração. d Reator Contínuo de Tanque Agitado. e Reator de Leito Fixo. f Reator de Leito Fluidizado (Illanes e Altamirano, 2008).

Reatores em batelada, mostrado na Figura 3a, são normalmente utilizados com enzimas livres e possuem um fácil controle de pH e temperatura. Podem também ser utilizados com enzimas imobilizadas, mas nesse caso devem possuir agitação e um sistema de recuperação de enzima, como uma peneira de aço, por exemplo (Illanes e Altamirano, 2008).

O Reator em Batelada com Recirculação, mostrado na Figura 3b, é uma forma de utilizar enzimas imobilizadas. Uma solução com substrato é circulada com um leito de esferas imobilizadas, este leito pode ser fixo ou fluidizado (Diano *et al.*, 2008), cada tipo de leito possui suas particularidades que serão descritas a seguir. Comparado com os reatores contínuos de leito fixo ou fluidizado este reator permite um melhor controle de processo. É recomendável para reações que liberam ou consomem prótons e necessitam de ajuste de pH. A solução de correção, ácido ou base forte, é adicionada no tanque de forma que não entre em contato direto com a enzima (Illanes e Altamirano, 2008).

Reatores em batelada com ultrafiltração, mostrado na Figura 3c, permitem que enzimas sejam reutilizadas sem a necessidade de imobilização. Além disso, este reator consiste em uma fase homogênea com fácil transferência de massa (Blatt *et al.*, 1968). Ainda que enzimas livres costumem ser menos estáveis e o processo de ultrafiltração torne o processo mais trabalhoso, este reator é empregado em algumas aplicações industriais (Bódalo *et al.*, 2001).

O reator contínuo de tanque agitado, mostrado na Figura 3d, é vantajoso do ponto de vista de homogeneização, além de possuir fácil controle de pH. Pode ser utilizado com enzimas livres ou imobilizadas. Quando utilizado com enzimas livres é necessária a reposição contínua de enzima, tornando o processo mais caro. Já com enzima imobilizada é importante usar um suporte com alta resistência mecânica devido à agitação (Frick e Schügerl, 1986).

Reatores de leito fixo, mostrado na Figura 3e, são formados por uma coluna imóvel de biocatalisadores por onde uma solução de substrato é bombeada continuamente. Estes reatores destacam-se devido à tecnologia simples, alta capacidade catalítica, fácil aumento de escala e operação. Estes reatores causam um estresse mecânico mínimo resultando em uma estabilidade duradoura e reduzindo custos de processo. No entanto são susceptíveis ao entupimento da coluna e criação de caminhos preferenciais (Jakobsen, 2008; Hama *et al.*, 2011).

Reatores de leito fluidizado, mostrado na Figura 3f, são parecidos com reatores de leito fixo, com a diferença de que o fluxo de substrato é sempre ascendente e em uma velocidade suficiente para suportar as partículas, porém sem arrastá-las junto com o fluido. Nesse caso as partículas permanecem em equilíbrio entre as forças de empuxo e arraste e a força da gravidade (Van Zessen *et al.*, 2005; Illanes e Altamirano, 2008). Estes reatores necessitam de um volume útil maior e em alguns casos, quando o fluxo é baixo, necessitam de uma bomba de recirculação para manter a velocidade mínima de fluidização na coluna. As vantagens destes reatores são a melhor transferência de massa e menor susceptibilidade ao entupimento e criação de caminhos preferenciais com relação ao leito fixo (Gòdia e Solà, 1995; Hama *et al.*, 2011).

Devido às semelhanças e diferenças descritas acima o presente trabalho avaliou dois processos enzimáticos em reatores contínuos de leito fixo e leito fluidizado.

2.3 ***Fruto-oligossacarídeos***

Fruto-oligossacarídeos (FOS), como já mencionado anteriormente, são oligossacarídeos prebióticos formados por unidades de frutosil. Eles são encontrados na natureza, em uma ampla variedade de plantas e também em algas (Benkeblia, 2013). Comercialmente podem ser produzidos de duas formas, hidrólise enzimática da inulina, com endoinulinase, ou síntese enzimática com sacarose, utilizando β-frutofuranosidase (E.C.3.2.1.26, E.C.2.4.1.9). Fruto-oligossacarídeos obtidos de sacarose possuem uma cadeia mais curta que os de inulina, e possuem sabor mais doce, podendo ser utilizados por diabéticos (Mabel *et al.*, 2008). Além

disso a produção a partir da sacarose possui um custo de matéria prima muito menor que a produção a partir da inulina.

Seu uso foi testado, por exemplo, para substituir parcialmente o uso de sacarose em bebidas à base de suco de abacaxi, manga e laranja, resultando em uma bebida com fibras e menor valor energético (Renuka *et al.*, 2009). O uso de FOS também foi testado na formulação de suco de cenoura em conjunto com *Lactobacillus rhamnosus* e *Lactobacillus bulgaricus* resultando em um produto simbiótico estável, sem alterar as propriedades organolépticas do suco original (Nazzaro *et al.*, 2008). No mercado brasileiro é possível encontrar FOS em requeijão cremoso, bolachas, iogurtes, leites fermentados, (Aguiar-Oliveira, 2012) alimentos para bebês, paçoca diet e néctar de frutas.

Com relação à estrutura FOS podem ser classificados como tipo inulina, tipo levan, ou ramificados, como mostrado na Figura 4. Todas essas formas são encontradas na natureza. FOS do tipo inulina são formados principalmente por ligações (2→1) frutosil-frutose, FOS do tipo levan são formados principalmente por ligações (2→6) frutosil-frutose e FOS do tipo ramificado possuem ambas ligações em quantidades similares (Benkeblia, 2013).

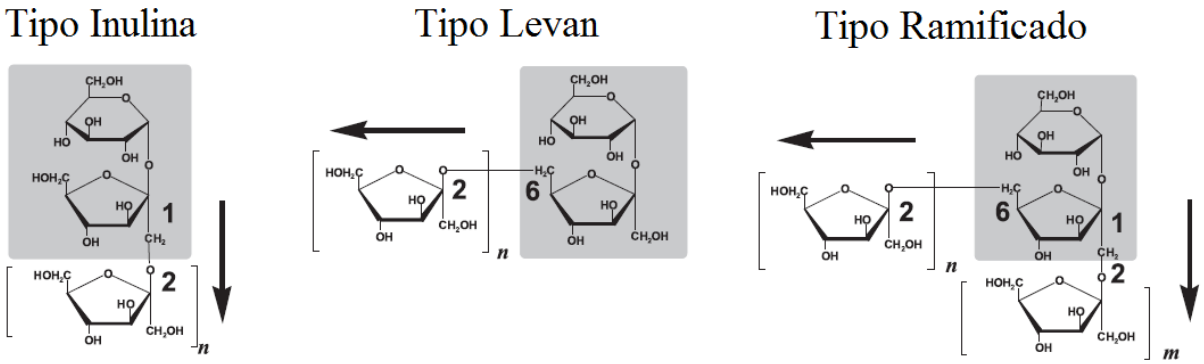

Figura 4 Tipos de Fruto-oligossacarídeos.

FOS produzidos por síntese enzimática da sacarose terão uma estrutura dependente da enzima utilizada na síntese. Por exemplo a β-fructofuranosidase de *Schwanniomyces occidentalis* produz 6-kestose (tipo levan) em quantidades maiores que 1-kestose (tipo inulina) (Álvaro-Benito *et al.*, 2007). Já β-fructofuranosidases provenientes de *Trichoderma reesei*, *Aspergillus oryzae* e *Aspergillus aculeatus* produzem apenas 1-kestose, nistose e em pequenas quantidades 1-frutofuranosilnistose todos do tipo inulina com 1, 2 ou 3 unidades de frutosil ligadas à uma sacarose por ligação (2→1) (Nemukula *et al.*, 2009; Vega-Paulino e Zúniga-Hansen, 2012; Lorenzoni *et al.*, 2014).

A maioria do trabalhos envolvendo a produção de FOS a partir de sacarose envolve também a etapa de produção da enzima, uma vez que não há enzima comercializada para esta

função (Chen e Liu, 1996; Nguyen *et al.*, 1999; Álvaro-Benito *et al.*, 2007; Hernalsteens e Maugeri, 2008; Hernalsteens e Maugeri, 2010). No entanto alguns trabalhos utilizam extratos enzimáticos comerciais com outras funções para produzir FOS, já que vários extratos possuem atividade de β-fructofuranosidase como uma função secundária (Vega-Paulino e Zúniga-Hansen, 2012). Por exemplo, o extrato comercial Pectinex Ultra SP-L, proveniente de *A. aculeatus*, que é comercializado como uma poligalacturonase, para produção de sucos, já foi utilizado em vários trabalhos para produzir FOS (Ghazi *et al.*, 2005; Tanriseven e Aslan, 2005; Csanádi e Sisak, 2006). Ghazi *et al.* (2007) purificaram a enzima β-fructofuranosidase deste extrato e obtiveram uma enzima com atividade específica 107 vezes maior que no extrato inicial. Eles também mostraram que a β-fructofuranosidase de *A. aculeatus* é proteína dimérica de 135 kDa.

Recentemente Vega-Paulino e Zúniga-Hansen (2012) mostraram que diversos extratos comerciais possuem atividade de β-fructofuranosidase, entre eles o extrato Viscozyme L, também proveniente de *A. aculeatus* que é comercializado como uma endo-β-glicanase, também utilizado na produção de sucos, possui uma atividade de β-fructofuranosidase ainda maior que a Pectinex Ultra SP-L. Em função disso a Viscozyme L foi utilizada neste trabalho para produção de FOS, sendo parcialmente purificada a fim de aumentar a atividade específica de β-fructofuranosidase.

2.4 *Açúcar Invertido*

A invertase foi uma das primeiras enzimas estudadas na história. Em 1890 foi publicado um artigo de revisão sobre esta enzima (O'sullivan e Tompson, 1890). Naquela época o grau de hidrólise da sacarose era medido pela rotação específica. A sacarose possui uma rotação específica positiva, ao passo que uma mistura de glicose e frutose possui uma rotação específica negativa. Tal fato foi observado pela primeira vez por Biot em 1836 que chamou a substância de açúcar invertido (Jordan, 1924).

A invertase também foi utilizada no estudo que determinou o modelo cinético de Michaelis-Menten, utilizado até hoje para a maioria das enzimas (Michaelis e Menten, 1913). Apenas em 1935 um método de análise alternativo ao polarimétrico foi proposto, envolvendo o ácido dinitrosalicílico visando diminuir o tempo de análise que podia chegar a 2 horas (Sumner e Howell, 1935). Este método foi aperfeiçoado por Miller (1959) e é utilizado até os dias atuais, inclusive foi utilizado neste trabalho.

O açúcar invertido é mais doce, mais higroscópico e menos susceptível à cristalização em comparação com a sacarose. Devido à esses fatores ele é usado em diversas aplicações

industriais desde o início do século XX até os dias de hoje, entre elas na fabricação de bombons, doces e bolos (Jordan, 1924; Podadera, 2007).

Além de ser obtido por hidrólise enzimática o açúcar invertido também pode ser obtido por hidrólise ácida sob altas temperaturas. No entanto, além de gastar mais energia, o processo gera hidroximetilfurfural, um subproduto indesejável (De Almeida, 2005). Mesmo possuindo desvantagens o processo químico ainda é uma opção industrial devido ao custo da enzima. Este custo poderia ser substancialmente reduzido com o uso de enzimas imobilizadas, contudo não há invertase comercial imobilizada disponível no mercado.

Assim, considerando que a imobilização de enzimas pode trazer diferentes benefícios, como o aumento da estabilidade, o aperfeiçoamento de processos e possibilidade de redução de custos de produção, a seguir serão apresentados dois trabalhos nos quais realizou-se a imobilização de enzimas para a produção de açúcar invertido e fruto-oligossacarídeos em batelada e em processo contínuo em reatores de leito fixo e fluidizado.

3. Artigos:

A seguir serão apresentados dois artigos. O primeiro, submetido em outubro de 2013, foi publicado na revista Carbohydrate Polymers, volume 103, páginas 193 à 197. O segundo será submetido para publicação na mesma revista.

3.1 **Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from *Aspergillus aculeatus***

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Abstract

The enzymatic synthesis of fructooligosaccharides (FOS) was carried out using a partially purified β-fructofuranosidase from the commercial enzyme preparation Viscozyme L. Partial purification of β-fructofuranosidase from Viscozyme L was done by batch adsorption using ion-exchange resin DEAE-Sepharose, showed a 6-fold increase in specific activity. The biocatalyst was then covalently immobilized on glutaraldehyde-activated chitosan particles. Thermal stability of the biocatalyst was evaluated at 50 ºC and 60 ºC, being around 100 times higher at 60 ºC when compared to the free enzyme. The immobilized biocatalyst was reused 50 times for FOS production (100 min per batch at 50 ºC and pH 5.5) without significant loss of activity. The average yield (grams of FOS per grams of initial sucrose) was 55 %. The immobilization process combined with partial purification method resulted in a derivative with activity of 1230 U_t/g (Transfructosylation activity), which is among the highest activities found in literature for FOS production.

Keywords: Enzyme, Immobilization, Fructooligosaccharides, Viscozyme L, Chitosan.

1 Introduction

Fructooligosaccharides (FOS), also known as oligofructose, are prebiotic food ingredients. This classification refers to their non digestibility, fermentation by intestinal microflora and selective stimulation of intestinal bacteria that contribute to a better health and well-being (Roberfroid, 2007). They can be obtained by inulin hydrolysis, using endoinulinase, or by synthesis, using β-fructofuranosidase (E.C.3.2.1.26, E.C.2.4.1.9). The FOS obtained from sucrose (β-D-Fructofuranosyl(2→1)α-D-glucopyranoside) has a shorter chain than those from inulin, having more sweetening power, and could be used as a sweetener by diabetics (Mabel, Sangeetha, Platel, Srinivasan & Prapulla, 2008).

Although the synthesis of FOS from sucrose could be economical because sucrose is cheaper than inulin, the use of enzymes as catalysts for large-scale industrial processes is costly and once they have been made soluble, their recovery for reuse is not economically practical. The immobilization of enzymes allows an easy reuse and other advantages, for example, better stability, continuous operation and reducing overall costs (Liese & Hilterhaus, 2013; Sheldon & Van Pelt, 2013).

Chitosan [(1→4)-2-amino-2-deoxy-β-D-glucan] has been used as a carrier for enzyme immobilization since the 70s (Kasumi, Tsuji, Hayashi & Tsumura, 1977; Muzzarelli, Barontini & Rocchetti, 1976). In recent studies, protocols for enzyme immobilization on chitosan were developed, resulting in stable biocatalysts, for β-galactosidase (Klein et al., 2012), invertase (Valerio, Alves, Klein, Rodrigues & Hertz, 2013) and for chitinase (Seo, Jang, Park & Jung, 2012). Chitosan is obtained from chitin [(1→4)-2- acetamido-2-deoxy-β-D-glucan], that is subjected to N-deacetylation followed by purification procedures. Its production is an economically attractive use for crustacean shells (Krajewska, 2004).

The commercial enzyme preparation from *Aspergillus aculeatus* (Pectinex Ultra SP-L) has been the aim of several studies for FOS production in both free and immobilized form (Csanádi & Sisak, 2006; Ghazi et al., 2007; Tanriseven & Aslan, 2005). Recent study has shown that Viscozyme L, other preparation from *A. aculeatus* has even more β-fructofuranosidase activity than Pectinex Ultra SP-L (Vega-Paulino & Zúniga-Hansen, 2012). It is interesting to note that both enzyme preparations have other key enzyme activities apart from β-fructofuranosidase. Pectinex Ultra SP-L is marketed as polygalacturonase that hydrolyzes (1→4)-α-D-galactosiduronic linkages in pectate and other galacturonans, while Viscozyme L is marketed as endo-β-glucanase that hydrolyzes (1→3)- or (1→4)- linkages in β-D-glucans.

In this context, the aim of this work is to prepare a highly stable and active derivative of β-fructofuranosidase from *A. aculeatus* immobilized on chitosan particles for FOS synthesis. The commercial preparation Viscozyme L was partially purified to improve the β-fructofuranosidase activity, and the immobilized preparation was evaluated for optimal pH and temperature, thermal stability, and operational stability in FOS synthesis.

2 Materials and Methods

2.1 Materials

Viscozyme L, a commercial enzyme preparation from *A. aculeatus*, produced by Novozymes, was kindly donated by LNF Latino Americana. Centrifugal Filter Unit (30 kDa) was acquired from Millipore. DEAE-Sepharose fast flow® was acquired from Sigma-Aldrich. Sucrose was acquired from Vetec Química Fina Ltda (Brazil). Enzymatic glucose (D-glucose) determination kit was from In Vitro Diagnóstica Ltda (Brazil) and glutaraldehyde 25 % was from Nuclear (Brazil). The high liquid chromatography (HPLC) column used in these tests was an Aminex® HPX-87C. All other chemicals were analytical or HPLC grade obtained from readily available commercial sources.

2.2 Enzyme activity

The enzymatic activity assay was carried out using a substrate solution of 100 g/L of sucrose in 50 mM sodium acetate buffer, pH 5.5 at 50 ºC. Samples were taken at regular intervals and the reaction was stopped by the addition of 0.1 M sodium carbonate buffer, pH 10.0. Glucose release was measured with the enzymatic glucose determination kit. One unit of enzymatic activity measured (U) was defined as the amount of enzyme that releases 1 μmol of glucose from sucrose per minute at test conditions. All the activities of immobilized enzymes were carried out under agitation.

2.3 Enzyme purification

Viscozyme L was diluted in 20 mM sodium phosphate buffer (pH 5.8) to a final protein concentration of 20 mg/mL and then applied to flasks containing DEAE-Sepharose previously equilibrated with the same buffer. Desorption was carried out washing the resin with the same buffer containing increasing concentrations of sodium chloride. Most of enzyme was desorbed at 0.4 M of sodium chloride solution. After desorption, protein solution was filtrated using a centrifugal filter (30 kDa; 4000 g; 40 min; 4 ºC) to concentrate the β-fructofuranosidase and to remove the salt.

2.4 Preparation and Activation of Chitosan Particles

The chitosan particles were prepared using the protocol described by Klein et al. (2012). Briefly, a chitosan solution was added dropwise into an alkaline coagulation solution, and the activation was performed by incubating the chitosan particles obtained with a glutaraldehyde solution (4% v/v) prepared in 0.1 M phosphate-sodium buffer (pH 7.0), at room temperature for 3 h in an orbital shaker at 120 rpm with 12 mm of orbital diameter. The activated support was exhaustively washed with 50 mM sodium acetate buffer (pH 5.5) to remove the excess glutaraldehyde.

2.5 Enzyme Immobilization

Chitosan particles were incubated with partially purified enzyme solution previously diluted in 50 mM of sodium acetate buffer (pH 5.5), for 3 h at room temperature and under gentle shaking. The immobilized enzyme was washed with, buffer, NaCl (1.0 M) and ethylene glycol (30 % v/v) to eliminate unbounded and ionic and hydrophobic bounded enzymes.

The immobilization yield (IY) and immobilization efficiency (EF) were calculated by the same equations previously described by Valerio et al. (2013).

2.6 Thermal stability

Soluble and immobilized enzymes were incubated at 50 ºC and 60 ºC for up to 9 days. Samples were withdrawn at intervals, placed in ice bath to stop thermal inactivation and enzyme activity was then measured.

In general, thermal inactivation of enzymes can be described by a first order reaction,

$$A / A_0 = \exp(-kt) \quad (1)$$

where A is the enzyme activity at time t, A_0 the initial enzyme activity, t is the treatment time, and k is the constant inactivation rate at the studied temperature. In this work, k was determined by fitting the first order model to the data of the residual enzyme activity plot (A/A_0) versus time (min) using nonlinear regression (Statistica 7.0, StatSoft, Inc., Tulsa, OK, USA).

The enzyme half-life ($t_{1/2}$) represents the time required for the enzyme to decay to 50% of its initial value of activity and was calculated from the values of k, following this equation:

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

2.7 Determination of optimal pH and temperature

The optimal pH and temperature were assayed for both partially purified (soluble) and immobilized enzymes. The optimal pH tests ranged from 3 to 8, maintaining the temperature constant at 50 ºC and the optimal temperature was assayed from 42 ºC to 80 ºC maintaining

pH at 5.5. The buffers (50 mM) used were glicyne-HCl pH 3.0, sodium acetate pH 4.0, 5.0 and 5.5, and sodium phosphate pH 6.0, 6.5, 7.0, 7.5 and 8.0.

2.8 Fructooligosaccharides production

The FOS production was carried out at 50 ºC using a solution of sucrose 600 g/L in 50 mM sodium acetate buffer pH 5.5. Fifty chitosan particles (22 U) were incubated in 2 mL of sucrose solution for 160 min. Samples were taken at 15 min intervals, for kinetic evaluation.

Reactions were carried out for 100 min at the same conditions, for operational stability evaluation. The particles were washed between batches with 50 mM sodium acetate buffer pH 5.5 to remove any trace of product. Samples were taken after each batch.

The samples were assayed for reducing sugars by the DNS method (Miller, 1959) and enzymatically for glucose. Fructose (D-fructose) was calculated as the difference between the concentrations of reducing sugars and glucose, FOS molar concentration was calculated as difference between the molar concentrations of glucose and fructose.

The samples were also analyzed by an HPLC system (Shimadzu, Tokyo, Japan) equipped with refractor index and Aminex HPX-87C (300 mm × 7.8 mm) column. Ultra-pure water was used as eluting solvent at a flow rate of 0.6 mL min^{-1}, at 85 ºC. The concentration of saccharides [sucrose, glucose, fructose, kestose (β-D-Fructofuranosyl(2→1)β-D-Fructofuranosyl(2→1)α-D-glucopyranoside) and nystose (β-D-Fructofuranosyl(2→1)β-D-Fructofuranosyl(2→1)β-D-Fructofuranosyl(2→1)α-D-glucopyranoside)] was determined by interpolation using external standards. HPLC analysis was carried out to confirm the assessment method and for direct measurement of transfructosylation activity of the immobilized biocatalyst. Transfructosylation activity (U_t) was defined as the amount of enzyme that produces 1 μmol of kestose or nystose from sucrose per minute.

3 Results and Discussion

3.1 Enzyme partial purification

Crude Viscozyme L has a β-fructofuranosidase specific activity of 4.2 U/mg and volumetric activity of 500 U/mL, at 50 ºC and pH 5.5. The purification process resulted in an enzyme solution with a specific activity of 25.0 U/mg resulting in a purification fold of 6.0 with an activity recovery of 60 %. After centrifugal filtration, the enzyme was concentrated to over 330 U/mL.

This purification is only partial, and can be improved as shown by Ghazi et al. (2007), that purified a β-fructofuranosidase from *A. aculeatus* (Pectinex Ultra SP-L) and obtained a purification fold of 107, obtaining a specific activity of 2635 U/mg, which is 105 times higher

than the activity obtained in our work. On the other hand, the activity recovery was only 36.8 % after four purification steps, which is smaller than 60 % obtained in our work by one-step purification. For the purification of Pectinex Ultra SP-L, a two step process resulted in a purification fold of 7, recovering 22.3 % of initial activity (Nemukula, Mutanda, Wilhelmi & Whiteley, 2009). The increase in enzyme purity can improve the immobilization conditions. However, enzyme isolation and purification can be expensive and time consuming (de Carvalho, 2011), and the enzyme immobilization should be a relatively simple operation not requiring a highly pure enzyme preparation that not always is commercially available (Sheldon & Van Pelt, 2013).

3.2 Enzyme immobilization

The results of the different enzyme load applied to the support are shown in Figure 1. The best biocatalyst activity was obtained with an amount of 120 mg of enzyme per gram of dry support. This amount resulted in an immobilization yield of 90 % and immobilization efficiency of 33 %. The biocatalyst obtained has shown a hydrolytic activity of 880 U per gram of dry support, equivalent to 0.44 U per chitosan particle.

As reported by other authors, the low immobilization efficiency could be due to linkages with groups related to active site and those responsible for maintaining the tertiary structure of the enzyme (Tanriseven & Aslan, 2005). One possible solution, is the immobilization in the presence of substrate that can protect the active site of the enzyme, improving efficiency (Cadena et al., 2010). In this work, the presence of sucrose at 100 g/L and 600 g/L in the immobilization solution did not improve the immobilization efficiency, as also reported by Valerio et al. (2013) for the β-fructofuranosidase from *S. cerevisiae*. Moreover, other reports showed similar behavior for different enzymes (Csanádi & Sisak, 2006; Ghazi et al., 2005; Platková, Polakovič, Štefuca, Vandáková & Antošová, 2006; Tanriseven & Aslan, 2005).

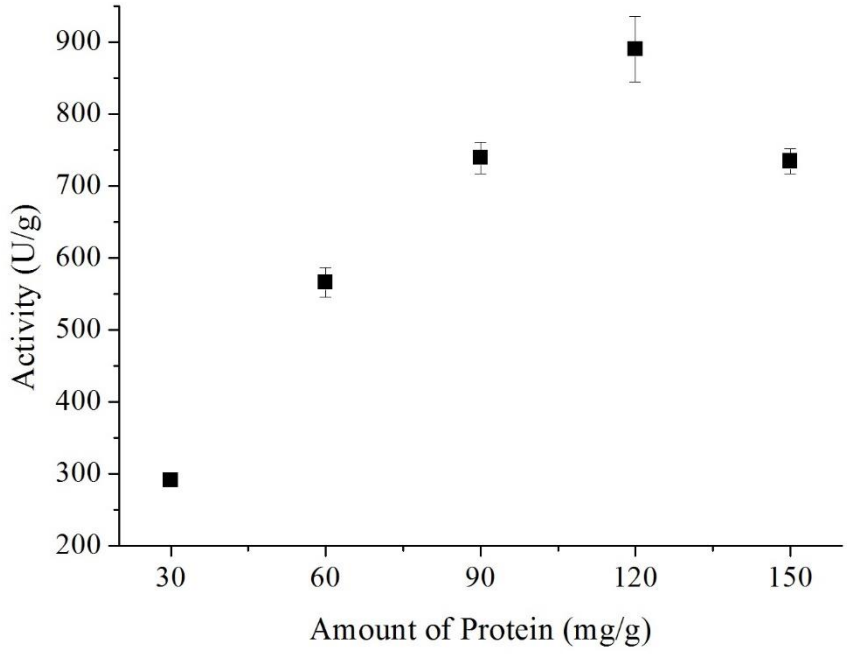

Figure 1: Activity (■) measured on the immobilized biocatalyst for each amount of protein tested for immobilization.

3.3 Thermal stability

The kinetic inactivation for both free and immobilized β-fructofuranosidase is shown in Figure 2 where the symbols refer to the average experimental values, whereas lines correspond to fitting Eq. (1) for the experimental data. The inactivation rate constant (k) and the half-life ($t_{1/2}$) for the free enzyme at 50 ºC and 60 ºC are given in Table 1 with the correlation coefficient r^2.

Table 1. Kinetic Parameters of Thermal Inactivation of Free β-frutofuranosidase at 50 and 60 ºC.

Biocatalyst	k (min^{-1})	$t_{1/2}$ (h)	r^2
Free β-frutofuranosidase at 50 ºC	0.0169	41.01	0.98
Free β-frutofuranosidase at 60 ºC	0.2221	3.12	0.9627

It was not possible to quantify the half-life of the immobilized enzymes, because after 200 h the prepared biocatalyst presented 83 % of its initial activity at 50 ºC, and 50 % at 60 ºC, as depicted in the Figure 2. According to Gardossi et al. (2010) the experiment must be carried out until 10 % of residual activity to allow clear distinction of kinetic inactivation model and proper half-life determination.

Due to the high thermal stability, 10 % of residual activity would be reached only after longer times. Figure 2 clearly shows that the immobilized enzyme is much more stable than

the free enzyme at both temperatures, proving that the immobilization onto chitosan particles has a positive effect in the thermal stability of the enzyme.

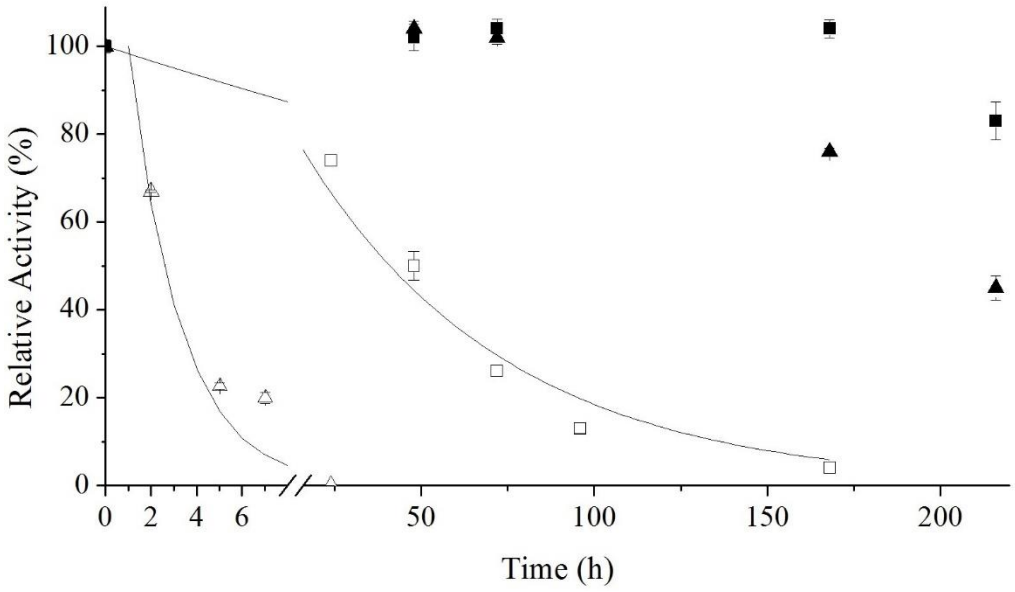

Figure 2: Relative activity of the free enzyme at 50 ºC (□), immobilized enzyme at 50 ºC (■), free enzyme at 60 ºC (∆) and immobilized enzyme at 60 ºC (▲).

An increase in the number of enzyme-support bonds is suggested as the most likely explanation for the stabilization achieved in the immobilized enzymes (Pedroche et al., 2007). However, when using supports at non-alkaline pH values, the immobilization occurs only via the amino terminal group (Mateo et al., 2005). Since in this work the enzyme is a dimeric glycoprotein (Ghazi et al., 2007), such high stability could be due to chitosan attachment of amino terminal group of both monomers of the enzyme, and then stabilizing the enzyme quaternary structure.

This high thermal stability provides several advantages for the biocatalyst, such as: the sucrose is easier solubilized at higher temperatures (Young & Jones, 1949); microbial growth of pathogenic microorganisms such as *Staphylococcus aureus* (Schmitt, Schuler-Schmid & Schmidt-Lorenz, 1990), *Escherichia coli* and *Salmonella enterica* (Bronikowski, Bennett & Lenski, 2001) is avoided at 50 ºC; in addition, thermal stability of enzymes can limit the long term application of the biocatalyst in the process (Liese & Hilterhaus, 2013).

3.4 Determination of optimal pH and temperature

The results of optimal pH and temperature are shown in Figures 3 and 4, respectively. It was not observed changes in the optimum pH comparing immobilized and free enzyme. However, the immobilized enzyme is slightly more active at lower pH values (pH 3.0). Sometimes the support may be an ionic exchanger, and may behave as a buffer, generating a pH inside the biocatalyst bead that may greatly differ from the pH value in the reaction

medium (Rodrigues, Ortiz, Berenguer-Murcia, Torres & Fernández-Lafuente, 2013). However, this explanation could be discarded since chitosan is a polyaminosaccharide without ionic strength and the enzyme molecules probably get immobilized mostly in their outer surfaces (Klein et al., 2012). This wide pH range of activity above 80 % might be a result of the stability of the enzyme 3D structure caused by the attachment on the support.

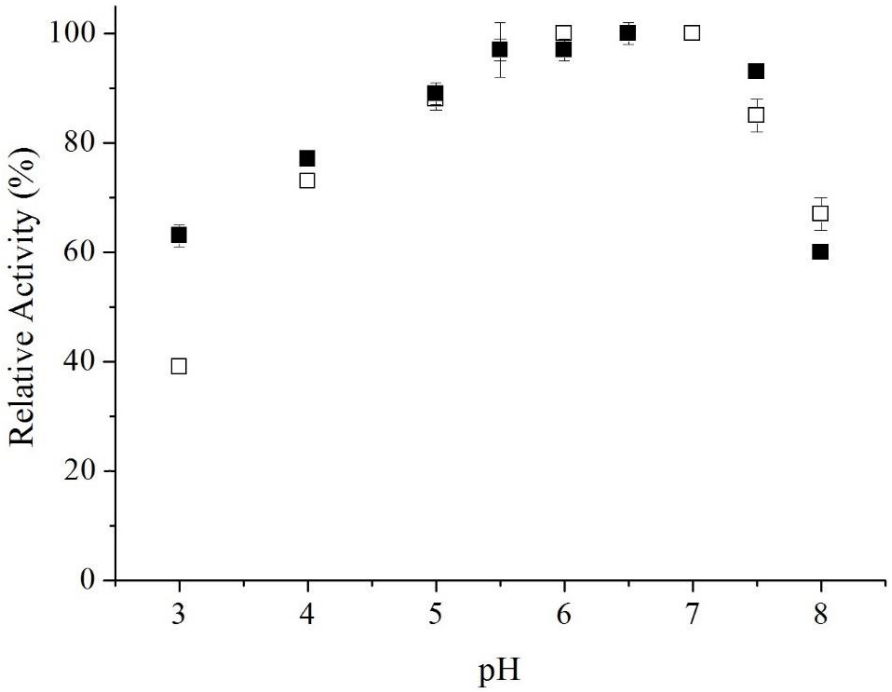

Figure 3: Effect of pH on the free (□) and immobilized (■) enzyme at 50 ºC.

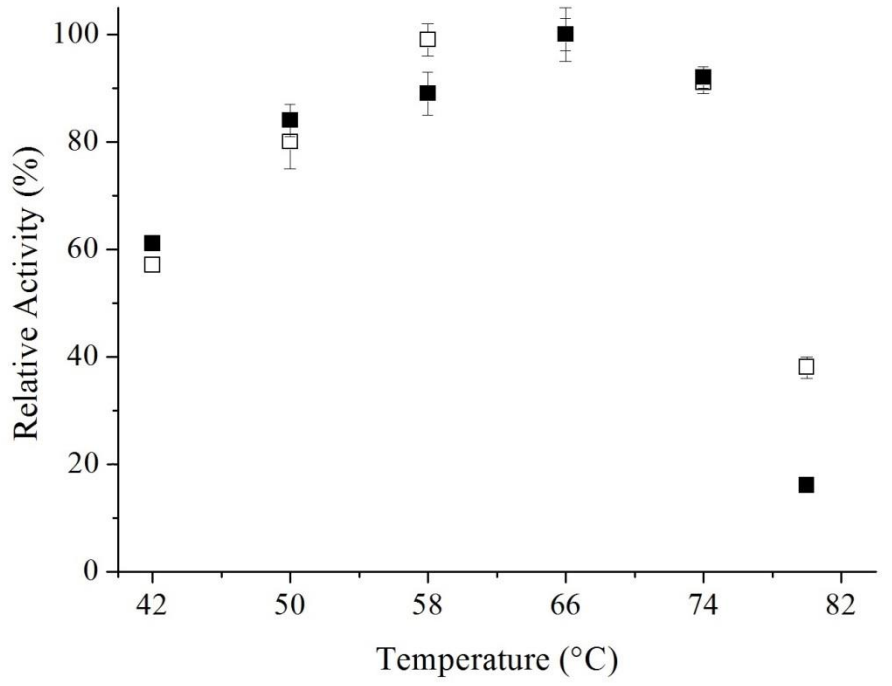

Figure 4: Effect of the temperature on free (□) and immobilized (■) enzyme at pH 5.5.

The optimum temperature for free enzyme was between 58 ºC and 66 ºC, presenting 100 % of relative activity on both temperatures. Nemukula et al. (2009) reported an optimal temperature of 60 ºC in a reaction of 40 minutes; on the other hand, Tanriseven & Aslan (2005), reported an optimal temperature of 65 ºC in a 1 minute reaction. These differences of optimal temperatures found for the same enzyme can be explained by different reaction times. The optimal temperature measured will be higher for shorter reaction times since thermal inactivation occurs during enzymatic reaction.

As can be seen on Figure 4, the immobilized enzyme showed a higher relative activity at 66 ºC than at 58 ºC. This result could be probably attributed to the higher thermal stability of the immobilized enzyme compared to free enzyme. The thermal stability is a key factor for studying the operational temperature. The enzyme is more active at 66 ºC, however, is more stable at 50 ºC. Then, for long-term use, it was selected 50 ºC for the experiments of FOS production.

3.5 Fructooligosaccharides production

Figure 5 shows the kinetics of FOS production from sucrose catalyzed by the chitosan-immobilized enzyme. The maximum yield of 55 % of FOS (including kestose and nystose) was achieved at 100 min. It can be also noted that after 100 min, the concentrations of kestose decrease and nystose increase while the sum of them remained constant. This can be due to the fact that kestose is used as a substrate to produce nystose (Jung, Yun, Kang, Lim & Lee, 1989). The stabilization on FOS overall concentration, and reduced rates of sucrose transfructosylation observed at the end of reaction can be due to the fact that glucose is a competitive inhibitor of the transfructosylating reaction of sucrose and kestose (Alvarado-Huallanco & Maugeri-Filho, 2010). The glucose concentration increased constantly and reached to 145 g/L after 160 min of reaction.

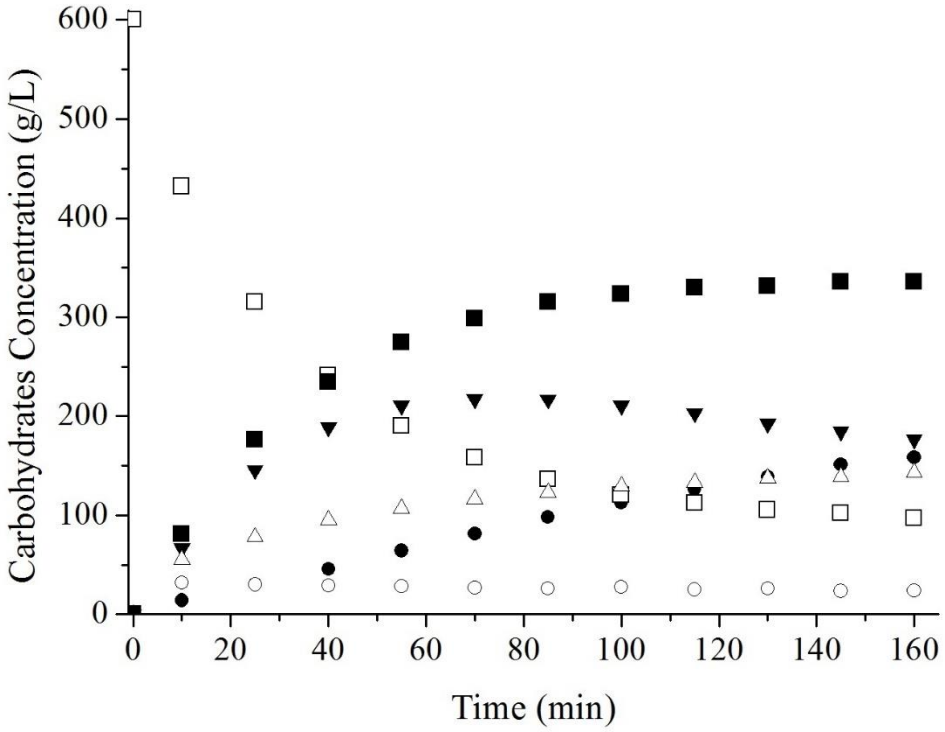

Figure 5: Kinetc evaluation of carbohydrates: Nystose (●), Kestose (▼), Sucrose (□), Glucose (∆), Fructose (○), Total FOS (■), during reaction with a new biocatalyst.

Figure 6 shows the operational stability of the biocatalyst. It can be noted that the yield of FOS varied from 40 % to 65 % and no significant losses of activity were observed over the batches. After 50 uses, a new complete kinetic was performed, where samples were taken at 15 min intervals, as for the first batch, and carbohydrates concentration were assayed on HPLC. The comparison between those batches showed similar concentrations for FOS and other sugars, and only small differences were observed, which means that there was no activity loss after 50 reuses of the biocatalyst.

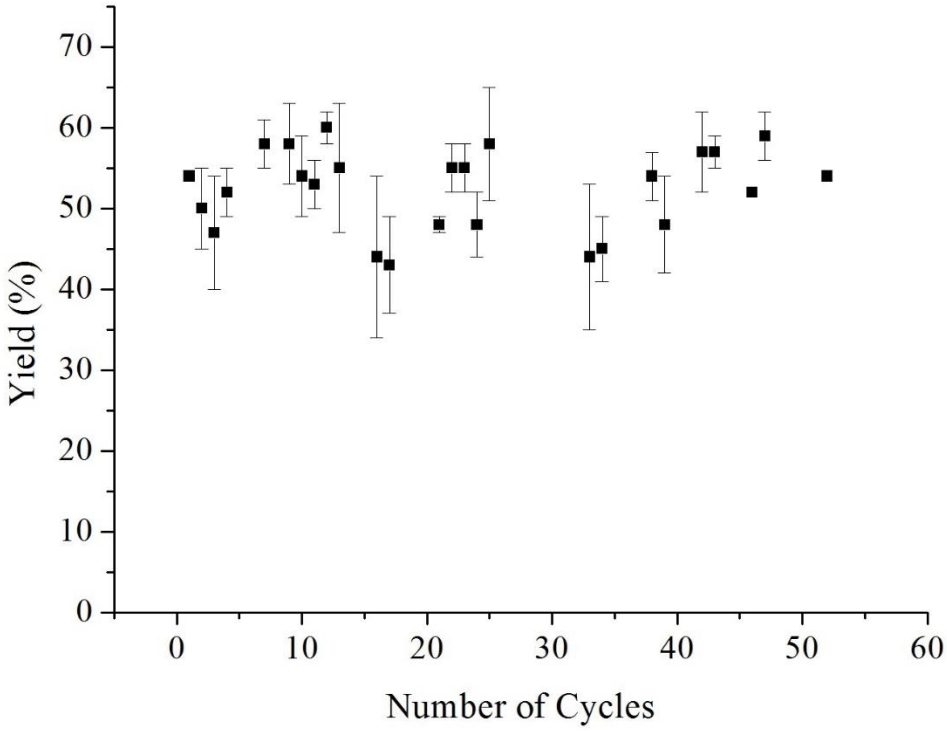

Figure 6: Operational stability of β-frutofuranosidase immobilized on chitosan particles in the FOS synthesis.

The transfructosylation activity was 0.61 Ut per chitosan particle and 1230 Ut per g of dry support. An immobilized biocatalyst presenting a transfructosylation activity of 1230 Ut/g of dry support has the highest activity found in literature so far. Tanriseven & Aslan (2005) obtained a biocatalyst with transfructosylation activity of 129 Ut/g, in a preparation using Eupergit C, an epoxy-activated acrylic bead, as support. Csanádi & Sisak (2006) obtained an immobilized biocatalyst with 14.8 Ut/g on Amberlite IRA900 Cl, an anion exchange resin. Recently, Fernandez-Arrojo et al. (2013) obtained a biocatalyst with 40.7 U/mL using a calcium alginate bead produced from sodium alginate SG300, which has similar properties than chitosan particles. A possible explanation for our high transfructosylation activity is that the enzyme initially used for immobilization had higher specific activity than the other works, since it was partially purified.

4 Conclusions

The immobilization of β-frutofuranosidase on glutaraldehyde-activated chitosan by covalent attachment produced a high active and stable biocatalyst, with a good immobilization yield and efficiency. Combined with the partial purification method, the immobilization process resulted in a derivative with activity of 1230 Ut/g, which is among the highest found in literature for FOS production. The biocatalyst also showed a high operational stability allowing its use for at least 50 times without significant losses of activity. The obtained

biocatalyst presents promising potential for industrial production of fructooligosaccharides from sucrose.

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The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) of the Brazilian government that supported this work.

References:

Alvarado-Huallanco, M. B., & Maugeri-Filho, F. (2010). Kinetics and modeling of fructo-oligosaccharide synthesis by immobilized fructosyltransferase from Rhodotorula sp. *Journal of Chemical Technology and Biotechnology, 85*, 1654-1662.

Bronikowski, A. M., Bennett, A. F., & Lenski, R. E. (2001). Evolutionary adaptation to temperature. VIII. Effects of temperature on growth rate in natural isolates of Escherichia coli and Salmonella enterica from different thermal environments. *Evolution, 55*, 33-40.

Cadena, P. G., Jeronimo, R. A. S., Melo, J. M., Silva, R. A., Lima Filho, J. L., & Pimentel, M. C. B. (2010). Covalent immobilization of invertase on polyurethane, plast-film and ferromagnetic Dacron. *Bioresource Technology, 101*, 1595-1602.

Csanádi, Z., & Sisak, C. (2006). Immobilization of Pectinex Ultra SP-L pectinase and its application to production of fructooligosaccharides. *Acta Alimentaria, 35*, 205-212.

de Carvalho, C. C. C. R. (2011). Enzymatic and whole cell catalysis: Finding new strategies for old processes. *Biotechnology Advances, 29*, 75-83.

Fernandez-Arrojo, L., Rodriguez-Colinas, B., Gutierrez-Alonso, P., Fernandez-Lobato, M., Alcalde, M., Ballesteros, A. O., & Plou, F. J. (2013). Dried alginate-entrapped enzymes (DALGEEs) and their application to the production of fructooligosaccharides. *Process Biochemistry, 48*, 677-682.

Gardossi, L., Poulsen, P. B., Ballesteros, A., Hult, K., Švedas, V. K., Vasić-Rački, Đ., Carrea, G., Magnusson, A., Schmid, A., Wohlgemuth, R., & Halling, P. J. (2010). Guidelines for reporting of biocatalytic reactions. *Trends in Biotechnology, 28*, 171-180.

Ghazi, I., De Segura, A. G., Fernández-Arrojo, L., Alcalde, M., Yates, M., Rojas-Cervantes, M. L., Plou, F. J., & Ballesteros, A. (2005). Immobilisation of fructosyltransferase from Aspergillus aculeatus on epoxy-activated Sepabeads EC for the synthesis of fructo-oligosaccharides. *Journal of Molecular Catalysis B: Enzymatic, 35*, 19-27.

Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Ferrer, M., Ballesteros, A., & Plou, F. J. (2007). Purification and kinetic characterization of a fructosyltransferase from Aspergillus aculeatus. *Journal of Biotechnology, 128*, 204-211.

Jung, K. H., Yun, J. W., Kang, K. R., Lim, J. Y., & Lee, J. H. (1989). Mathematical model for enzymatic production of fructo-oligosaccharides from sucrose. *Enzyme and Microbial Technology, 11*, 491-494.

Kasumi, T., Tsuji, M., Hayashi, K., & Tsumura, N. (1977). Preparation and some properties of chitosan bound enzymes. *Agricultural and Biological Chemistry, 41*, 1865-1872.

Klein, M. P., Nunes, M. R., Rodrigues, R. C., Benvenutti, E. V., Costa, T. M. H., Hertz, P. F., & Ninow, J. L. (2012). Effect of the support size on the properties of β-galactosidase immobilized on chitosan: Advantages and disadvantages of macro and nanoparticles. *Biomacromolecules, 13*, 2456-2464.

Krajewska, B. (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and Microbial Technology, 35*, 126-139.

Liese, A., & Hilterhaus, L. (2013). Evaluation of immobilized enzymes for industrial applications. *Chemical Society Reviews, 42*, 6236-6249.

Mabel, M. J., Sangeetha, P. T., Platel, K., Srinivasan, K., & Prapulla, S. G. (2008). Physicochemical characterization of fructooligosaccharides and evaluation of their suitability as a potential sweetener for diabetics. *Carbohydrate Research, 343*, 56-66.

Mateo, C., Abian, O., Bernedo, M., Cuenca, E., Fuentes, M., Fernandez-Lorente, G., Palomo, J. M., Grazu, V., Pessela, B. C. C., Giacomini, C., Irazoqui, G., Villarino, A., Ovsejevi, K., Batista-Viera, F., Fernandez-Lafuente, R., & Guisán, J. M. (2005). Some special features of glyoxyl supports to immobilize proteins. *Enzyme and Microbial Technology, 37*, 456-462.

Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry, 31*, 426-428.

Muzzarelli, R. A. A., Barontini, G., & Rocchetti, R. (1976). Immobilized enzymes on chitosan columns: α Chymotrypsin and acid phosphatase. *Biotechnology and Bioengineering, 18*, 1445-1454.

Nemukula, A., Mutanda, T., Wilhelmi, B. S., & Whiteley, C. G. (2009). Response surface methodology: Synthesis of short chain fructooligosaccharides with a fructosyltransferase from Aspergillus aculeatus. *Bioresource Technology, 100*, 2040-2045.

Pedroche, J., del Mar Yust, M., Mateo, C., Fernández-Lafuente, R., Girón-Calle, J., Alaiz, M., Vioque, J., Guisán, J. M., & Millán, F. (2007). Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: Correlation between enzyme–support linkages and thermal stability. *Enzyme and Microbial Technology, 40*, 1160-1166.

Platková, Z., Polakovič, M., Štefuca, V., Vandáková, M., & Antošová, M. (2006). Selection of carrier for immobilization of fructosyltransferase from Aureobasidium pullulans. *Chemical Papers, 60*, 469-472.

Roberfroid, M. (2007). Prebiotics: The Concept Revisited. *The Journal of Nutrition, 137*, 830S-837S.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, A., Torres, R., & Fernández-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews, 42*, 6290-6307.

Schmitt, M., Schuler-Schmid, U., & Schmidt-Lorenz, W. (1990). Temperature limits of growth, TNase and enterotoxin production of Staphylococcus aureus strains isolated from foods. *International Journal of Food Microbiology, 11*, 1-19.

Seo, D.-J., Jang, Y.-H., Park, R.-D., & Jung, W.-J. (2012). Immobilization of chitinases from Streptomyces griseus and Paenibacillus illinoisensis on chitosan beads. *Carbohydrate Polymers, 88*, 391-394.

Sheldon, R. A., & Van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews, 42*, 6223-6235.

Tanriseven, A., & Aslan, Y. (2005). Immobilization of Pectinex Ultra SP-L to produce fructooligosaccharides. *Enzyme and Microbial Technology, 36*, 550-554.

Valerio, S. G., Alves, J. S., Klein, M. P., Rodrigues, R. C., & Hertz, P. F. (2013). High operational stability of invertase from Saccharomyces cerevisiae immobilized on chitosan nanoparticles. *Carbohydrate Polymers, 92*, 462-468.

Vega-Paulino, R. J., & Zúniga-Hansen, M. E. (2012). Potential application of commercial enzyme preparations for industrial production of short-chain fructooligosaccharides. *Journal of Molecular Catalysis B: Enzymatic, 76*, 44-51.

Young, F. E., & Jones, F. T. (1949). Sucrose hydrates: The sucrose-water phase diagram. *Journal of Physical & Colloid Chemistry, 53*, 1334-1350.

3.2 **Continuous production of fructooligosaccharides and invert sugar by chitosan immobilized enzymes: comparison between fluidized and packed bed reactors**

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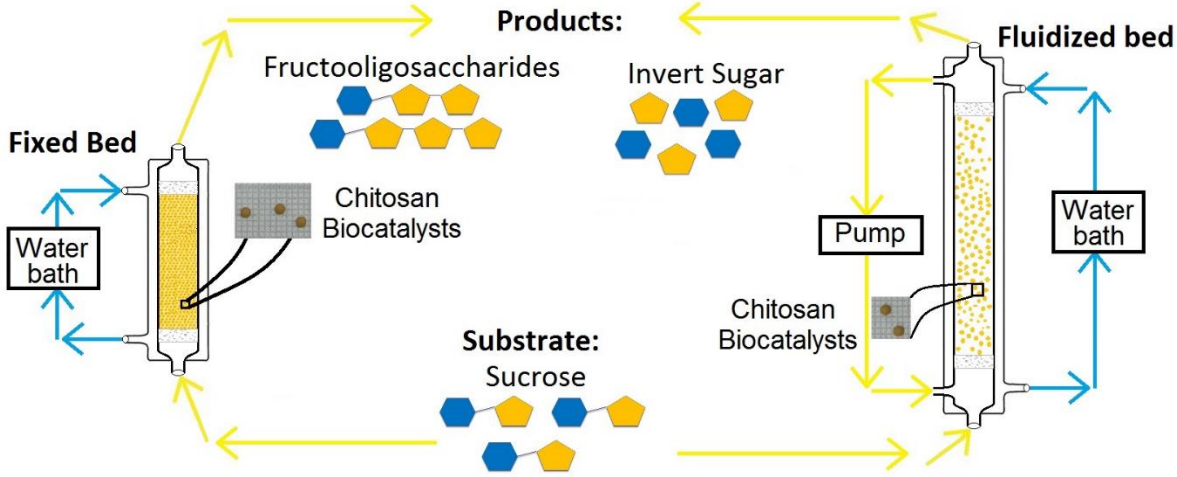
Abstract:

In this work, β-fructofuranosidase and β-fructosyltransferase were covalently immobilized on chitosan spheres, using glutaraldehyde as a coupling agent, in order to produce invert sugar and fructooligosaccharides (FOS). Maxinvert L was used to make β-fructofuranosidase biocatalyst yielding 7000 U/g. A partial purified β-fructosyltransferase from Viscozyme L was used to prepare the other biocatalyst yielding 1500 U/g. The production of invert sugar and FOS was evaluated using different continuous enzymatic reactors: two packed bed reactors (PBR) and two fluidized bed reactors (FBR). The invert sugar production achieved a yield of 98 % (grams of product per grams of initial sucrose) in the PBR and 94 % in the FBR, whereas FOS production achieved a yield of 59 % in the PBR and 54 % in the FBR. The operational stability of FOS produced in the PBR was evaluated for 40 days showing no loss of activity.

Keywords: Invert Sugar, Fructooligosaccharides, Packed bed enzyme reactor, Fluidized bed enzyme reactor, Chitosan, Enzyme Immobilization.

Graphical abstract:



1 Introduction

The commercial viability of industrial biotransformations is heavily dependent on the cost of the enzyme. Industrial applications of enzymes are often hampered by the lack of long-term operational stability, difficult recovery and reuse of the enzyme. These drawbacks can be overcome by immobilization of the enzyme (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente & Rodrigues, 2011; Sheldon & Van Pelt, 2013).

Notwithstanding all these advantages compared to the free enzymes, immobilized enzymes usually have their activities reduced, producing unfavorable effects on their overall catalytic performances. This alteration can result from mass transfer limitations, which may be reduced by applying appropriate reactor designs and immobilization techniques (Krajewska, 2004; Liese & Hilterhaus, 2013). Among immobilization techniques, the use of chitosan [(1→4)-2-amino-2-deoxy-β-D-glucan] as a support for covalent attachment is widely used for a multiplicity of purposes, mostly in industrial applications. This fact is due to several qualities of chitosan, such as the presence of reactive functional groups for direct reactions and for chemical modifications resulting in high affinity to proteins, hydrophilicity, good biocompatibility, non-toxicity, improved resistance to chemical degradation and ease of preparation in a variety of physical forms (Krajewska, 2004; Ravi Kumar, 2000). Recently, several works reported the development of packed bed or fluidized bed reactors filled with chitosan biocatalysts for industrial purposes (Klein et al., 2013; Schöffer, Klein, Rodrigues & Hertz, 2013; Yewale, Singhal & Vaidya, 2013; Zhou, Chen & Yan, 2014).

Packed bed reactors (PBR) consist of an immobile stack of particles within a column, through which a reactant solution is pumped. These reactors are preferred over other reactor types because of simpler technology, high bed volume, ease of operation, and scaling up (Hama et al., 2011; Jakobsen, 2008). Fluidized bed reactors (FBR) consist of particles within a column, which are kept in movement (fluidization), by the liquid flow. The particles are retained by a hydrodynamic balance between gravity and drag forces promoted by the upflow substrate stream (Illanes & Altamirano, 2008; van Zessen, Tramper, Rinzema & Beeftink, 2005).

Both types of reactors enable continuous production without the need of a prior separation of the enzyme resulting in lower process costs (Klein et al., 2013; van Zessen et al., 2005). In comparison to PBR, FBR are less susceptible to column clogging, formation of preferential flow paths and compression due to bed weigh (Gòdia & Solà, 1995; Hama et al., 2011). FBR also present higher axial dispersions than PBR. On the other hand, PBR present

lower shear stress on immobilized enzymes, generally leading to long-term enzyme stability (Hama et al., 2011).

Invertase (or β-fructofuranosidase, E.C. 3.2.1.26) is a suitable enzyme for carriyng out studies concerning enzymatic processes due to its stability, no need for any cofactors and its commercial significance (Danisman, Tan, Kacar & Ergene, 2004; Milovanović, Božić & Vujčić, 2007). This enzyme is used for sucrose (β-D-fructofuranosyl(2→1)β-D-glucopyranoside) hydrolysis, resulting in an equimolar mixture of glucose (α-D-glucose) and fructose (β-D-fructose) known as invert sugar. This product is widely used in food and beverage industries due to its higher sweetness and lower susceptibility to crystallization (Kotwal & Shankar, 2009). However, the application of immobilized invertase in enzyme reactors for sucrose hydrolysis, on the industrial scale, is still in the development phase (Andjelković, Pićurić & Vujčić, 2010).

β-fructosyltransferase (E.C.2.4.1.9) is another suitable enzyme for studying the reactor design in enzymatic processes. This enzyme is used for short chain fructooligosaccharides (FOS) synthesis from sucrose. FOS are prebiotic food ingredients (Roberfroid, 2007), and short chain FOS present sweet taste and may be used as a sweetener by diabetics (Mabel, Sangeetha, Platel, Srinivasan & Prapulla, 2008). Although the enzymatic synthesis of FOS also requires sucrose as substrate, the concentration of sucrose required is higher than for sucrose hydrolysis. This solution is more viscous, and the use of a highly viscous liquid in PBR may lead to the fatal problem of column clogging (Hama et al., 2011), therefore this effect must be evaluated.

In this context, the aim of this work is to evaluate the best reactor design to be used for invert sugar and FOS production catalyzed by immobilized invertase and β-fructosyltransferase, respectively. Both enzymes have been recently immobilized in chitosan particles yielding stable biocatalysts (Lorenzoni, Aydos, Klein, Rodrigues & Hertz, 2014; Valerio, Alves, Klein, Rodrigues & Hertz, 2013). Packed-bed and Fluidized-bed reactors were compared for sucrose hydrolysis and FOS synthesis.

2 Experimental

2.1 Materials

Invertase from *Saccharomyces cerevisiae* (Maxinvert L 10000) was kindly donated by DSM Food Specialties (The Netherlands). Commercial enzyme preparation from *Aspergillus aculeatus* with β-fructosyltransferase activity (Viscozyme L), produced by Novozymes, was kindly donated by LNF Latino Americana (Brazil). Chitosan from shrimp shells (>75%

deacetylated) was purchased from Sigma–Aldrich (USA). Sucrose and glutaraldehyde 25 % were purchased from Vetec Química Fina Ltda (Brazil). Enzymatic glucose (D-glucose) determination kit was purchased from In Vitro Diagnóstica Ltda (Brazil). All other chemicals were analytical or HPLC grade obtained from readily available commercial sources.

2.2 Enzymatic activity

The enzymatic hydrolytic activities of Maxinvert L (invertase) and Viscozyme L (β-fructosyltransferase) were evaluated using a substrate solution of 100 g/L of sucrose in 50 mM sodium acetate buffer at 50 ºC. The pH of the buffer was 4.5 for invertase and 5.5 for β-fructosyltransferase. Samples were taken at regular intervals and the reaction was stopped by the addition of 0.1 M sodium carbonate buffer, pH 10.0. Glucose released was measured with the enzymatic glucose determination kit. One hydrolytic unit of enzymatic activity (U) was defined as the amount of enzyme that produces 1 μmol of glucose per minute at test conditions. All the activities of immobilized enzymes were carried out under agitation. Protein content of the enzyme solutions was determined by the Lowry assay.

2.3 Chitosan spheres synthesis

The chitosan particles were prepared adding dropwise a chitosan solution into an alkaline coagulation solution, previously described by Klein *et al.* (2012). The prepared particles had a spherical shape with a diameter of approximately 2 mm and a dry weight of 0.343 mg ± 0.008 mg per sphere.

Activation of chitosan particles was carried out incubating the chitosan particles with glutaraldehyde solution as it was previously described in Lorenzoni *et al.* (2014).

2.4 Enzyme Immobilization

Invertase immobilization was carried out incubating the particles with a solution of enzyme diluted in activity buffer (50 mM sodium acetate, pH 4.5) at room temperature under gentle shaking for 3 h. The amount of enzyme applied to the support was 50 mg per gram of support.

β-fructosyltransferase, was partially purified and immobilized as previously described by Lorenzoni *et al.* (2014). The amount of enzyme applied to the support was 180 mg per gram of support.

After immobilization, both biocatalysts were washed with buffer NaCl (1.0 M) and ethylene glycol (30%, volume fraction) in order to eliminate non-covalently bonded enzymes. The immobilization yield and immobilization efficiency were calculated by the same equations previously described in Valerio *et al.* (2013), and Sheldon and Van Pelt (2013).

2.5 Reactors setup

The reactors consisted of a water-jacketed glass column, each filled with 930 chitosan spheres (280 mg of dry weight). Both enzymatic preparations were evaluated in packed bed and fluidized bed reactors. The reactors were flow rate controlled with adjustable peristaltic pumps.

The packed bed column (height, 85 mm; inner diameter, 10 mm; volume, 6.67 mL) has an entrance at the bottom and an exit at the top, which were fitted with a sintered glass disc to retain the particles within the column. The fluidized bed column (height, 230 mm; inner diameter, 10 mm; volume, 18.0 mL) was equipped with one additional exit at the top and one additional entrance at the bottom for external liquid recirculation, also fitted with sintered glass disc. This design enables fluidization of the biocatalysts independently of the residence time. The bed height was kept constant at 230 mm. Schematics of reactors configurations are illustrated in Figure 1.

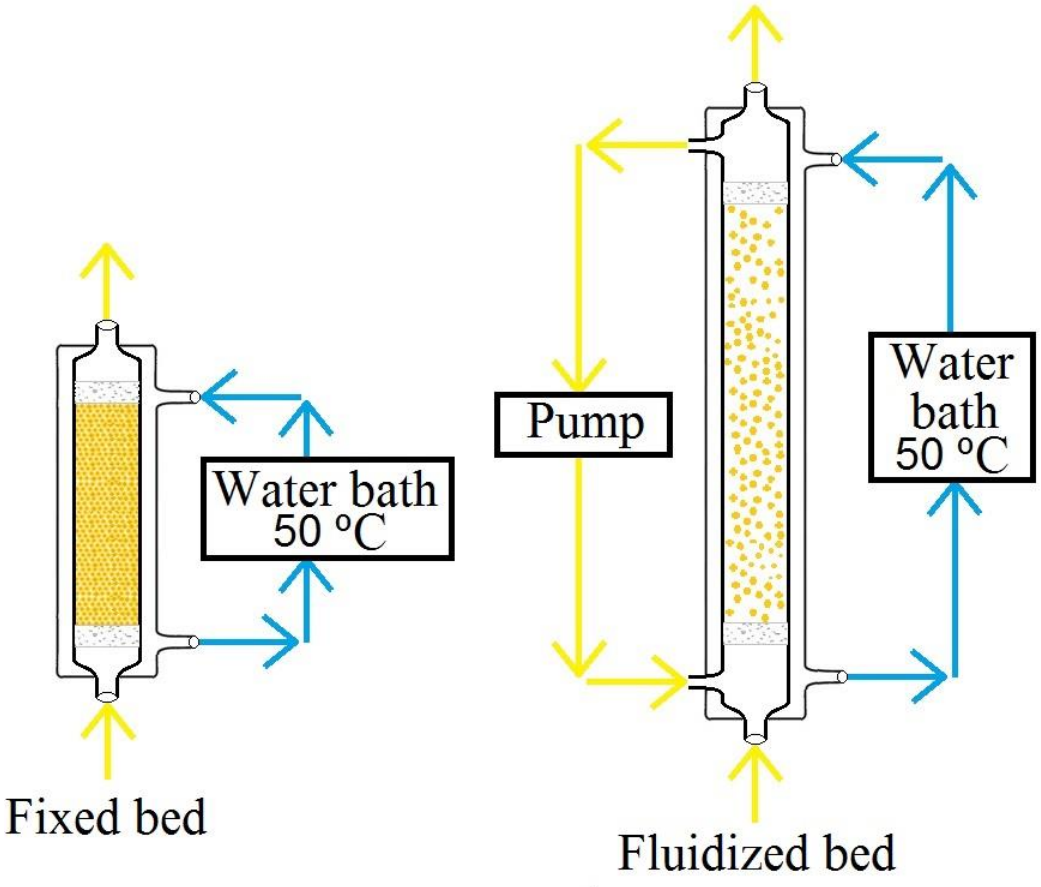


Figure 1 Schematics of reactors configurations.

2.5.1 *Invert sugar production*

Production of invert sugar was carried using a solution of sucrose 150 g/L diluted in 50 mM sodium acetate buffer pH 4.5. The solution was pumped at flow rates of (0.5, 1.0, 1.5,

2.0, 2.5, 3.0, 3.5, 4.0, 4.7) mL/min in both fluidized and packed bed reactors. Four bed volumes of solution were passed through the column before taking the sample to achieve stationary state. These experiments were carried out in duplicate, changing the column bed at each experiment.

2.5.2 *Fructooligosaccharides production*

Production of fructooligosaccharides was carried using a solution of sucrose 600 g/L diluted in 20 mM sodium acetate buffer pH 5.5. The solution was pumped at flow rates of (0.052, 0.082, 0.11, 0.17, 0.23, 0.29, 0.44) mL/min in both fluidized and packed bed reactors. Four bed volumes of solution were passed through the column before taking the sample to achieve stationary state and the experiments were carried out in duplicate, changing the column bed at each experiment.

2.5.3 *HPLC analysis*

All the samples collected in the reactors were analyzed in HPLC system (Shimadzu, Tokyo, Japan) equipped with refractor index and Aminex HPX-87C (300 mm x 7.8 mm) column. Ultra-pure water was used as eluting solvent at a flow rate of 0.6 mL/min, at 85 ºC.

The concentration of saccharides (sucrose, glucose and fructose) was determined in invert sugar samples by interpolation using external standards. In the fructooligosaccharides samples, kestose (β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1)α-D-glucopyranoside) and nystose (β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1) α-D-glucopyranoside) concentrations were also determined.

3 Results and Discussion

3.1 Enzymatic activity

Maxinvert L contains a specific hydrolytic activity of 3000 U/mg at 50 ºC and pH 4.5. The enzymatic solution obtained from partial purification of Viscozyme L, contained specific hydrolytic activity of 25.3 U/mg, at 50 ºC and pH 5.5. It is important to note that the specific activity of the enzyme solution used in this work, after partially purification is much lower compared to Maxinvert L. This difference is due to the fact that Maxinvert L is a commercial enzyme preparation specific for sucrose hydrolysis, whereas Viscozyme L is marketed as endo-β-glucanase that hydrolyzes (1→3)- or (1→4)- linkages in β-D-glucans, and the secondary activity recovered in this preparation is more specific for β-fructosyltransferase activity. Is important to note, despite its high hydrolytic activity, Maxinvert L contains little β-fructosyltransferase activity and therefore is not suitable for FOS synthesis (Farine et al., 2001; Vega-Paulino & Zúniga-Hansen, 2012).

3.2 Enzyme Immobilization

Maxinvert L immobilization resulted in a biocatalyst with 2.4 U per sphere (7000 U per gram of dry support), immobilization yield of 42 % and an immobilization efficiency of 12 %. β-fructosyltransferase immobilization resulted in a biocatalyst with 0.52 U per sphere (1500 U per gram of dry support), immobilization yield of 82 % and an immobilization efficiency of 42 %. A higher immobilization yield was observed for β-fructosyltransferase than for Maxinvert L, despite the higher protein load applied, but several other factors may cause this result. Different immobilization pH may change immobilization yields when using glutaraldehyde as a coupling agent (Barbosa et al., 2014; Monsan, 1978). In this case, the optimal pH of each enzyme was used for immobilization to avoid protein inactivation during this step. The difference between the enzymes structures can also interfere in enzyme immobilization; Maxinvert L is a highly glycosylated octameric protein with 428 kDa (Sainz-Polo et al., 2013), while β-fructosyltransferase from *A. aculeatus* is a dimeric glycoprotein with 135 kDa (Ghazi et al., 2007). However, both enzymes are not completely purified and is not possible to assert something without knowing all other proteins in the mixture.

A β-fructosyltransferase biocatalyst with a better activity than in a previous work was obtained (Lorenzoni et al., 2014). This fact can be attributed to the smaller size of spheres used in this work, since smaller particles have a greater superficial area and therefore can carry a higher protein load (Klein et al., 2012). A remarkable achievement since that biocatalyst was among the highest active found in literature so far (Lorenzoni et al., 2014).

3.4 Invert sugar and Fructooligosaccharides production

Results of yields of invert sugar production (grams of invert sugar per grams of initial sucrose) and FOS production (grams of FOS per grams of initial sucrose) for different flow rates are shown in Figure 2 and 3, respectively.

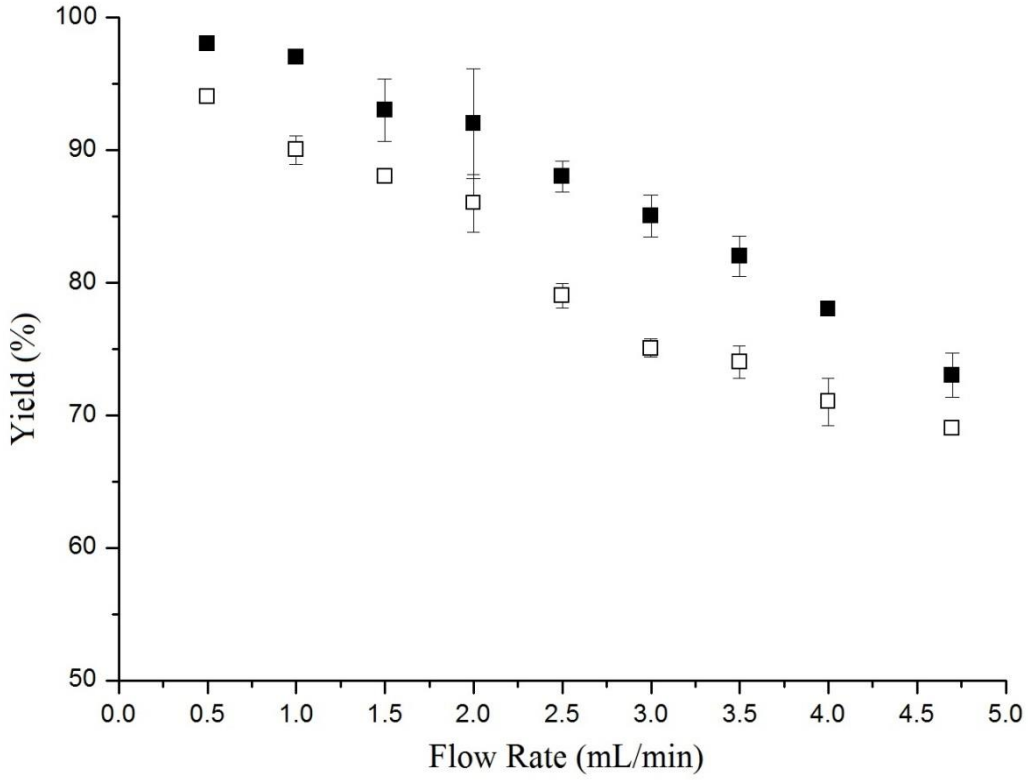


Figure 2 Yields of invert sugar production on packed bed (■) and fluidized bed (□) at pH 4.5, 50 ºC and 150 g/L of sucrose.

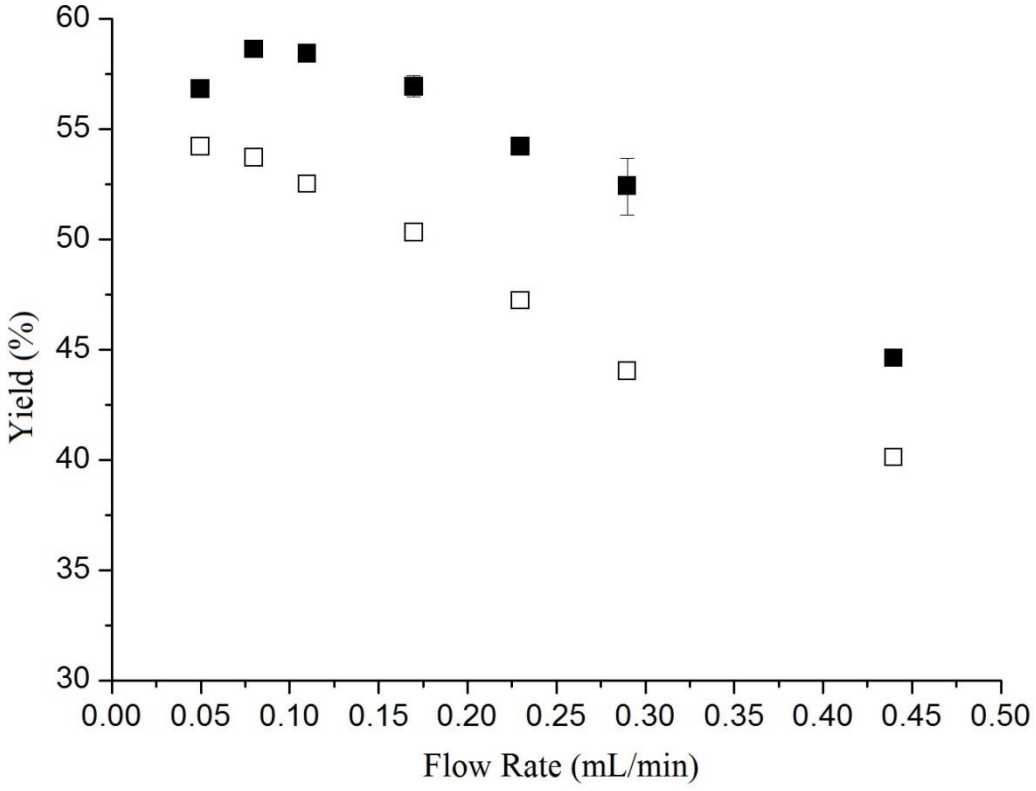


Figure 3 Yields of FOS production on packed bed (■) and fluidized bed (□) at pH 5.5, 50 ºC and 600 g/L of sucrose.

Packed bed reactors showed higher yields than fluidized bed in both reactions. For invert sugar production, the highest yields were observed at the lowest flow rates due to higher residence times inside the reactor.

For FOS production, the best yield was 59 %, at 0.08 mL/min, and at 0.05 mL/min the yield decreased to 57 %. This fact is due to the higher hydrolytic activity of the enzyme in the presence of nystose (Vega & Zuniga-Hansen, 2014). In addition, a higher nystose concentration 240 g/L ± 2 g/L and a lower kestose concentration 101 g/L ± 2 g/L were detected in the samples collected at 0.05 mL/min. At 0.08 mL/min, the nystose and kestose concentrations were 218 g/L ± 2 g/L and 133 g/L ± 1 g/L respectively. Both conditions could be chosen to a further scale up, although their different product composition.

To discuss these results, is important to consider immobilized enzyme systems as being two-phase systems, and thus, they suffer from inevitable mass transfer limitations, producing unfavorable effects on their overall catalytic performances (Krajewska, 2004). Since fluidized bed reactors present better axial dispersion rates, it is reasonable to predict that they will result in better yields in comparison to packed bed reactors. However, in this work, the packed bed reactor was kinetically favorable resulting in better yields for both reactions evaluated. A possible reason is the fact that in both reactions glucose is produced, which, in turn, causes product inhibition (Michaelis & Menten, 1913; Vega & Zuniga-Hansen, 2014). In the fluidized bed, the product is pumped from the exit, on the top of the reactor, to the entrance, in the bottom of the reactor. This system leads to higher glucose concentrations in the bottom. Therefore, glucose concentration in the bottom of the fluidized bed is higher than in packed bed, inhibiting enzymatic activity. This fact would not be noted on a non-recirculating fluidized bed, but in such case, a higher flow rate would be needed for particles suspension leading to lower residence times and lower yields.

Despite kinetically better, packed bed reactors can have some drawbacks in comparison to fluidized bed, such as column clogging, creation of preferential flow paths and particle compression (Gòdia & Solà, 1995). These drawbacks occur after some operation time, thus to evaluate them we measured the operational stability of the packed bed reactor for FOS production. The FOS production medium is also much more viscous than invert sugar production medium as well as the susceptibility to clogging. Operational stability was evaluated for 40 days at the flow rate of 0.15 mL/min and no loss of activity was detected, as shown in Figure 4.

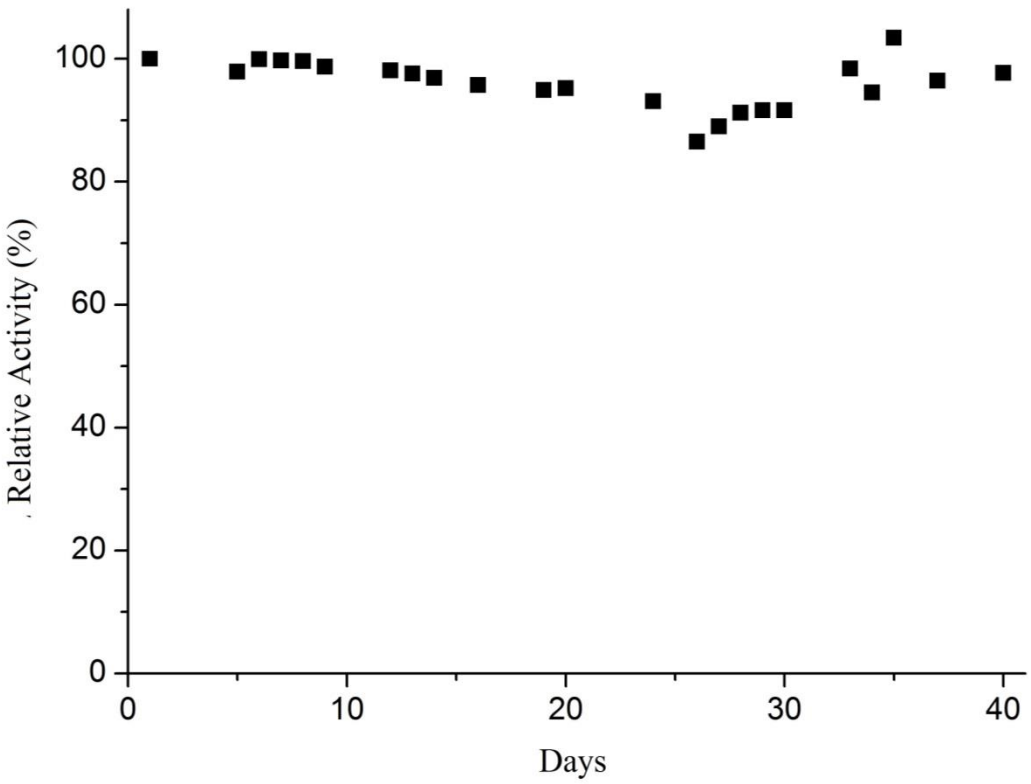


Figure 4 Yields of FOS production in the packed bed operated continuously for 40 days at 50 ºC, pH 5.5 and 600 g/L of sucrose.

4 Conclusions

The evaluation of reactor design for enzymatic production of invert sugar and FOS has proved that packed bed reactors are the best choice for the synthesis of those products. Packed bed reactors are not only kinetically favorable but also present a simpler technology and requires smaller volumes. A high operational stability was observed, no column clogging or preferential flows were detected on the packed bed reactor after 40 days of operation for FOS production. The packed bed reactors made in this work presents great potential for industrial production of either fructooligosaccharides or invert sugar from sucrose.

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References:

Andjelković, U., Pićurić, S., & Vujčić, Z. (2010). Purification and characterisation of Saccharomyces cerevisiae external invertase isoforms. *Food Chemistry, 120*, 799-804.

Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Torres, R., Rodrigues, R. C., & Fernandez-Lafuente, R. (2014). Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. *RSC Advances, 4*, 1583-1600.

Danisman, T., Tan, S., Kacar, Y., & Ergene, A. (2004). Covalent immobilization of invertase on microporous pHEMA–GMA membrane. *Food Chemistry, 85*, 461-466.

Farine, S., Versluis, C., Bonnici, P. J., Heck, A., L'Homme, C., Puigserver, A., & Biagini, A. (2001). Application of high performance anion exchange chromatography to study invertase-catalysed hydrolysis of sucrose and formation of intermediate fructan products. *Applied Microbiology and Biotechnology, 55*, 55-60.

Garcia-Galan, C., Berenguer-Murcia, A., Fernandez-Lafuente, R., & Rodrigues, R. C. (2011). Potential of different enzyme immobilization strategies to improve enzyme performance. *Advanced Synthesis and Catalysis, 353*, 2885-2904.

Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Ferrer, M., Ballesteros, A., & Plou, F. J. (2007). Purification and kinetic characterization of a fructosyltransferase from Aspergillus aculeatus. *Journal of Biotechnology, 128*, 204-211.

Gòdia, F., & Solà, C. (1995). Fluidized-Bed Bioreactors. *Biotechnology Progress, 11*, 479--497.

Hama, S., Tamalampudi, S., Yoshida, A., Tamadani, N., Kuratani, N., Noda, H., Fukuda, H., & Kondo, A. (2011). Enzymatic packed-bed reactor integrated with glycerol-separating system for solvent-free production of biodiesel fuel. *Biochemical Engineering Journal, 55*(1), 66-71.

Illanes, A., & Altamirano, C. (2008). Enzyme Reactors. *Enzyme Biocatalysis* (pp. 205-251): Springer Netherlands.

Jakobsen, H. (2008). Fluidized Bed Reactors. *Chemical Reactor Modeling* (pp. 867-952): Springer Berlin Heidelberg.

Klein, M. P., Fallavena, L. P., Schöffer, J. d. N., Ayub, M. A. Z., Rodrigues, R. C., Ninow, J. L., & Hertz, P. F. (2013). High stability of immobilized β-d-galactosidase for lactose hydrolysis and galactooligosaccharides synthesis. *Carbohydrate Polymers, 95*, 465-470.

Klein, M. P., Nunes, M. R., Rodrigues, R. C., Benvenutti, E. V., Costa, T. M. H., Hertz, P. F., & Ninow, J. L. (2012). Effect of the support size on the properties of β-galactosidase immobilized on chitosan: Advantages and disadvantages of macro and nanoparticles. *Biomacromolecules, 13*, 2456-2464.

Kotwal, S. M., & Shankar, V. (2009). Immobilized invertase. *Biotechnology Advances, 27*, 311-322.

Krajewska, B. (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and Microbial Technology, 35*, 126-139.

Liese, A., & Hilterhaus, L. (2013). Evaluation of immobilized enzymes for industrial applications. *Chemical Society Reviews, 42*, 6236-6249.

Lorenzoni, A. S. G., Aydos, L. F., Klein, M. P., Rodrigues, R. C., & Hertz, P. F. (2014). Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from Aspergillus aculeatus. *Carbohydrate Polymers, 103*, 193-197.

Mabel, M. J., Sangeetha, P. T., Platel, K., Srinivasan, K., & Prapulla, S. G. (2008). Physicochemical characterization of fructooligosaccharides and evaluation of their suitability as a potential sweetener for diabetics. *Carbohydrate Research, 343*, 56-66.

Michaelis, L., & Menten, M. L. (1913). Die kinetik der invertinwirkung. *Biochemische Zeitschrift, 49*, 333-369.

Milovanović, A., Božić, N., & Vujčić, Z. (2007). Cell wall invertase immobilization within calcium alginate beads. *Food Chemistry, 104*, 81-86.
Monsan, P. (1978). Optimization of glutaraldehyde activation of a support for enzyme immobilization. *Journal of Molecular Catalysis, 3*, 371-384.
Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers, 46*, 1-27.
Roberfroid, M. (2007). Prebiotics: The Concept Revisited. *The Journal of Nutrition, 137*, 830S-837S.
Sainz-Polo, M. A., Ramirez-Escudero, M., Lafraya, A., Gonzalez, B., Marin-Navarro, J., Polaina, J., & Sanz-Aparicio, J. (2013). Three-dimensional structure of Saccharomyces invertase: role of a non-catalytic domain in oligomerization and substrate specificity. *J Biol Chem, 288*, 9755-9766.
Schöffer, J. d. N., Klein, M. P., Rodrigues, R. C., & Hertz, P. F. (2013). Continuous production of β-cyclodextrin from starch by highly stable cyclodextrin glycosyltransferase immobilized on chitosan. *Carbohydrate Polymers, 98*, 1311-1316.
Sheldon, R. A., & Van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews, 42*, 6223-6235.
Valerio, S. G., Alves, J. S., Klein, M. P., Rodrigues, R. C., & Hertz, P. F. (2013). High operational stability of invertase from Saccharomyces cerevisiae immobilized on chitosan nanoparticles. *Carbohydrate Polymers, 92*, 462-468.
van Zessen, E., Tramper, J., Rinzema, A., & Beeftink, H. H. (2005). Fluidized-bed and packed-bed characteristics of gel beads. *Chemical Engineering Journal, 115*, 103-111.
Vega, R., & Zuniga-Hansen, M. E. (2014). A new mechanism and kinetic model for the enzymatic synthesis of short-chain fructooligosaccharides from sucrose. *Biochemical Engineering Journal, 82*, 158-165.
Vega-Paulino, R. J., & Zúniga-Hansen, M. E. (2012). Potential application of commercial enzyme preparations for industrial production of short-chain fructooligosaccharides. *Journal of Molecular Catalysis B: Enzymatic, 76*, 44-51.
Yewale, T., Singhal, R. S., & Vaidya, A. A. (2013). Immobilization of inulinase from Aspergillus niger NCIM 945 on chitosan and its application in continuous inulin hydrolysis. *Biocatalysis and Agricultural Biotechnology, 2*, 96-101.
Zhou, G.-x., Chen, G.-y., & Yan, B.-b. (2014). Biodiesel production in a magnetically-stabilized, fluidized bed reactor with an immobilized lipase in magnetic chitosan microspheres. *Biotechnology Letters, 36*, 63-68.

4. Considerações Finais:

A imobilização de β-frutosiltransferase em partículas de quitosana ativadas com glutaraldeído resultou em um biocatalisador ativo e muito estável. A atividade do biocatalisador obtido foi de 1230 U_t/g (unidades de transfrutosilação por grama de suporte seco), estando entre os melhores encontrados na literatura para produção de fruto-oligossacarídeos. Além de aumentar a estabilidade térmica a estabilidade operacional foi surpreendente, podendo ser utilizado em 50 bateladas sem perda de atividade. Seu potencial para utilização na indústria foi testado em um segundo trabalho avaliando o uso em reatores.

No segundo trabalho, a aplicação do biocatalisador com atividade de β-frutosiltransferase em reatores para produção de fruto-oligossacarídeos testada em reator de leito fixo e leito fluidizado, mostrou que o reator de leito fixo é mais vantajoso. Além de ocupar menos espaço e ser mais simples de operar, o reator de leito fixo apresentou maior rendimento, 59 % frente aos 54 % do reator de leito fluidizado. Efeitos como o entupimento da coluna e a criação de caminhos preferenciais foram testados ao longo de sua operação contínua durante 40 dias, a manutenção da atividade durante este período mostrou que o sistema é muito estável.

A produção de açúcar invertido em reatores enzimáticos também foi testada utilizando a enzima comercial Maxinvert L que possui alta atividade de β-frutofuranosidase. O processo de imobilização covalente em esferas de quitosana resultou em um biocatalisador com 7000 U/g (unidades de hidrólise por grama de suporte seco). Testes em reatores de leito fixo e leito fluidizado mostraram que para a produção de açúcar invertido o reator de leito fixo também é mais favorável resultando em 98 % de rendimento frente aos 94 % de rendimento do reator de leito fluidizado.

5. Perspectivas:

Como perspectivas futuras desse trabalho, no âmbito mais tecnológico, pode-se realizar testes para avaliar a possível presença de resíduos de glutaraldeído nos produtos obtidos pelos biocatalisadores. Em caso negativo poderia ser avaliado um aumento de escala objetivando aplicações industriais. Em caso positivo poderia ser testado o uso de genipina como agente de ligação entre enzima e suporte uma vez que a toxicidade da genipina é muito inferior à do glutaraldeído.

Pode-se também realizar experimentos envolvendo mutagênese para modificar a enzima β-frutosiltransferase de *A. aculeatus* de forma a obter proteínas mais estáveis de fácil purificação e imobilização. Esta mesma abordagem pode ser utilizada para a obtenção de uma invertase mutante de *S. cerevisae* com alta atividade de transfrutosilação e alta estabilidade.

O desenvolvimento de reatores enzimáticos utilizando mais de uma enzima também é uma possibilidade de estudo. O uso de uma β-frutosiltransferase em conjunto com uma glicose isomerase poderia reduzir o efeito de inibição competitiva com a glicose. Também, o uso de β-frutosiltransferase, glicose isomerase e inulinase em conjunto, poderiam proporcionar altos rendimentos de produção de FOS, uma vez que a β-frutosiltransferase produz glicose como subproduto, a glicose isomerase transforma a glicose em frutose e algumas inulinases são capazes de produzir FOS com frutose.

6. Referências:

AGUIAR-OLIVEIRA, E. **Estudos sobre as propriedades catalíticas da frutosiltransferase de *Rhodotorula* sp. livre e imobilizada em suporte inorgânico.** 2012. 209f. Tese (Doutorado em Engenharia de Alimentos) – Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas. 2012.

ÁLVARO-BENITO, M. et al. Characterization of a β-fructofuranosidase from Schwanniomyces occidentalis with transfructosylating activity yielding the prebiotic 6-kestose. **Journal of Biotechnology,** v. 132, n. 1, p. 75-81, 2007. ISSN 0168-1656. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0168165607014198>. Acesso em 20, Fev. 2014.

BENKEBLIA, N. Fructooligosaccharides and fructans analysis in plants and food crops. **Journal of Chromatography A,** v. 1313, n. 0, p. 54-61, 2013. ISSN 0021-9673. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0021967313012429>. Acesso em 20, Fev. 2014.

BLATT, W. F. et al. A modified ultrafiltration cell for separating the products of proteolysis. **Analytical Biochemistry,** v. 22, n. 1, p. 161-165, 1968. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-0014232254&partnerID=40&md5=7892f30f5e0896c11f7852c57db00da0>. Acesso em 20, Fev. 2014.

BORNSCHEUER, U. T. et al. Engineering the third wave of biocatalysis. **Nature,** v. 484, n. 7397, p. 185-194, 2012. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-84860741240&partnerID=40&md5=7b4370e852f543a22ec7c610091d52d9>. Acesso em 20, Fev. 2014.

BÓDALO, A. et al. Ultrafiltration membrane reactors for enzymatic resolution of amino acids: design model and optimization. **Enzyme and Microbial Technology,** v. 28, n. 4–5, p. 355-361, 2001. ISSN 0141-0229. Disponível em: <http://www.sciencedirect.com/science/article/pii/S014102290000329X>. Acesso em 20, Fev. 2014.

CAO, L. Introduction: Immobilized Enzymes: Past, Present and Prospects. p. 1-52, 2006c. ISSN 9783527607662. Disponível em: <http://dx.doi.org/10.1002/3527607668.ch1>. Acesso em 20, Fev. 2014.

_____. Covalent Enzyme Immobilization. p. 169-316, 2006b. ISSN 9783527607662. Disponível em: <http://dx.doi.org/10.1002/3527607668.ch3>. Acesso em 20, Fev. 2014.

_____. Adsorption-based Immobilization. p. 53-168, 2006a. ISSN 9783527607662. Disponível em: <http://dx.doi.org/10.1002/3527607668.ch2>. Acesso em 20, Fev. 2014.

CAO, L.; LANGEN, L. V.; SHELDON, R. A. Immobilised enzymes: carrier-bound or carrier-free? **Current Opinion in Biotechnology,** v. 14, n. 4, p. 387-394, 2003. ISSN 0958-1669. Disponível em: <http://www.sciencedirect.com/science/article/pii/S095816690300096X>. Acesso em 20, Fev. 2014.

CAO, L.; VAN RANTWIJK, F.; SHELDON, R. A. Cross-linked enzyme aggregates: A simple and effective method for the immobilization of penicillin acylase. **Organic Letters,** v. 2, n. 10, p. 1361-1364, 2000. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-0034682159&partnerID=40&md5=ea6d838e09f1ed07700c79482c8e0e1a>. Acesso em 20, Fev. 2014.

CHEN, W.-C.; LIU, C.-H. Production of β-fructofuranosidase by Aspergillus japonicus. **Enzyme and Microbial Technology,** v. 18, n. 2, p. 153-160, 1996. ISSN 0141-0229. Disponível em: <http://www.sciencedirect.com/science/article/pii/0141022995000992>.

CHIOU, S.-H.; WU, W.-T. Immobilization of Candida rugosa lipase on chitosan with activation of the hydroxyl groups. **Biomaterials,** v. 25, n. 2, p. 197-204, 2004. ISSN 0142-9612. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0142961203004824>.

CSANÁDI, Z.; SISAK, C. Immobilization of Pectinex Ultra SP-L pectinase and its application to production of fructooligosaccharides. **Acta Alimentaria,** v. 35, n. 2, p. 205-212, 2006. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-33746003896&partnerID=40&md5=0a1a26520296ba69de0bdba2fb2700d4>.

DE ALMEIDA, A. C. S. et al. Sucrose hydrolysis catalyzed by auto-immobilized invertase into intact cells of Cladosporium cladosporioides. **Electronic Journal of Biotechnology**, Valparaíso, v. 8, n. 1, Abr. 2005. Disponível em: <http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0717-34582005000100009&lng=es&nrm=iso>. Acesso em 19 Fev. 2014.

DIANO, N. et al. Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors. **Journal of Agricultural and Food Chemistry,** v. 56, n. 23, p. 11471-11477, 2008. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-58949104770&partnerID=40&md5=ea2f2baa68e32c0e822a3a0419083f8d>. Acesso em 20, Fev. 2014.

FESSNER, W.-D. et al. Immobilized Enzymes: Methods and Applications. In: (Ed.). **Biocatalysis - From Discovery to Application**: Springer Berlin Heidelberg, v.200, 1999. p.95-126. (Topics in Current Chemistry). ISBN 978-3-540-64942-7.

FRICK, C.; SCHÜGERL, K. Continuous acetone-butanol production with free and immobilized Clostridium acetobutylicum. **Applied Microbiology and Biotechnology,** v. 25, n. 3, p. 186-193, 1986. ISSN 0175-7598. Disponível em: <http://dx.doi.org/10.1007/BF00253646>. Acesso em 20, Fev. 2014.

GARCIA-GALAN, C. et al. Potential of different enzyme immobilization strategies to improve enzyme performance. **Advanced Synthesis and Catalysis,** v. 353, n. 16, p. 2885-2904, 2011. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-81555222964&partnerID=40&md5=3306501554b28e08f1941b4945f950ef>. Acesso em 20, Fev. 2014.

GHAZI, I. et al. Immobilisation of fructosyltransferase from Aspergillus aculeatus on epoxy-activated Sepabeads EC for the synthesis of fructo-oligosaccharides. **Journal of Molecular Catalysis B: Enzymatic,** v. 35, n. 1–3, p. 19-27, 2005. ISSN 1381-1177. Disponível em: <http://www.sciencedirect.com/science/article/pii/S1381117705000627>.

_____. Purification and kinetic characterization of a fructosyltransferase from Aspergillus aculeatus. **Journal of Biotechnology,** v. 128, n. 1, p. 204-211, 2007. ISSN 0168-1656. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0168165606007905>.

GÒDIA, F.; SOLÀ, C. Fluidized-Bed Bioreactors. **Biotechnology Progress,** v. 11, n. 5, p. 479-497, 1995. ISSN 1520-6033. Disponível em: <http://dx.doi.org/10.1021/bp00035a001>. Acesso em 20, Fev. 2014.

HAMA, S. et al. Enzymatic packed-bed reactor integrated with glycerol-separating system for solvent-free production of biodiesel fuel. **Biochemical Engineering Journal,** v. 55, n. 1, p. 66-71, 2011. ISSN 1369-703X. Disponível em: <http://www.sciencedirect.com/science/article/pii/S1369703X11000787>. Acesso em 20, Fev. 2014.

HERNALSTEENS, S.; MAUGERI, F. Purification and characterisation of a fructosyltransferase from Rhodotorula sp. **Applied Microbiology and Biotechnology,** v. 79, n. 4, p. 589-596, 2008. ISSN 0175-7598. Disponível em: <http://dx.doi.org/10.1007/s00253-008-1470-x>.

_____. Partial Purification and Characterization of Extracellular Fructofuranosidase with Transfructosylating Activity from Candida sp. **Food and Bioprocess Technology,** v. 3, n. 4, p. 568-576, 2010. ISSN 1935-5130. Disponível em: <http://dx.doi.org/10.1007/s11947-008-0089-3>.

ILLANES, A. Enzyme Production. In: (Ed.). **Enzyme Biocatalysis**: Springer Netherlands, 2008. p.57-106. ISBN 978-1-4020-8360-0.

ILLANES, A.; ALTAMIRANO, C. Enzyme Reactors. In: (Ed.). **Enzyme Biocatalysis**: Springer Netherlands, 2008. p.205-251. ISBN 978-1-4020-8360-0.

JAKOBSEN, H. Fluidized Bed Reactors. In: (Ed.). **Chemical Reactor Modeling**: Springer Berlin Heidelberg, 2008. p.867-952. ISBN 978-3-540-25197-2.

JORDAN, S. Commercial Invert Sugar. **Industrial & Engineering Chemistry,** v. 16, n. 3, p. 307-310, 1924.

KATZBAUER, B.; NARODOSLAWSKY, M.; MOSER, A. Classification system for immobilization techniques. **Bioprocess Engineering,** v. 12, n. 4, p. 173-179, 1995. ISSN 0178-515X. Disponível em: <http://dx.doi.org/10.1007/BF01767463>. Acesso em 20, Fev. 2014.

KASUMI, T. et al. Preparation and some properties of chitosan bound enzymes. **Agricultural and Biological Chemistry,** v. 41, n. 10, p. 1865-1872, 1977. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-0017743343&partnerID=40&md5=6365c964f9764f4cbcec9abe7156b433>.

KLEIN, M. P. et al. Effect of the support size on the properties of β-galactosidase immobilized on chitosan: Advantages and disadvantages of macro and nanoparticles. **Biomacromolecules,** v. 13, n. 8, p. 2456-2464, 2012. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-84864966807&partnerID=40&md5=6010076dae421549b5aefc8eeb763087>.

KLEIN, M. P. et al. High stability of immobilized β-d-galactosidase for lactose hydrolysis and galactooligosaccharides synthesis. **Carbohydrate Polymers,** v. 95, n. 1, p. 465-470, 2013. ISSN 0144-8617. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0144861713001835>.

KOTWAL, S. M.; SHANKAR, V. Immobilized invertase. **Biotechnology Advances,** v. 27, n. 4, p. 311-322, 2009. ISSN 0734-9750.

KRAJEWSKA, B. Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. **Enzyme and Microbial Technology,** v. 35, n. 2–3, p. 126-139, 2004. ISSN 0141-0229. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0141022904001231>. Acesso em 20, Fev. 2014.

LORENZONI, A. S. G. et al. Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from Aspergillus aculeatus. **Carbohydrate Polymers,** v. 103, p. 193-197, 2014. ISSN 0144-8617. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0144861713012526>. Acesso em 20, Fev. 2014.

MABEL, M. J. et al. Physicochemical characterization of fructooligosaccharides and evaluation of their suitability as a potential sweetener for diabetics. **Carbohydrate Research,** v. 343, n. 1, p. 56-66, 2008. ISSN 0008-6215. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0008621507004387>. Acesso em 20, Fev. 2014.

MATEO, C. et al. Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment. **Enzyme and Microbial Technology,** v. 26, n. 7, p. 509-515, 2000. ISSN 0141-0229. Disponível em: <http://www.sciencedirect.com/science/article/pii/S014102299900188X >.

MICHAELIS, L.; MENTEN M. L. Die kinetik der invertinwirkung. **Biochemische Zeitschrift,** v. 49, p. 333-369, 1913.

MILLER, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. **Analytical Chemistry,** v. 31, n. 3, p. 426-428, 1959.

MIGNEAULT, I. et al. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. **BioTechniques,** v. 37, n. 5, p. 790-802, 2004. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-8444230519&partnerID=40&md5=6600321ffc9a1098a16f506f071d53c9>.

MUZZARELLI, R. A. A.; BARONTINI, G.; ROCCHETTI, R. Immobilized enzymes on chitosan columns: α Chymotrypsin and acid phosphatase. **Biotechnology and Bioengineering,** v. 18, n. 10, p. 1445-1454, 1976. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-0017141854&partnerID=40&md5=00a4ea62db8a527f9c58dadecd6e7efa>.

NAZZARO, F. et al. Synbiotic potential of carrot juice supplemented with Lactobacillus spp. and inulin or fructooligosaccharides. **Journal of the Science of Food and Agriculture,** v. 88, n. 13, p. 2271-2276, 2008. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-55449111254&partnerID=40&md5=d823b4c00cf8850e9977982ee777904d>. Acesso em 20, Fev. 2014.

NEMUKULA, A. et al. Response surface methodology: Synthesis of short chain fructooligosaccharides with a fructosyltransferase from Aspergillus aculeatus. **Bioresource Technology,** v. 100, n. 6, p. 2040-2045, 2009. ISSN 0960-8524. Disponível em: <http://www.sciencedirect.com/science/article/pii/S096085240800878X>. Acesso em 20, Fev. 2014.

NGUYEN, Q. et al. Production, purification and identification of fructooligosaccharides produced by β-fructofuranosidase from Aspergillus niger IMI 303386. **Biotechnology Letters,** v. 21, n. 3, p. 183-186, 1999. ISSN 0141-5492. Disponível em: <http://dx.doi.org/10.1023/A%3A1005429525865>.

NOYORI, R. Synthesizing our future. **Nature Chemistry,** v. 1, n. 1, p. 5-6, 2009. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-67651248658&partnerID=40&md5=062f614fa0476e5beff26236fd5a3d7f>. Acesso em 20, Fev. 2014.

OKUDA, K. et al. Reaction of glutaraldehyde with amino and thiol compounds. **Journal of Fermentation and Bioengineering,** v. 71, n. 2, p. 100-105, 1991. ISSN 0922-338X. Disponível em: <http://www.sciencedirect.com/science/article/pii/0922338X91902315>.

O'SULLIVAN, C.; TOMPSON, F. W. LX. - Invertase: A contribution to the history of an enzyme or unorganised ferment. **Journal of the Chemical Society, Transactions,** v. 57, p. 834-931, 1890. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-0001675978&partnerID=40&md5=891aa9c9df73e8421fbba98729b68620>. Acesso em 20, Fev. 2014.

PASSOS, L. M. L.; PARK, Y. K. Frutooligossacarídeos: implicações na saúde humana e utilização em alimentos. **Ciência Rural**, v. 33, n. 2, 2003. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0103-84782003000200034&lng=en&nrm=iso>. Acesso em 11, Fev. 2014.

PODADERA, P. **Estudo Das Propriedades Do Açúcar Líquido Invertido Processado Com Radiação Gama E Feixe De Elétrons**. 2007. 99f. Tese (Doutorado em Ciências da Área de Tecnologia Nuclear - Aplicações) – Instituto de Pesquisas Energéticas e Nucleares, Universidade de São Paulo, São Paulo, 2007.

RAVI KUMAR, M. N. V. A review of chitin and chitosan applications. **Reactive and Functional Polymers,** v. 46, n. 1, p. 1-27, 2000. ISSN 1381-5148. Disponível em: <http://www.sciencedirect.com/science/article/pii/S1381514800000389>. Acesso em 20, Fev. 2014.

RENUKA, B. et al. Fructooligosaccharide fortification of selected fruit juice beverages: Effect on the quality characteristics. **LWT - Food Science and Technology,** v. 42, n. 5, p. 1031-1033, 2009. ISSN 0023-6438. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0023643808002892>. Acesso em 20, Fev. 2014.

SEO, D.-J. et al. Immobilization of chitinases from Streptomyces griseus and Paenibacillus illinoisensis on chitosan beads. **Carbohydrate Polymers,** v. 88, n. 1, p. 391-394, 2012. ISSN 0144-8617. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0144861711011118>.

SHELDON, R. A.; VAN PELT, S. Enzyme immobilisation in biocatalysis: Why, what and how. **Chemical Society Reviews,** v. 42, n. 15, p. 6223-6235, 2013. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-84880122011&partnerID=40&md5=aad1eceb313f192d47069436eb2ebba2>. Acesso em 20, Fev. 2014.

SCHÖFFER, J. D. N. et al. Continuous production of β-cyclodextrin from starch by highly stable cyclodextrin glycosyltransferase immobilized on chitosan. **Carbohydrate Polymers,** v. 98, n. 2, p. 1311-1316, 2013. ISSN 0144-8617. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0144861713007303>.

SUMNER, J. B.; HOWELL S. F. A method for determination of saccharase activity. **Journal of Biological Chemistry,** v. 108, n.1, p. 51-54. 1935.

TANRISEVEN, A.; ASLAN, Y. Immobilization of Pectinex Ultra SP-L to produce fructooligosaccharides. **Enzyme and Microbial Technology,** v. 36, n. 4, p. 550-554, 2005. ISSN 0141-0229. Disponível em: <http://www.sciencedirect.com/science/article/pii/S014102290400362X>.

VAAJE-KOLSTAD, G. et al. The chitinolytic machinery of Serratia marcescens – a model system for enzymatic degradation of recalcitrant polysaccharides. **FEBS Journal,** v. 280, n. 13, p. 3028-3049, 2013. ISSN 1742-4658. Disponível em: <http://dx.doi.org/10.1111/febs.12181>.

VALERIO, S. G. et al. High operational stability of invertase from Saccharomyces cerevisiae immobilized on chitosan nanoparticles. **Carbohydrate Polymers,** v. 92, n. 1, p. 462-468, 2013. ISSN 0144-8617. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0144861712009095>.

VAN ZESSEN, E. et al. Fluidized-bed and packed-bed characteristics of gel beads. **Chemical Engineering Journal,** v. 115, n. 1–2, p. 103-111, 2005. ISSN 1385-8947. Disponível em: <http://www.sciencedirect.com/science/article/pii/S1385894705003074>. Acesso em 20, Fev. 2014.

VEGA-PAULINO, R. J.; ZÚNIGA-HANSEN, M. E. Potential application of commercial enzyme preparations for industrial production of short-chain fructooligosaccharides. **Journal of Molecular Catalysis B: Enzymatic,** v. 76, p. 44-51, 2012. ISSN 1381-1177. Disponível em: <http://www.sciencedirect.com/science/article/pii/S1381117711003249>. Acesso em 20, Fev. 2014.

WOHLGEMUTH, R. Biocatalysis - key to sustainable industrial chemistry. **Current Opinion in Biotechnology,** v. 21, n. 6, p. 713-724, 2010. ISSN 0958-1669. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0958166910001850>. Acesso em 20, Fev. 2014.

Apêndice A – Ilustrações

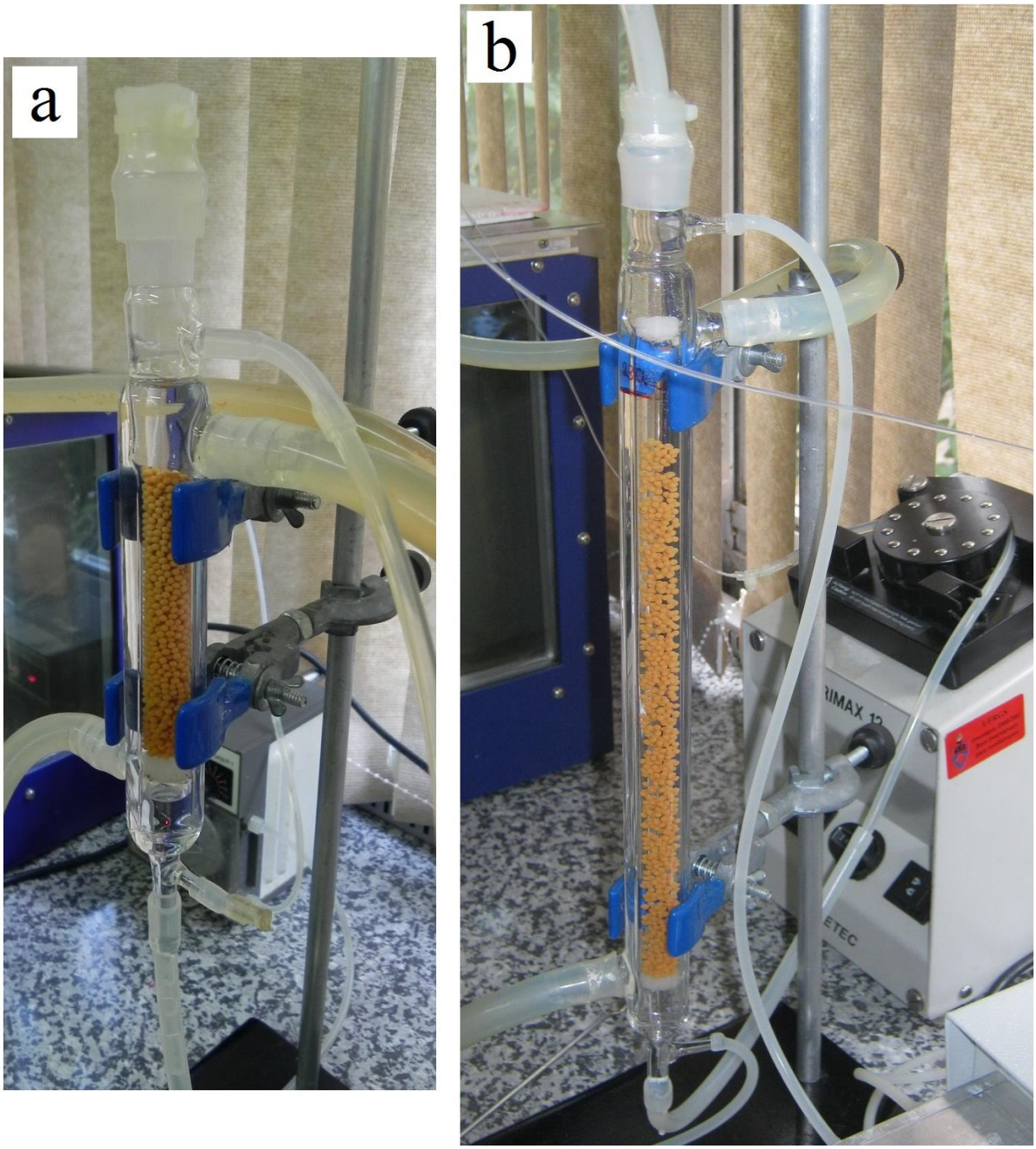

Figura 16 Reatores em operação: a Leito Fixo. b Leito Fluidizado.