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RODRIGO LIRA DE OLIVEIRA

DESENVOLVIMENTO DE PROCESSOS DE PRODUÇÃO DE FRUTO-OLIGOSSACARÍDEOS EM REATOR ENZIMÁTICO UTILIZANDO ENZIMAS COM ATIVIDADE DE TRANSFRUTOSILAÇÃO IMOBILIZADAS

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Rodrigo Lira de Oliveira

Desenvolvimento de processos de produção de fruto-oligossacarídeos em reator enzimático utilizando enzimas com atividade de transfrutosilação imobilizadas

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Comissão Julgadora da Tese para obtenção do título de Doutor

Profa. Dra. Tatiana Souza Porto – Presidente/Orientadora
Unidade Acadêmica de Garanhuns – UFRPE

Professor Dr. Adalberto Pessoa Júnior
Universidade de São Paulo – USP

Profa. Dra. Michele Rigon Spier
Universidade Federal do Paraná – UFPR

Professor Dr. Ranilson de Souza Bezerra
Universidade Federal de Pernambuco – UFPE

Profa. Dra. Maria Tereza dos Santos Correia
Universidade Federal de Pernambuco – UFPE

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LISTA DE ABREVIATURAS E SÍMBOLOS

a_w	Atividade de água, adimensional
CHP	Ponto crítico de umidade, (%)
D	Tempo de redução decimal, (min)
E^*_a	Energia de ativação, (kJ mol^{-1})
E^*_d	Energia de ativação para inativação térmica, (kJ mol^{-1})
EC	Comissão Internacional de Enzimas
FES	Fermentação em Estado Sólido
FFase	β-fructofuranosidase
FSm	Fermentação Submersa
FOS	Fruto-oligossacarídeos
FTase	Frutosiltransferase
GF_2	1-kestose
GF_3	Nistose
GF_4	β-fructofuranosil nistose
h	Constante de Planck, (J min)
K	Coeficiente de partição, adimensional
k_{cat}	Constante catalítica ou número de rotatividade (min^{-1})
k_b	Constante de Boltzmann, (J K^{-1})
K_m	Constante de Michaelis-Menten, (mM)
k_d	Constante de inativação térmica de primeira ordem, (min^{-1})
PEG	Polietilenoglicol
Q_{10}	Coeficiente de temperatura, adimensional
R	Constante universal dos gases, (J mol^{-1} K^{-1})
SDFA	Sistema de Duas Fases Aquosas
$t_{1/2}$	Tempo de meia vida, (min)
U_H	Atividade hidrolítica, (U mL^{-1})
U_{TF}	Atividade de transfrutosilação, (U mL^{-1})
V_{max}	Velocidade máxima da reação, (mM min^{-1})
WAI	Índice de Absorção de Água, (g de água/g de substrato seco)
Z	Constante de resistência térmica (°C)
ΔG^*	Energia livre de Gibbs de ativação da reação enzimática, (kJ mol^{-1})

ΔG^*_d	Energia livre de Gibbs de inativação térmica, (kJ mol^{-1})
ΔH^*	Entalpia de ativação da reação enzimática, (kJ mol^{-1})
ΔH^*_d	Entalpia de ativação de inativação térmica, (kJ mol^{-1})
ΔS^*	Entropia de ativação da reação enzimática, (J K^{-1} mol^{-1})
ΔS^*_d	Entropia de ativação da inativação térmica, (J K^{-1} mol^{-1})
ψ	Coeficiente de atividade residual, adimensional

RESUMO

Fruto-oligossacarídeos (FOS) correspondem a uma importante classe de prebióticos que apresentam diversos efeitos benéficos ao consumidor e propriedades tecnológicas adequadas para aplicação em diversos produtos alimentícios. Diante disso, o presente trabalho teve como objetivo desenvolver processo de produção de fruto-oligossacarídeos utilizando enzimas formadoras de FOS em sua forma imobilizada. Primeiramente, foram investigadas diferentes linhagens produtoras de β-frutofuranosidase (FFase) por Fermentação em Estado Sólido (FES), sendo a melhor produção observada pela linhagem *Aspergillus tamarii* URM4634 utilizando farelo de soja como substrato (U_H = 209,1 U mL^{-1} e U_{TF} = 53,9 U mL^{-1}). A enzima foi caracterizada bioquimicamente e avaliada em relação a seus parâmetros cinéticos e termodinâmicos, verificou-se que de fato a enzima se tratava de uma FFase com atividade de transfrutosilação sendo termoestável em temperaturas comumente empregadas na produção de FOS (50°C). Também foram realizados estudos envolvendo a imobilização preparação comercial com atividade de transfrutosilação Pectinex Ultra SP-L (U_H = 378,0 U mL^{-1} e U_{TF} = 301,7 U mL^{-1}) em quitosana (CS) e em nanopartículas magnéticas recobertas com quitosana (MNPs-CS). As diferentes técnicas de imobilização mostraram-se efetivas, apresentando elevados rendimentos de imobilização de 95,9% e 94,8%, em CS e MNP-CS, respectivamente. Ambos os métodos de imobilização conferiram maior termoestabilidade à enzima, conforme pode ser observado pelos parâmetros cinéticos e termodinâmicos. Ambas as formas imobilizadas da enzima se apresentaram boa capacidade de reutilização e estáveis ao armazenamento. Foram realizadas a produção de FOS pelas enzimas imobilizadas em ambos os suportes, sendo os melhores resultados obtidos pela preparação imobilizada em MNP-CS (101.56 g L^{-1}) por processo descontínuo em relação a imobilizada em CS via processo contínuo em reator enzimático de leito empacotado (25.73 g L^{-1}). Com base nos resultados obtidos pode se concluir que foram desenvolvidos dois biocatalisadores imobilizados com comprovada aplicação na produção e FOS utilizando a enzima comercial Pectinex Ultra SP-L e outro com resultados promissores para aplicações industriais, principalmente para produção de açúcar invertido, envolvendo a FFase de *A. tamarii* imobilizada por ligação covalente em quitosana.

Palavras-chave: Fruto-oligossacarídeos; Frutosiltransferase; β-frutofuranosidase; *Aspergillus tamarii*; Imobilização de enzimas.

ABSTRACT

Fructooligosaccharides (FOS) correspond to an important class of prebiotics that present several beneficial effects to the consumer and technological properties suitable for application in different food products. In view of this, the present work aimed to develop a production process for fructo-oligosaccharides using FOS-forming enzymes in their immobilized form. First, different strains producing β-fructofuranosidase (FFase) by Solid State Fermentation (SSF) were investigated, with the best production observed by the *Aspergillus* tamarii strain URM4634 using soy bran as a substrate (U_H = 209.1 U mL^{-1} and U_{TF} = 53.9 U mL^{-1}). The enzyme was biochemically characterized and evaluated in relation to its kinetic and thermodynamic parameters, it was found that in fact the enzyme was a phase with transfructosylating activity, being thermostable at temperatures commonly used in the production of FOS (50°C). Studies involving the immobilization of commercial preparation Pectinex Ultra SP-L with transfructosylating activity (U_H = 378.0 U mL^{-1} and U_{TF} = 301.7 U mL^{-1}) were also carried out in chitosan (CS) and in Fe_3O_4–chitosan-magnetic nanoparticles (MNPs-CS). The different immobilization techniques proved to be effective, with immobilization yields of 95.9% and 94.8%, in CS and MNP-CS, respectively. Both methods of immobilization conferred greater thermostability to the enzyme, as can be observed by the kinetic and thermodynamic parameters. Both immobilized forms of the enzyme showed good reuse capacity and are stable to storage. FOS production was carried out by enzymes immobilized on both supports, the best results being obtained by the preparation immobilized in MNP-CS (101.56 g L^{-1}) by a batch process compared to immobilized in CS via continuous process in an enzymatic bed reactor packaged (25.73 g L^{-1}). Based on the results obtained, it can be concluded that two immobilized biocatalysts were developed with proven application in production and FOS using the commercial enzyme Pectinex Ultra SP-L and another with promising results for industrial applications, mainly for inverted sugar production, involving the FFase from *A. tamarii* immobilized by covalent bond in chitosan.

Keywords: Fructooligosaccharides; Fructosyltransferase; β-fructofuranosidase; *Aspergillus tamarii*, Enzyme immobilization.

OBJETIVOS

Objetivo geral

Desenvolver processos de produção de fruto-oligossacarídeos a partir de enzimas com atividade de transfrutosilação imobilizadas.

Objetivos específicos

- Selecionar linhagens fúngicas do gênero *Aspergillus* produtoras de enzimas com atividade de transfrutosilação e realizar a produção por Fermentação em Estado Sólido (FES) investigando as variáveis envolvidas no processo por meio de planejamentos experimentais;
- Caracterizar bioquimicamente a β-frutofuranosidase produzida por FES em relação ao pH e temperatura ótimos, estabilidade ao pH e temperatura e estimar os parâmetros cinéticos e termodinâmicos relacionados as reações catalisadas e à inativação térmica da enzima;
- Imobilizar a β-frutofuranosidase por ligação covalente em partículas de quitosana;
- Imobilizar a preparação enzimática comercial Pectinex Ultra SP-L em partículas de quitosana e em nanopartículas magnéticas de Fe_3O_4 com quitosana, investigando através de planejamentos experimentais a influência das principais variáveis envolvidas nos processos de imobilização;
- Caracterizar bioquimicamente a preparação livre e imobilizada em relação ao pH e temperatura ótimos, estabilidade ao pH e temperatura e estimar os parâmetros cinéticos e termodinâmicos relacionados as reações catalisadas e à inativação térmica da enzima;
- Avaliar a capacidade da reutilização e a estabilidade ao armazenamento da preparação comercial imobilizada nos dois suportes;
- Realizar a produção do fruto-oligossacarídeos (FOS) com as formas imobilizadas da Pectinex, em batelada e através do processo contínuo em reator enzimático de leito empacotado;

INTRODUÇÃO

A sociedade contemporânea tem apresentado uma preocupação crescente com a qualidade de vida e saúde, sobretudo referente ao consumo de alimentos. Diante disso, tem-se aumentado a busca contínua por uma maior compreensão do papel da ampla gama de componentes e nutrientes alimentares na melhoria da saúde ou na prevenção de doenças crônicas. As pesquisas nesse sentido, resultaram em uma infinidade de novos produtos que indicam benefícios distintos, tais alimentos são denominados alimentos funcionais (PANDEY; NAIK; VAKIL, 2015). Entre estes encontram-se os prebióticos, que que são principalmente fibras não digeríveis que afetam beneficamente a saúde do hospedeiro estimulando seletivamente o crescimento de bactérias benéficas para o hospedeiro. Entre os principais prebióticos, tem-se os fruto-oligossacarídeos (FOS) que são oligômeros de frutose compostos principalmente por 1-kestose. nistose e 1-frutofuranosil-nistose, e amplamente empregados em diversos produtos da indústria de alimentos sorvetes e sobremesas lácteas, iogurtes, biscoitos, produtos de panificação e mais recentemente sucos de fruta.

Os FOS podem ser naturalmente obtidos em frutas, grãos e vegetais, e através da hidrólise enzimática da inulina ou a transfrutosilação dos resíduos de sacarose. A produção dos FOS em larga escala é preferencialmente realizada pelo processo de transfrutosilação, uma vez que a sacarose apresenta custo inferior que a inulina. As enzimas envolvidas neste processo são a frutosiltransferase (FTase, EC 2.4.1.9) e a β-frutofuranosidase (FFase, EC 3.2.1.26) que podem ser obtidas de origem vegetal e microbiana, sendo preferencialmente obtidas através de micro-organismos, uma vez que a extração destas enzimas a partir de vegetais é limitada pela sazonalidade. É importante ressaltar que as frutosiltrasferases são preferencialmente utilizadas em relação às β-frutofuranosidases, pois a atividade de transfrutosilação nesta última só é mais evidente em altas concentrações de sacarose (GANAIE; GUPTA; KANGO, 2013).

A síntese em escala industrial de fruto-oligossacarídeos através de frutosiltransferases em sua forma solúvel apresenta como principal inconveniente a dificuldade de recuperação e reutilização da enzima, o que vem a ocasionar um aumento de custos ao processo podendo comprometer a viabilidade econômica deste. Este inconveniente pode ser superado através da imobilização das enzimas, que surge como uma alternativa interessante, uma vez que confere uma capacidade de reutilização ao biocatalisador além de outras vantagens, por exemplo, melhoria na estabilidade à temperatura e interferentes presentes no meio reacional, aumento na produtividade, além de permitir a realização de processos contínuos (SHELDON; VAN PELT, 2013).

Os processos contínuos envolvendo enzimas imobilizadas ocorrem através de reatores enzimáticos, que são equipamentos onde ocorrem a reação de conversão de substratos em produtos, sendo estes considerados o coração dos processos biotecnológicos. Existem muitas configurações de reatores disponíveis para operar de forma contínua, tais como os reatores tubulares, que podem ser de leito empacotado ou fluidizado, e os de cesto contínuo (ILLANES, 2008). A decisão sobre que tipo de reator a se utilizar é o primeiro passo no projeto, sendo que a seleção tipo de reator mais apropriado para um determinado bioprocesso depende das características da bioconversão e de condições reacionais, cinéticas e ligadas ao biocatalisador, que vão determinar o modo de operação e características do fluxo.

Diante disso, o presente trabalho teve como objetivo desenvolver processos de produção de FOS utilizando enzimas formadoras destes oligossacarídeos de diferentes fontes microbianas, imobilizadas em diferentes suportes por processos contínuos e descontínuos. Para tanto, o presente trabalho foi dividido em capítulos redigidos na forma de artigos, com exceção do Capítulo I que consiste em uma revisão bibliográfica sobre o tema abordado na tese. Os capítulos II e III dizem respeito a produção, caracterização bioquímica e estudo cinético/termodinâmico de uma nova β-fructofuranosidase de *Aspergillus tamarii* URM4634 com atividade de transfrutosilação. Os capítulos IV e V dizem respeito a imobilização da preparação comercial Pectinex Ultra SP-L por ligação covalente em quitosana e em nanopartículas magnéticas com quitosana e aplicação destes biocatalisadores imobilizados na produção de FOS por processos contínuo e descontínuo, respectivamente.

CAPÍTULO I

1. REVISÃO DE LITERATURA

1.1. Prebióticos

A sociedade contemporânea tem apresentado uma preocupação crescente com a qualidade de vida e saúde, sobretudo referente ao consumo de alimentos. Essa maior conscientização do público em geral nas últimas décadas tem levado a indústria de alimentos a dar uma maior atenção para o desenvolvimento de novos produtos que apresentam benefícios comprovados para a saúde do consumidor, sendo assim, denominados alimentos funcionais. Estes podem ser agrupados em três categorias: (1) alimentos usuais com substâncias bioativas que ocorrem naturalmente como as fibras alimentares, (2) alimentos suplementados com substâncias bioativas como os probióticos e antioxidantes e (3) ingredientes alimentares derivados de alimentos convencionais como os prebióticos (AL-SHERAJI et al., 2013; BALI et al., 2015).

Os prebióticos, agrupados na terceira categoria, correspondem a uma parte importante dos alimentos funcionais e foram inicialmente definidos por Gibson e Roberfroid (1995) como sendo ingredientes alimentares não digeríveis, que afetam beneficamente o hospedeiro, estimulando seletivamente o crescimento e/ou atividade de um ou de um número limitado de bactérias no cólon e, portanto, melhora a saúde do hospedeiro. Posteriormente, esse conceito foi atualizado e foram definidas três diretrizes que necessitam de demonstração científica para classificação de um ingrediente alimentar como prebiótico: (1) resistência à acidez gástrica, hidrólise enzimática e absorção gastrointestinal; (2) ser fermentado pela microbiota intestinal e (3) estimular seletivamente o crescimento e/ou atividade de bactérias intestinais associadas à saúde e bem-estar (GIBSON et al., 2004).

Em 2008 a FAO (Food and Agricultural Organization) das Nações Unidas organizou uma reunião para definir o novo conceito de prébiotico, como resultado a definição foi redefinida como "um componente alimentar não viável que confere um benefício à saúde do hospedeiro associado à modulação da microbiota", removendo o critério da capacidade de fermentação seletiva (PINEIRO et al., 2008). Posteriormente, Gibson et al. (2010) desenvolveram uma definição mais estrita para prebióticos alimentares como sendo “um ingrediente fermentado seletivamente que resulta em alterações específicas na composição e/ou atividade da microbiota gastrointestinal, conferindo benefício (s) à saúde do hospedeiro".

Bindels et al. (2015) propuseram que os requisitos de especificidade fossem removidos com base em relatórios que mostrassem que múltiplos táxons, em vez de espécies específicas, fossem enriquecidos por prebióticos. Essa proposta levou a outra definição de um prebiótico como "um composto não digerível que, através da sua metabolização por micro-organismos no intestino, modula a composição e/ou atividade da microbiota intestinal, conferindo assim um efeito fisiológico benéfico ao hospedeiro". Esta definição limitou os prebióticos às interações com a microbiota intestinal, excluindo locais extra-intestinais, e eliminou a necessidade de fermentação seletiva.

Em 2016, durante um painel de especialistas em microbiologia, nutrição e pesquisa clínica promovida pela Associação Científica Internacional de Probióticos e Prebióticos (ISAPP) foi definido prebiótico como sendo “um substrato que é utilizado seletivamente por microrganismos hospedeiros que conferem um benefício à saúde”. Nesta definição revisada e a mais atual, o termo continua a ser aplicável a outros locais do corpo colonizados por bactérias, além do trato gastrointestinal (por exemplo, trato vaginal e pele); considera-se também que eles devem cumprir o requisito de ação por mecanismos seletivos mediados pela microbiota (GIBSON et al., 2017).

Os prebióticos apresentam potencial de melhorar a saúde humana e animal, controlando o desequilíbrio da microbiota intestinal através da estimulação do crescimento de bactérias benéficas como as pertencentes aos gêneros *Lactobacillus* e *Bifidobacterium*, responsáveis por inibir a proliferação de bactérias nocivas e permitir que bactérias saudáveis aumentem a produção de ácidos graxos de cadeia curta. Resultando na melhoria da integridade da membrana intestinal e na absorção de nutrientes, diminuindo os níveis glicêmicos e o peso corporal, aumento da inibição da toxicidade de carcinógenos, maior imunidade e modulação de biomarcadores metabólicos, cardiovasculares e inflamatórios (FARIAS et al., 2019; MOHANTY et al., 2018). Além disso, são capazes de influenciar o metabolismo e a obesidade, pois regulam a expressão de hormônios anoréticos intestinais e diminuem os níveis de hormônios orogênicos. Também foi observado que a suplementação prebiótica evita sinais pró-inflamatórios que são prejudiciais aos processos cognitivos, levando a efeitos positivos na aprendizagem e na memória, sendo considerada uma alternativa potencial para o tratamento da esquizofrenia (KAO; BURNET; LENNOX, 2018).

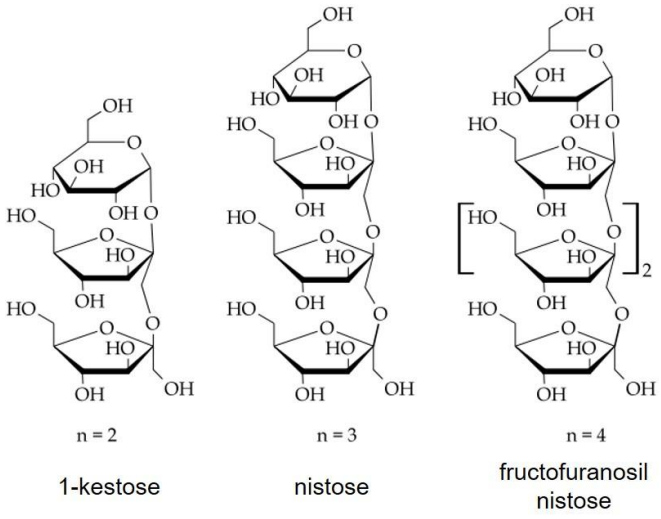
Diante dos benefícios apresentados, os prebióticos podem ser amplamente utilizados em vários tipos de produtos alimentícios, incluindo lácteos, bebidas, fórmulas para lactentes, produtos cárneos, ração animal e como suplementos. Diante destas possibilidades de aplicações, muitas empresas, especialmente no mundo ocidental e nos países europeus,

produzem e fornecem prebióticos para a preparação de alimentos saudáveis, alguns exemplos são Orafti e Cosucra na Bélgica e a Beghin-Say na França (ASHWINI et al., 2019; MARKOWIAK; SLIZEWSKA, 2017). As projeções para o mercado de prébioticos são animadoras e de acordo com a Global Market Insights (Delaware, EUA), tendo em vista que o mercado mundial de prebióticos está aumentando o faturamento previsto para esse nicho de mercado deve ultrapassar US $ 8,5 bilhões em 2024. As pesquisas e o desenvolvimento de novos produtos que utilizam prebióticos está direcionada principalmente para oligossacarídeos não digestíveis como, inulina, fruto-oligossacarídeos (FOS) e galacto-oligossacarídeos (GOS) que são caracterizados como prebióticos estabelecidos (PATEL; GOYAL, 2012).

1.2. Fruto-oligossacarídeos: definições, propriedades e aplicações

Fruto-oligossacarídeos (FOS) correspondem a oligossacarídeos pertencentes ao grupo frutano, que é a denominação geral utilizada para polímeros de frutose com uma estrutura geral de unidade de glicose ligada a múltiplas unidades de frutose. Os FOS, especificamente, podem ser definidos como sendo frutanas que apresentam de 2-4 unidades de frutosil (F) ligadas a uma glicose terminal (G) por meio de ligações glicosídicas do tipo β (2→1). Estes apresentam a forma geral GF_n e são principalmente compostos por 1-kestose (GF_2), nistose (GF_3) e fructofuranosil-nistose (GF_4) (ANTOŠOVÁ; POLAKOVIČ, 2001). As estruturas químicas destes principais FOS podem ser visualizadas na Figura 1.

Figura 1. Estrutura química dos principais fruto-oligossacarídeos (FOS). Fonte: Adaptado de BALI et al. (2015).



Dependendo do tipo de ligação entre os monossacarídeos, diferentes tipos de séries de FOS podem ser produzidos. Neo-FOS consiste principalmente de neo-kestose (neo-GF_2) e neo-nistose (neo-GF_3), em que as unidades frutosil são ligadas na posição β (2→6) da molécula de sacarose (FLORES-MALTOS et al., 2014). Devido a sua estrutura ramificada particular, os Neo-FOS apresentam funções prebióticas, estabilidade química e térmica superiores em comparação com os FOS convencionais, entretanto os Neo-FOS ainda não se encontram comercialmente disponíveis.

Com relação às propriedades físico-químicas dos FOS, estes podem ser considerados carboidratos de baixa caloria (1,0–1,7 kcal/g), apresentam maior viscosidade e higroscopicidade em comparação à sacarose, são estáveis em uma faixa de pH comumente observada em diferentes produtos alimentícios (4,0-7,0) e altas temperaturas (>140°C) e apresentam grau de polimerização (DP) inferior a 10. Além disso, os FOS apresentam perfil de doçura e propriedades de retenção de água similares a sacarose e sorbitol. Por suas propriedades tecnológicas semelhantes à sacarose, também atuam aumentando os aromas e mascarando sabores estranhos provenientes de adoçantes intensos (KUMAR; SRIPADA; POORNACHANDRA, 2018).

Como mencionado acima, a ampla utilização dos FOS e demais prebióticos está associada aos benefícios que estes proporcionam para a saúdo do consumidor. Diante disso, os FOS apresentam os seguintes potenciais efeitos benefícios à saúde: estímulo seletivo ao crescimento e/ou atividade de micro-organismos benéficos como os gêneros *Bifidobacterium* e *Lactobacillus*, ativação do sistema imune, redução da morbidade e duração da diarreia infecciosa associada aos antibióticos, redução dos riscos de câncer de cólon, alívio dos sintomas da síndrome do intestino irritável e doença intestinal inflamatória, redução dos riscos de distúrbios relacionados à obesidade e melhora da biodisponibilidade e absorção de minerais como Ca^{2+} e Mg^{2+} (CHEN; LI; CHEN, 2016).

Os FOS podem ser encontrados naturalmente em alguns vegetais ou podem ser obtidos por síntese enzimática utilizando sacarose ou inulina como substratos. Com relação às fontes vegetais de obtenção de FOS destacam-se as seguintes: cebola, trigo, centeio, cebolinha, tomate, banana, alho, alcachofra de Jerusalém. O teor de FOS presente nestas fontes é variável e em sua maioria varia entre 0,3 e 6%, sendo a chicória e a alcachofra de Jerusalém exceções a essa regra e apresentam percentual de FOS variando entre 5-10% e até 20%, respectivamente (DOMINGUEZ et al., 2013). A produção por via enzimática pode ser realizada através da hidrólise da inulina e da transfrutosilação de resíduos de sacarose e apresenta rendimento máximo teórico entre 55–60%. Os mecanismos de ação das enzimas envolvidas na síntese dos

FOS serão mais bem descritos na Seção 1.3. Além disso, os processos de síntese enzimática desses oligossacarídeos podem ser conduzidos utilizando enzimas isoladas em suas formas livres e imobilizadas operando descontínua e continuamente em reatores enzimáticos (Seção 1.9), respectivamente, e através de processos fermentativos utilizando células microbianas (Seção 1.10).

O início da industrialização e comercialização dos FOS ocorreu no início da década de 1980 devido principalmente à demanda dos consumidores por produtos com calorias reduzidas. A empresa japonesa Meiji Seika Kaisha Co. em 1984 foi a pioneira na produção industrial de FOS e comercialização, atualmente são os principais produtores de FOS, obtidos pelo processo de transfrutosilação, que é comercializado utilizando a marca Meioligo ®. Além desta empresa, podem-se mencionar outras que atuam na produção e comercialização de FOS em vários países tais como: Actilight (Beghin-Meiji Industries, França), Nutraflora (GTC Nutrition, EUA), FortiFeed (GTC Nutrition, EUA), Prebiovis scFOS (Victory Biology Engineering Co., China). Geralmente as misturas de FOS comercialmente disponíveis apresentam um custo de aproximadamente € 150/kg e apresentam pureza superior a 95% (DOMINGUEZ et al., 2013; NOBRE et al., 2015). Para obtenção deste alto grau de pureza, diversas técnicas têm sido utilizadas para separar FOS de outros açúcares, tais como ultrafiltração e nanofiltração, sistemas de carvão ativado ou cromatografia de troca iônica (NOBRE; SUVAROV; DE WEIRELD, 2014).

Os FOS são aditivos alimentares amplamente utilizados na indústria de alimentos, suas aplicações mais comuns são em formulações de sorvetes e sobremesas lácteas que levam no rótulo "açúcar reduzido", "sem adição de açúcar", "calorias reduzidas", "produto sem açúcar", etc., em formulações para diabéticos, iogurtes, biscoitos e produtos de panificação, substituindo carboidratos e gerando produtos de teor reduzido de açúcar, barras de cereais, produtos de confeitaria, sucos e néctares (YUN, 1996). Nestes produtos a porcentagem de FOS varia de 2% a 50%.

A utilização de FOS em sucos de fruta é uma alternativa interessante para o desenvolvimento de alimentos funcionais, uma vez que os sucos já são naturalmente ricos em compostos bioativos e atendem às alegações do consumidor de alimentos saudáveis, saborosos e práticos. Todavia, os estudos envolvendo o desenvolvimento a fortificação de sucos com FOS ainda são escassos e poucas frutas já foram utilizadas para essa finalidade tais como: laranja, manga, abacaxi e cranberry (FONTELES; RODRIGUES, 2018). Outra alternativa para o aproveitamento de FOS em matrizes vegetais é a elaboração de geleias enriquecidas com estes oligossacarídeos conforme reportado por Davim et al. (2015).

Outra importante aplicação dos FOS está no desenvolvimento de produtos simbióticos, que consistem basicamente em uma formulação sinérgica de probióticos e prebióticos que aumentam a contagem de bactérias bifidogênicas no cólon, muito mais do que quando o prebiótico ou probiótico é usado sozinho (KUMAR; SRIPADA; POORNACHANDRA, 2018). Para esta finalidade os FOS têm sido empregados como material de parede para microencapsulação de probióticos como culturas do gênero *Lactobacillus* via *spray drying* (RAJAM; ANANDHARAMAKRISHNAN, 2015). Partindo desse princípio, diferentes sucos de fruta simbióticos que utilizam FOS têm sido desenvolvidos (KALITA et al., 2018). Além disso, outra aplicação interessante envolvendo produtos simbióticos é o desenvolvimento de leite com baixo teor de gordura simbiótico como descrito por Oliveira et al. (2013).

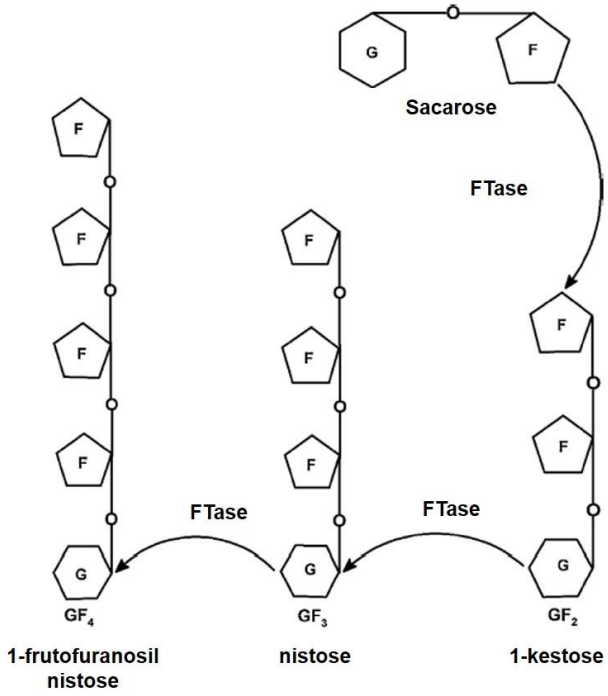
Além das aplicações de FOS voltadas para alimentação humana é importante mencionar que estes prebióticos têm sido estudados para aplicações na nutrição animal, sobretudo na avicultura e aquicultura. Em geral, a suplementação de FOS na dieta de frangos de corte pode melhorar a imunidade e reduzir a suscetibilidade à colonização por patógenos, sem prejudicar seu desempenho de crescimento podendo ser uma alternativa viável para substituir antibióticos promotores do crescimento (SHANG et al., 2018). Além disso, o complemento de FOS em dietas de frango de corte têm surgido como um método alternativo de produção de aves e que pode ser estendido para outros tipos de operações envolvendo outros tipos de produção animal (RICKE, 2015). Em relação à aquicultura, autores tem relatado a influência positiva dos FOS no crescimento e na hemato-imunologia de peixes, bem como resistência a infecções (GUERREIRO; OLIVA-TELES; ENES, 2017). Também foi reportado efeitos benéficos no crescimento, imunidade, capacidade antioxidante e alteração da microbiota intestinal em caranguejos devido a suplementação de FOS na dieta (JIA et al., 2017).

1.3. Enzimas envolvidas na síntese de fruto-oligossacarídeos

A síntese dos FOS a partir de sacarose pode ocorrer pela ação das enzimas frutosiltransferase (FTase, EC 2.4.1.9) e β-frutofuranosidase (FFase, EC 3.2.1.26) também conhecidas como invertases, ambas pertencentes a família das 32 das glicosil hidrolases (GH32). As FTases possuem, quase que exclusivamente atividade de transfrutosilação, sendo as FTases microbianas as únicas a apresentarem ambas atividades hidrolítica e de transfrutosilação, já as FFases geralmente só apresentam a atividade de transfrutosilação sob condições especiais (ANTOŠOVÁ; POLAKOVIČ, 2001).

As FTases são enzimas que catalisam a transferência de uma unidade de frutose proveniente de uma sacarose ou frutana para outra proporcionando a formação de um fruto-oligossacarídeo ou outra frutana. A nomenclatura das FTases é baseada tanto na especificidade do substrato quanto na ação catalítica da própria enzima. A única FTase presente em fungos e a envolvida na formação de FOS a partir da sacarose é a sacarose-1-frutosiltransferase (1-STT, EC 2.4.1.99) que atua clivando a ligação glicosídica da sacarose e transfere o resíduo de frutose para a posição C-1 da extremidade frutosil de outra molécula de sacarose formando a 1-kestose. Esta enzima também pode transferir um ou dois resíduos de frutose para a 1-kestose formada e, desse modo, formar nistose ou 1-fructofuranosil nistose, respectivamente (TOLEDO et al., 2019). A Figura 2 ilustra de modo simplificado esse mecanismo que irá depender da fonte de obtenção (vegetal ou microbiana) e pode ser influenciada pela pureza da enzima (MAIORANO et al., 2008).

Figura 2. Produção dos principais FOS utilizando sacarose como substrato pela ação da FTase. Fonte: Adaptado de SINGH et al. (2016).



Cabe mencionar que em FTases extraídas de vegetais, além da presença da sucrose 1-frutosiltransferase também é observada a presença da frutano-1-frutosiltransferase (1-FTT, EC 2.4.1.100) que atua transferindo o resíduo terminal de frutose de um frutano, geralmente 1-kestose, para a posição C-1 da extremidade frutosil de outro frutano receptor, permitindo o

alongamento da cadeia. Plantas como a chicória (*C. intybus*) e alcachofra (*H. tuberosus*) apresentam altos níveis destas duas FTases (MUTANDA et al., 2014; TOLEDO et al., 2019).

As propriedades e os mecanismos de ação das FTases microbianas têm sido mais estudados e mais bem compreendidos em relação às obtidas de origem microbiana. Chuankhayan et al. (2010) reportaram que a estrutura dessas enzimas possui dois domínios dobrados, compostos por 632 resíduos. Esses resíduos constituem uma hélice de cinco pás na posição N-terminal (resíduos 21-468) e um domínio de sanduíche C-terminal (480-653), e ambas as estruturas estão ligadas por uma hélice curta de 9 resíduos (469-479). Jitonnom, Ketudat-Cairns e Hannongbua (2018) utilizando técnicas de mecânica quântica e molecular avaliaram as estruturas de transição e gastos energéticos presentes nas reações de hidrólise e transfrutosilação da FTase de *A. japonicus*. Estes autores reportaram que a primeira metade da reação é composta pela formação de um intermediário frutosil-enzima que requer a superação de uma barreira energética de 15,7 kcal mol^{-1} e para a segunda meia reação (hidrólise e transfrutosilação) as barreiras reacionais apresentam valores semelhantes (14,0 e 13,2 kcal mol^{-1}) o que sugere a coexistência dos dois mecanismos competindo pelos produtos da catálise.

As FFases são principalmente conhecidas por catalisarem a hidrólise das ligações β (2→1) da sacarose em uma mistura equimolar de glicose e frutose que é denominada açúcar invertido. O xarope deste açúcar é mais doce do que a sacarose e mais fácil de incorporar em preparações alimentícias e farmacêuticas porque não apresenta problemas de cristalização, como a sacarose em soluções concentradas. Além dessa aplicação, as FFases também são empregadas na fermentação de melaço de cana-de-açúcar, produção de etanol, alimentos para abelhas e mel artificial (KULSHRESTHA et al., 2013; NADEEM et al., 2015). Sabe-se que sob condições apropriadas, altas concentrações de sacarose, algumas FFases também apresentam a atividade de transfrutosilação. A principal diferença observada entre as FFase que apresentam atividade de transfrutosilação e as demais é a maior afinidade para sacarose ou FOS como aceptores dos resíduos de frutosil do que a água. A produção dos FOS a partir desta enzima pode ocorrer por dois mecanismos: hidrólise reversa ou transglicosilação (ANTOŠOVÁ; POLAKOVIČ, 2001).

No processo de hidrólise reversa o equilíbrio da reação é deslocado da hidrólise para a síntese de FOS. A primeira etapa da reação envolve a formação de um complexo enzima-doador ativado, que reage com o grupo hidroxila da molécula receptora, que é a sacarose. A quantidade de FOS sintetizada vai depender das constantes de equilíbrio das reações de decomposição do complexo enzima-doador em enzima e frutose ou 1-kestose. O aumento na concentração de substrato e consequentemente diminuição da atividade irão possibilitar aumentos no

rendimento final de FOS. Já na transglicosilação, as reações de decomposição do complexo enzima-doador ocorrem em água, enquanto a sacarose e a água competem pelo grupo frutosil no complexo. Sendo este processo cineticamente controlado, uma vez que a kestose produzida na reação é um substrato potencial para hidrólise pela FFase, logo a concentração final de FOS irá depender diretamente das velocidades de síntese e hidrólise (ANTOŠOVÁ; POLAKOVIČ, 2001; KUMAR; SRIPADA; POORNACHANDRA, 2018).

As definições envolvendo a atividade enzimática das FTases e das FFases são motivos de discussão entre pesquisadores, sendo que até o momento não existe um consenso em como expressá-las. Chen e Liu (1996) foram os primeiros a reportar uma forma simplificada para determinar as atividades hidrolítica e de transfrutosilação da FFase e definiram a atividade hidrolítica como sendo a quantidade de enzima necessária para liberar 1 μmol de glicose por minuto e de transfrutosilação como sendo a quantidade de enzima necessária para transferir 1 μmol de frutose por minuto.

Sangeetha, Ramesh e Prapulla (2004) definiram a atividade de FTase como sendo a quantidade de enzima necessária para liberar 1 μmol de glicose, havendo claramente uma confusão entre a definição das atividades da FFase. Huang et al. (2016) também são inespecíficos na definição da atividade de FTase, pois definem a atividade de FTase como sendo a quantidade de enzima necessária para liberar 1 μmol de açúcares redutores. Outra definição observada foi reportada por Onderková et al. (2010) que definiram a atividade de FTase como sendo a quantidade de μmoles de kestose. Muñiz-Márquez et al. (2015) seguiram uma definição semelhante uma vez que definiram como sendo a quantidade de enzima necessária para formar 1 μmol de kestose por minuto.

Como pode ser observado, diante do impasse envolvendo as diferentes definições das atividades da FFase e FTase, pode-se observar claramente que a falta de padronização é um problema para os pesquisadores que atuam estudando essas enzimas. Além deste problema, outra questão antiga que tem sido debatida é a nomenclatura da enzima (MAIORANO et al., 2008). Principalmente envolvendo enzimas de origem fúngica, uma vez que estas apresentam ambas atividades hidrolítica e de transfrutosilação. Como sugestão para esse impasse, uma alternativa confiável seria definir a nomenclatura com base na cinética das reações catalisadas pelas enzimas. Uma vez que as FTases apresentam menores valores para a o K_m e maiores valores para o V_{max} para a reação de transfrutosilação em relação à de hidrólise, indicando maior afinidade para primeira reação, ao passo que as FFases apresentam comportamento oposto, conforme pode ser visto dos resultados reportados por Huang et al. (2016) e Xu et al. (2015),

avaliando FTase e FFase, respectivamente. Com isso, esta última abordagem foi a escolhida para definir as atividades e nomenclaturas das enzimas estudadas no presente trabalho de tese.

Cabe ainda mencionar que os FOS também podem ser sintetizados por via enzimática pela hidrólise da inulina através da ação das inulinases. Estas são divididas em duas subclasses diferentes com base em seu modo de ação em exoinulinases e endoinulinases. As exoinulinases (EC: 3.2.1.80) hidrolisam a inulina, clivando a frutose da extremidade não redutora da inulina. Enquanto endoinulinases (EC: 3.2.1.7) que atuam na extremidade não redutora β (2→1) da inulina produzindo uma mistura de FOS com alto grau de polimerização principalmente compostos por inulotrioses (F3), inulotetraoses (F4) e inulopentaoses (F5). Os FOS produzidos a partir de inulina apresentam maiores cadeias em comparação com os obtidos a partir da sacarose, todavia apresentam propriedades funcionais e fisiológicas similares (SINGH; SINGH; KENNEDY, 2016).

1.4. Produção de enzimas envolvidas na produção de fruto-oligossacarídeos por processos fermentativos

Os micro-organismos são as principais fontes de enzimas produtoras de FOS a partir de sacarose (FTases e FFases) sendo preferencialmente utilizados em relação a extração por vegetais, uma vez que esta é limitada pela sazonalidade. As FFases e FTases microbianas podem ser de natureza intracelular e extracelular e é reportado na literatura que os fungos filamentosos pertencentes aos gêneros *Aspergillus, Aureobasidium e Penicillium* fornecem enzimas com maiores rendimentos de produção de FOS. Este fato pode ser observado na Tabela 1, a qual apresenta diferentes linhagens fúngicas e bacterianas utilizadas para produção dessas enzimas e as linhagens pertencentes a estes gêneros são os que aparecem em maior quantidade. Como os fungos filamentosos naturalmente possuem os genes que possibilitam a síntese de FOS, modificações genéticas utilizando estes já tem sido realizada para o desenvolvimento de FFases (BEDZO et al., 2019) e FTases (GUO et al., 2016) recombinantes. Diante disso, para facilitar a identificação de linhagens fúngicas potencialmente produtoras de enzimas com atividade de transfrutosilação existem métodos qualitativos, como o descrito por Dominguez et al. (2006) que se baseou na formação de halos de cor rosa e azul, indicando a liberação de glicose e frutose, respectivamente.

Tabela 1. Diferentes linhagens fúngicas e bacterianas produtoras de enzimas envolvidas na síntese de FOS a partir da sacarose (FTases e FFases).

Micro-organismo	Enzima	Referência
Arthrobacter arilaitensis	FFase	(CHU et al., 2014)
Aspergillus awamori	FFase	(SMAALI et al., 2012)
Aspergillus flavus	FTase	(GANAIE et al., 2017)
Aspergillus japonicus	FFase	(MUSSATTO et al., 2012)
Aspergillus niger	FFase	(GOOSEN et al., 2007)
Aspergillus niger	FTase	(LATEEF et al., 2012)
Aspergillus oryzae	FTase	(WEI et al., 2014)
Aspergillus tamarii	FTase	(CHOUKADE; KANGO, 2019)
Aureobasidium melanogenum	FFase	(CHI et al., 2019)
Aureobasidium pullulans	FTase	(ŠEDOVÁ et al., 2014)
Bacillus agaradhaerens	FTase	(KRALJ et al., 2018)
Bacillus cereus	FTase	(EL-BEIH et al., 2009)
Bacillus macerans	FTase	(NAM et al., 2000)
Bacillus subtilis	FTase	(BABU et al., 2008)
Candida sp.	FFase	(HERNALSTEENS; MAUGERI, 2010)
Cryptococcus sp.	FFase	(HERNALSTEENS; MAUGERI, 2008a)
Fusarium graminearum	FFase	(GONÇALVES et al., 2015)
Penicillium aurantiogriseum	FTase	(FARID et al., 2015)
Penicillium citronigrum	FFase	(NASCIMENTO et al., 2016)
Penicillium purpurogenum	FTase	(DHAKE; PATIL, 2007)
Penicillium oxalicum	FFase	(XU et al., 2015)
Penicillium simplicissimum	FTase	(MASHITAH; HATIJAH, 2014)
Rhodotorula sp.	FTase	(HERNALSTEENS; MAUGERI, 2008b)
Rhodotorula dairenensis	FFase	(GUTIÉRREZ-ALONSO et al., 2009)
Rhizopus stolonifer	FTase	(LATEEF; KANA, 2012)
Saccharomyces cerevisiae	FFase	(BHALLA et al., 2017)
Sclerotinia sclerotiorum	FFase	(MOUELHI; ABIDI; MARZOUKI, 2016)
Schwanniomyces occidentalis	FFase	(PIEDRABUENA et al., 2016)
Xanthophyllomyces dendrorhous	FFase	(MÍGUEZ et al., 2018)

Os processos fermentativos para produção de enzimas em geral são conduzidos utilizando meio líquido (Fermentação Submersa, FSm) ou sólido (Fermentação em Estado Sólido, FES). O cultivo via FSm pode ser definido como sendo a fermentação realizada em excesso de água, sendo o meio de cultivo composto essencialmente por água contendo nutrientes dissolvidos. Quase todos as instalações de produção de enzimas em escala industrial se utilizam desse tipo de processo devido às vantagens relacionada à instrumentação e controle do bioprocesso (monitoramento de pH, oxigênio dissolvido, temperatura, concentração de moléculas solúveis em água), facilidade de operação, separação da biomassa após a fermentação, mistura, aeração e aumento de escala. Todavia a FSm apresenta algumas desvantagens relacionadas ao maior espaço físico requerido, gasto energético e/ou necessidade de água (FARINAS, 2015; SADH et al., 2018; SINGHANIA et al., 2010). A produção de FFases e FTases via FSm já é bastante documentada na literatura, o meio de cultivo para essa finalidade geralmente é composto por sacarose suplementado com fonte de nitrogênio e micronutrientes.

Fermentação em Estado Sólido (FES) é definida como sendo o bioprocesso realizado na ausência ou quase ausência de água livre. No entanto, o substrato deve possuir umidade suficiente para suportar o crescimento e a atividade metabólica do micro-organismo. A matriz sólida pode ser a fonte de carbono (e outros nutrientes), ou pode ser um material inerte para suportar o crescimento dos microrganismos (com solução de crescimento impregnada) (THOMAS; LARROCHE; PANDEY, 2013). De acordo com Sadh, Duhan e Duhan (2018) a FES consiste em um processo em várias etapas que são: (1) seleção de substrato; (2) pré-tratamento do substrato por processamento mecânico, químico ou bioquímico para melhorar a disponibilidade dos nutrientes ligados e também para reduzir o tamanho dos componentes; (3) hidrólise de substratos poliméricos, por exemplo, polissacarídeos e proteínas e (4) processo de fermentação para utilização de produtos de hidrólise.

Esse tipo de fermentação geralmente apresenta maiores rendimentos em termos de atividade enzimática quanto comparados com a fermentação submersa, isso é justificado pelo fato de a FES simular o habitat da maioria dos micro-organismos, principalmente fungos. Outro fator importante atribuído a FES para elevados rendimentos é a resistência dos micro-organismos à repressão catabólica (inibição da síntese enzimática) na presença de substratos abundantes, como glicerol, glicose ou outras fontes de carbono (LIZARDI-JIMÉNEZ; HERNÁNDEZ-MARTÍNEZ, 2017).

Outras vantagens apresentadas pela FES são a menor energia requerida para esterilização (devido à menor atividade de água); menor susceptibilidade à contaminação

bacteriana; menor complexidade dos biorreatores para FES tanto para processos contínuos quanto descontínuos do que exigida pelos biorreatores para FSm; possui diversas vantagens ambientais, pois permite a utilização de resíduos sólidos agroindustriais como substrato e/ou fonte de energia em sua forma natural e facilita o gerenciamento de resíduos sólidos, além de menor produção de efluentes (SOCCOL et al., 2017). Estas vantagens podem compensar as desvantagens da FES, que são a lentidão na fermentação, dificuldade de controlar o processo com precisão e a dificuldade na transferência de calor de forma homogênea (NIGAM; PANDEY, 2009).

A utilização de resíduos agroindustriais como substratos para FES é um ponto que favorece esse tipo de processo, uma vez que fornece uma solução vantajosa para um problema ambiental. Todos os anos uma grande quantidade de resíduos agroindustriais é produzida, que se não houver o descarte adequado poderá causar poluição ambiental e efeitos prejudiciais à saúde humana e animal, além de possibilitar o aumento de vários gases de efeito estufa. A maior parte desses resíduos não é tratada e consequentemente é subutilizada uma vez que representam uma excelente fonte de produtos bioativos. Com isso a FES tem alcançando grande relevância no aproveitamento de biomassas inexploradas especialmente na produção de enzimas. Como os resíduos apresentam uma composição variável, uma grande diversidade de enzimas pode ser obtidas com base em sua composição (SADH et al., 2018; SADH; DUHAN; DUHAN, 2018). Estudos envolvendo a produção de FTases e FFases via FES utilizando resíduos agroindustriais ainda são escassos, todavia diferentes resíduos já foram explorados para essa finalidade, como pode ser observado na Tabela 2. Nesta, também pode ser observado, a confirmação do potencial dos fungos filamentosos do gênero *Aspergillus* para a produção dessas enzimas.

Tabela 2. Produção de FTases e FFases por Fermentação em Estado Sólido (FES) a partir de diferentes micro-organismos e substratos.

Micro-organismo	Enzima	Substratos	Referência
Aspergillus awamori	FTase	Sabugo de milho, farelo de trigo e farelo de arroz	(SATHISH; PRAKASHAM, 2013)
Aspergillus flavus	FTase	Farelo de trigo, palha de milho, bagaço de cana-de-açúcar, casca de mandioca, bagaço de maçã, casca de banana, casca de raiz de beterraba, casca de laranja, casca de goiabas, casca de abacaxi, casca de mamão, casca de manga, casca de maracujá, casca de jabuticaba e casca de caju.	(GANAIE et al., 2017)
Aspergillus japonicus	FFase	Tegumento de grãos de café	(MUSSATTO et al., 2012)
Aspergillus niger	FTase	Casca de banana e vagem de noz de cola	(LATEEF et al., 2012)
Aspergillus oryzae	FTase	Farelo de trigo, farelo de arroz, farelo de aveia, sabugo de milho, farelo de milho, farinha de milho, grãos de milho, espiga de milho integral, casca de café, polpa de café, resíduo de chá, resíduo de café, bagaço de cana-de-açúcar e bagaço de mandioca.	(SANGEETHA; RAMESH; PRAPULLA, 2004)
Aspergillus oryzae	FTase	Aguamiel em espuma de poliuretano	(MUÑIZ-MÁRQUEZ et al., 2015)
Rhyzopus stolonifer	FTase	Casca de mandioca	(LATEEF; KANA, 2012)

A escolha do substrato é um parâmetro fundamental da FES, sendo assim, uma abordagem interessante avaliar a produção de uma enzima de interesse é a utilização de planejamentos de modelagem de mistura que avaliam as proporções de diferentes substratos. Estes planejamentos experimentais são uma classe especial de superfícies de resposta, onde os fatores são os componentes de uma mistura e as respostas são influenciadas pela variação de proporções (SAHIN; DEMIRTAS; BURNAK, 2016) e na FES pode ser utilizado como um primeiro passo para otimização da produção. Tal abordagem para avaliar as interações de diferentes co-substratos agroindustriais na produção de enzimas por SSF foi relatada apenas para glutaminase (SATHISH et al., 2008), frutosiltransferase (SATHISH; PRAKASHAM, 2013), L-asparaginase (FURLAN et al., 2015), protease, α-amilase (DE CASTRO; SATO, 2013) e um complexo multi-enzimático composto por lipase, carboximetil celulase, β-glucosidase e α-amilase (OHARA et al., 2018).

Além da composição do substrato, único ou uma mistura, existem diversos fatores que podem influenciar a FES, entre eles destacam-se tamanho da partícula de substrato, atividade de água, umidade, temperatura, pH, agitação e aeração (SINGHANIA et al., 2009). Estes fatores estão relacionados diretamente com a fisiologia do micro-organismo e a produção de biomoléculas de interesse e devem ser otimizados com base em planejamentos fatoriais e metodologia de superfície de resposta, de modo a identificar os fatores críticos e suas interações (THOMAS; LARROCHE; PANDEY, 2013). Todavia, ainda não há relatos na literatura envolvendo estudos da otimização da produção de FTase ou FFase via FES.

O tamanho da partícula de substrato influencia diretamente a taxa de transferência de oxigênio, uma vez que à medida que o tamanho da partícula diminui, a razão entre a área superficial e o volume aumenta. Além disso, o espaço vazio também é determinado pelo tamanho da partícula de substrato que também impacta na transferência de oxigênio. Quando o tamanho da partícula é pequeno, os micro-organismos obtêm uma maior área superficial para o seu crescimento. Todavia, partículas muito pequenas podem resultar em aglomeração e baixo crescimento, enquanto partículas maiores proporcionam melhor espaço entre as partículas, mas superfície limitada para ataque microbiano. Logo, o tamanho adequado do substrato deve ser mantido para melhorar o crescimento microbiano e a transferência de massa, pois uma vez que o tamanho da partícula excede um valor específico a produção de enzimas é diretamente afetada (CASTRO; NISHIDE; SATO, 2014; DAS; BHAT M; SELVARAJ, 2019).

O conteúdo de água deve ser considerado um fator crítico na FES, pois é responsável pela difusão dos solutos adicionais bem como dos metabólitos produzidos pelo micro-organismo e apresenta influência direta nos parâmetros cinéticos e termodinâmicos relativos a

FES. Por isso, os parâmetros atividade de água (a_w) e umidade devem ser levados em consideração uma vez que apresentam grande influência na atividade microbiana. A a_w do substrato é um parâmetro fundamental no que diz respeito a transferência da massa de água e de solutos através das células microbianas além de determinar que tipos de micro-organismos podem crescer na FES. Bactérias crescem principalmente em valores mais altos de a_w enquanto fungos filamentos e algumas leveduras podem crescer em valores mais baixos (0,6-0,7). Os micro-organismos capazes de realizar suas atividades metabólicas em valores mais baixos de a_w são os mais adequados para FES (NIGAM; PANDEY, 2009; PANDEY, 2003).

O nível de umidade do substrato é um dos principais fatores que influenciam o processo e varia pela sua natureza, bem como os requisitos do micro-organismo para seu crescimento. Um alto nível de umidade resulta em diminuição da porosidade, baixa difusão do oxigênio, risco grande de contaminação, redução no volume de gás e redução de troca gasosa. Entretanto, um baixo nível de umidade leva a um crescimento abaixo do ideal e um menor grau de absorção pelo substrato (NIGAM; PANDEY, 2009). A temperatura é uma das variáveis mais importantes do processo que afeta a FES, uma vez que o crescimento microbiano sob condições aeróbicas resulta na liberação do calor metabólico. Em níveis extremos, isso pode causar desnaturação das enzimas produzidas, assim como outros efeitos deletérios no crescimento de micro-organismos e na produção de metabólitos. Como o processo de FES ocorre na ausência de água livre, é difícil remover o calor metabólico produzido durante o crescimento microbiano, devido à limitada condutividade térmica do substrato sólido e à baixa capacidade térmica do ar.

O pH por sua vez influencia diretamente o crescimento microbiano e na FES gradientes de pH surgem dentro das partículas de substrato, embora as correções do pH nas soluções nutritivas podem ser adicionadas para diminuir a faixa de variação e não prejudicar o crescimento celular e a produção da biomolécula. Entretanto, o controle desta variável, durante a FES, não é fácil de ser realizado (FARINAS, 2015; NIGAM; PANDEY, 2009). Na FES, a aeração desempenha um papel mais importante em comparação com a FSm, onde só auxilia na transferência de gás. Neste caso, a aeração facilita a transferência de calor, gás e umidade entre as partículas sólidas em fermentação e o ambiente de gasoso do sistema. A temperatura da fase gasosa atua suprindo ou removendo calor, mantendo a umidade relativa em equilíbrio com a fase líquida (NIGAM; PANDEY, 2009).

1.5. Extração e purificação de enzimas por Sistema de Duas Fases Aquosas (SDFA)

Os Sistemas de Duas Fases Aquosas (SDFA) também conhecidos como Sistemas Aquosos Bifásicos (SAB) correspondem a técnicas de extração líquido-líquido utilizadas para separação de produtos biológicos baseadas no particionamento da biomolécula para uma das fases. Estes sistemas, aparecem como alternativas aos métodos tradicionalmente utilizados na indústria biotecnológica para a recuperação, separação e purificação de biomoléculas, incluindo a tecnologia à base de membrana (microfiltração, ultrafiltração e nanofiltração), precipitação (*salting out*, isoelétrico e afinidade) e cromatografia líquida (troca iônica, fase reversa, interação hidrofóbica, gel de filtração e afinidade). Os SDFAs mais comuns são formados por dois polímeros (geralmente polietileno glicol (PEG) e dextrana) ou um polímero e um sal (por exemplo, fosfato, sulfato ou citrato), outros tipos incluem líquidos iônicos e álcoois de cadeia curta (IQBAL et al., 2016).

Em comparação com os métodos convencionais de purificação mencionados acima, a utilização de SDFA para separação de biomoléculas apresenta muitas vantagens, que incluem operação simples, rápida separação, alta seletividade, baixo consumo energético, baixo custo, impactos ambientais mais baixos e relativa confiabilidade para *scale-up* (NADAR; PAWAR; RATHOD, 2017). Além disso, o particionamento de biomoléculas por SDFA é considerado um método suave de purificação, uma vez que desnaturação ou perda de atividade biológica geralmente não são verificadas. Isto provavelmente se deve ao alto teor de água e à baixa tensão interfacial dos sistemas que protegerão as proteínas. Além disso, os próprios polímeros também podem ter um efeito estabilizador (ASENJO; ANDREWS, 2011).

A separação das biomoléculas de interesse é obtida através da manipulação do coeficiente de partição (*K*), alterando o peso molecular médio dos polímeros, o tipo de íons no sistema, a força iônica da fase sal ou adicionando um sal adicional (NaCl ou KCl). Além disso, o perfil de partição da biomolécula depende de diferentes interações físico-químicas entre a biomolécula e os produtos químicos formadores das fases. Interações como forças de van der Waals, ligação de hidrogênio, interações eletrostáticas, efeitos estéricos, hidrofobicidade, interações de bioespecificabilidade, bem como efeitos conformacionais entre os componentes da fase e as substâncias contribuem para o particionamento da biomolécula (ASENJO; ANDREWS, 2011; PHONG et al., 2018). Em sistemas PEG-Sal, a partição das biomoléculas depende principalmente dos efeitos de exclusão de volume e de *salting out*. O primeiro efeito pode ser resumidamente descrito pelo aumento do volume ocupado pelo polímero em decorrência do aumento da concentração do polímero e/ou do comprimento da cadeia ou massa

molar, o que resulta em um espaço reduzido na fase superior e consequentemente, as biomoléculas tendem a particionar na fase inferior. Já o efeito de *salting out* está relacionado com a diminuição da solubilidade da biomolécula devido ao aumento da concentração de sal o que favorece a migração das moléculas para a fase superior (ROSSO et al., 2012).

Os primeiros estudos envolvendo SDFA em processos *downstream* foram realizados por Albertsson . Desde então, uma extensa pesquisa sobre o uso de estratégias baseadas em SDFA para a recuperação e purificação de biomoléculas tem sido realizada. Nos últimos anos, mais de 100 estudos foram publicados por ano relatando o uso do SDFA para diferentes finalidades. Alguns estão focados em sua aplicação, enquanto outros no entendimento dos mecanismos de separação e separação de fases. Em geral, os estudos de aplicação do SDFA são divididos em duas categorias: SDFA utilizado em operações de processamento *downstream* (por exemplo, clarificação, separação, extração e purificação, alguns dos quais podem ser combinados) e SDFA utilizado em estratégias de pré-tratamento (principalmente como uma etapa de concentração) para melhor as caracterizações analíticas (PEREIRA; FREIRE; COUTINHO, 2020). Entretanto, até o momento não há estudos envolvendo a aplicação de SDFA envolvendo enzimas formadoras de FOS. Os relatos existentes correspondem apenas a β-frutofuranosidases (invertases) sem atividade de transfrutosilação (KARKAS; ÖNAL, 2012; SÁNCHEZ-TRASVIÑA et al., 2015; YÜCEKAN; ÖNAL, 2011).

1.6. Preparações enzimáticas comerciais com atividade de transfrutosilação

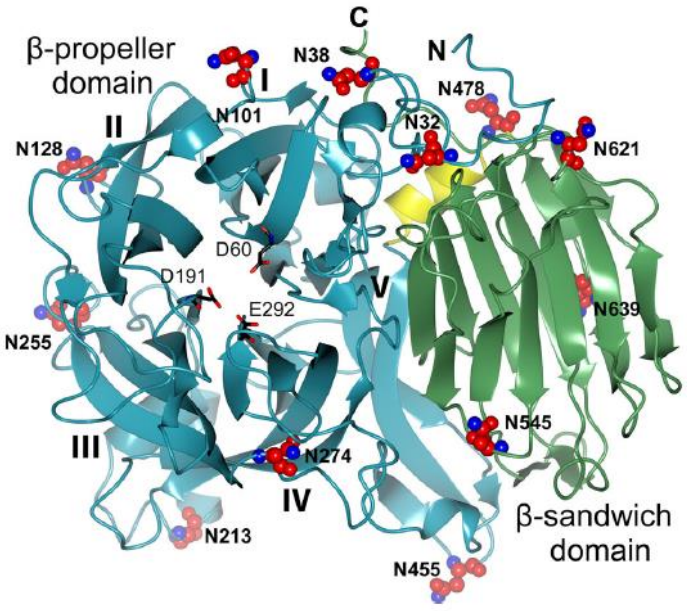
Enzimas isoladas envolvidas na produção de FOS (FTases ou FFases) ainda não se encontram disponíveis comercialmente o que impacta diretamente na disponibilidade deste aditivo alimentar no mercado. Uma alternativa para essa limitação é a utilização de preparações enzimáticas comerciais de grau alimentício que possuam atividade de transfrutosilação proveniente de uma FTase ou FFase (VIRGEN-ORTÍZ et al., 2016). Vega-Paulino e Zúniga-Hansen (2012) avaliaram o potencial de diferentes preparações comerciais para produção industrial de FOS, estes autores reportaram altas atividades de transfrutosilação nas preparações Viscozyme L, Pectinex Ultra SP-L, Rohapect CM, Viscozyme L e Pectinex Smash.

A Pectinex Ultra SP-L é uma preparação enzimática comercial destinada para aplicação no processamento de suco de fruta obtida de *Aspergillus aculeatus* e comercializada pela Sigma-Aldrich. Nesta é reportada a presença de diferentes atividades enzimáticas como pectinase, celulase, β-galactosidase e frutosiltransferase (TANRISEVEN; ASLAN, 2005). Além disso, a Pectinex Ultra SP-L já satisfaz as especificações de pureza para enzimas de grau

alimentício recomendada pelo Comitê Conjunto de Especialistas FAO/OMS sobre Aditivos Alimentares (JECFA) e o Food Chemichal Codex (FCC) o que favorece mais essa aplicação (REHMAN et al., 2016).

O primeiro relato da atividade de transfrutosilação presente na preparação comercial Pectinex Ultra SP-L foi realizado por Hang e Woodams (1995). Ghazi et al. (2007) realizaram o primeiro estudo envolvendo a purificação da FTase presente na preparação comercial e reportaram que a enzima apresenta um peso molecular estimado de 65 kDa. Virgen-Ortíz et al. (2016) também realizaram a purificação da FTase e através de modelos de homologia obtiveram uma representação da estrutura tridimensional da enzima (Figura 3). O modelo de estrutura tridimensional obtido consiste de um domínio N-terminal de cinco β-hélices (resíduos 21-468), um domínio C-terminal β-sanduíche (resíduos 478-654) e uma α-hélice curta de nove resíduos (469-477) que liga ambos os domínios.

Figura 3. Estrutura tridimensional da FTase de *Aspegillus aculeatus* purificada presente na preparação comercial Pectinex Ultra SP-L. Fonte: Virgen-Ortíz et al. (2016).



Os estudos envolvendo a produção de FOS utilizando a Pectinex Ultra SP-L ainda são escassos na literatura, no entanto já foram realizados utilizando a enzima em sua forma livre utilizando sacarose (NEMUKULA et al., 2009) e substratos alternativos como xarope de beterraba açucareira e melaço (GHAZI et al., 2006). Em sua forma imobilizada nos seguintes suportes: resinas de troca iônica (CSANADI; SISAK, 2006), Sepabeads (GHAZI et al., 2005), Eupergit C (TANRISEVEN; ASLAN, 2005) e alginato liofilizado (FERNANDEZ-ARROJO

et al., 2013). Além disso há relatos da aplicação dessa preparação enzimática na produção de oligossacarídeos baseados em lactulose (NGUYEN et al., 2018).

1.7. Cinética e termodinâmica de inativação térmica

A inativação ou desnaturação térmica é uma das principais limitações para o uso de enzimas em processos biotecnológicos. Portanto, a determinação dos parâmetros cinéticos e termodinâmicos relacionados a esse processo são bastante úteis, uma vez que auxilia na compreensão do mecanismo de desnaturação, bem como o efeito da temperatura e do tempo de exposição neste processo (HEIDTMANN et al., 2012). De acordo com Converti et al. (2002) o processo de inativação térmica de uma enzima ocorre por um processo de duas etapas, uma etapa reversível preliminar do desdobramento térmico que seria responsável pela formação de uma conformação menos ativa da enzima (E_I) que seria subsequentemente sujeita a uma etapa irreversível resultando em uma proteína agregada estável (inativa), como pode ser observado na equação abaixo:

$$E \overset{K_I}{\leftrightarrow} E_I \overset{k_d}{\rightarrow} E_D \quad (1)$$

Onde K_I é a constante de equilíbrio do desdobramento parcial da enzima e k_d é a constante de inativação térmica de primeira ordem. Se o estágio irreversível da desnaturação for muito mais lento que o anterior, E e E_I podem ser considerados em equilíbrio e o processo geral de desnaturação pode ser cineticamente descrito em termos de um modelo cinético de primeira ordem, como se segue:

$$v_d = k_d E \quad (2)$$

Onde v_d é a taxa de inativação enzimática e E a concentração da enzima em sua forma ativa. Partindo desta premissa os valores de k_d podem ser estimados a partir da inclinação da reta obtida do gráfico entre o lnψ *versus* tempo, onde ψ é o coeficiente de atividade e consiste na razão entre a concentração total de enzima ativa em um dado tempo e a no início do tratamento térmico, e o tempo. Uma vez obtida a k_d para uma dada temperatura é possível determinar parâmetros cinéticos e termodinâmicos que provêm diversas informações valiosas para realização de um bioprocesso.

Entre os principais parâmetros cinéticos relacionados à desnaturação térmica tem-se o tempo de meia vida ($t_{1/2}$) e os valores D e Z. O tempo de meia vida pode ser definido como sendo o tempo após o qual a atividade enzimática foi reduzida à metade da atividade inicial e é estimada a partir da Equação 3 (OLIVEIRA et al., 2018). O tempo de meia vida é especialmente útil quando se estuda enzimas imobilizadas, uma vez que pela razão entre o $t_{1/2}$ das enzimas imobilizada e livre é possível calcular o fator de estabilidade, que indica se houve uma melhoria real na estabilidade do biocatalisador. Os processos de imobilização serão mais bem descritos na Seção 1.6.

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{3}$$

O valor-*D* ou tempo de redução decimal é definido como sendo o tempo de exposição da enzima a uma determinada temperatura para manter 10% de atividade residual e é estimado pela Equação 4. Geralmente esse parâmetro é acompanhado do fator de sensibilidade ou valor Z, que é definido como o aumento da temperatura necessário para reduzir o valor D em um ciclo logarítmico, e pode ser calculado a partir da inclinação da reta obtida pelo gráfico log*D* *vs.* T (°C) (OLIVEIRA et al., 2018).

$$D = \frac{\ln 10}{k_d} \tag{4}$$

Com relação aos parâmetros termodinâmicos, a entalpia (ΔH_d^*) e entropia (ΔS_d^*) de inativação térmica fornecem o número de ligações não covalentes quebradas e a mudança na desordem enzima/solvente associada com a formação do estado de transição, respectivamente. Já energia livre de Gibbs (ΔG_d^*) de inativação térmica indica quanto da conformação inicial ainda está preservada ou ativa. Baixos valores de ΔG_d^* indicam que o processo de desnaturação ocorre de maneira mais espontânea, uma vez que o estado assumido apresenta menos energia disponível, em função da desnaturação, quando comparado com o estado inicial. Por incluir contribuições entálpicas e entrópicas, este parâmetro é uma das ferramentas mais precisas e confiáveis para prever e avaliar a estabilidade de diferentes tipos de enzimas (OLIVEIRA et al., 2018).

De modo geral, existem poucos dados cinéticos e termodinâmicos referentes a desnaturação térmica de FFases ou FTases na literatura científica, podendo apenas mencionar

os trabalhos de Aguiar-Oliveira e Maugeri (2011) envolvendo a FTase de *Rhodoturula* sp. imobilizada em nióbio e de Onderková et al. (2010) no qual foi estudada a FTase livre de *Aureobasidium pullulans*, sendo que este último estudo descreveu apenas os dados cinéticos relacionados ao processo de inativação térmica. Os estudos envolvendo FFases, envolvem apenas enzimas que possuem apenas atividade hidrolítica como o estudo realizado por Hussain et al. (2009) envolvendo a FFase vegetal obtida a partir de *Saccharum officinarum*.

1.8. Imobilização de enzimas

Como já é largamente conhecido, as enzimas são excelentes catalisadores e sua utilização apresenta vários benefícios em comparação com os catalisadores químicos, entretanto, a utilização destas apresenta algumas limitações tais como falta de estabilidade a longo prazo frente as condições operacionais e dificuldades no processo de recuperação e reutilização (SHELDON; VAN PELT, 2013). Para minimizar estes problemas e promover um maior aproveitamento do potencial catalítico da enzima o processo de imobilização surge como uma alternativa bastante atrativa. Por definição, o termo "enzimas imobilizadas" consiste em enzimas fisicamente confinadas ou localizadas em uma determinada região do espaço com retenção de suas atividades catalíticas e que podem ser usadas repetida e continuamente (MOHAMAD et al., 2015).

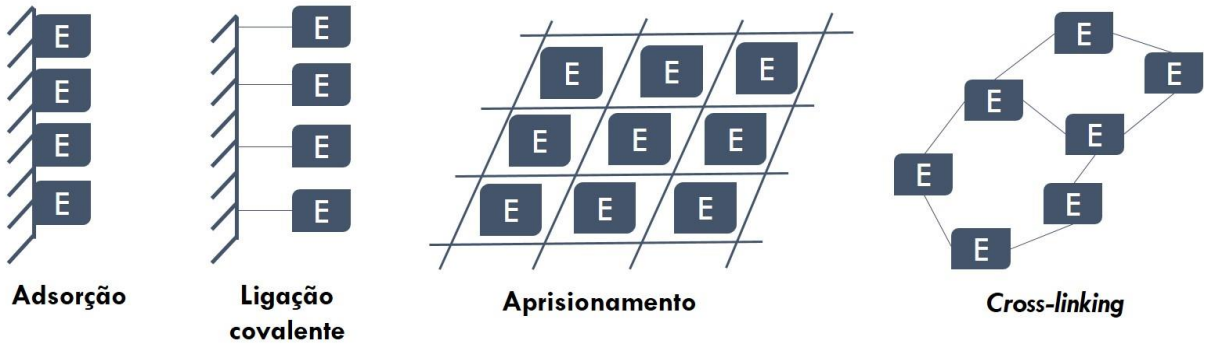
As principais vantagens associadas ao processo de imobilização são a possibilidade de reutilização das enzimas ao longo de vários ciclos catalíticos sucessivos e a melhora significativa na estabilidade das enzimas sob várias condições operacionais (pH, temperatura, solventes, contaminantes e impurezas). Além disso, com a imobilização surge a possibilidade de se interromper rapidamente a reação pela remoção da enzima do meio reacional (ou vice-versa), especialmente útil nas indústrias alimentícia e farmacêutica, e consequentemente obter um produto com maior grau de pureza, livre de contaminação pela presença da enzima (ZHANG; GE; LIU, 2015). Como desvantagens associadas à imobilização de enzimas, em alguns casos, podem ser observadas redução na atividade enzimática e limitação difusional, sendo que esta última afeta diretamente os parâmetros cinéticos da reação catalisada pela enzima (BRENA; BATISTA-VIERA, 2013).

Uma etapa crucial no processo de imobilização é a escolha do suporte, esta deve ser realizadas levando em consideração o tipo da enzima e as condições de processo no qual o biocatalisador será utilizado. Além disso, existem características ideais que devem ser buscadas em um suporte que incluem resistência física à compressão, estabilidade química e térmica,

possibilidade de regeneração e reutilização, hidrofilia, ser inerte, presença de grupos reativos, biocompatibilidade, alta afinidade pelas enzimas, resistência a ataques microbianos, alta disponibilidade e baixo custo. Uma grande variedade de materiais de diferentes origens pode ser utilizada como suporte para imobilização. Estes materiais podem, em geral, ser divididos em orgânicos, inorgânicos e híbridos ou compostos e podem ser agrupados em duas grandes categorias: materiais clássicos e novos materiais. Entre os materiais clássicos, inclui-se os óxidos metálicos, minerais, materiais a base de carbono, biopolímeros e polímeros sintéticos. Já os novos materiais incluem os nanomateriais (magnéticos ou não), materiais mesoporosos, materiais híbridos e membranas poliméricas (EDET; NTEKPE; OMEREJI, 2013; ZDARTA et al., 2018).

Com relação aos métodos de imobilização de enzimas podem ser agrupados em quatro categorias baseados no princípio geral de cada técnica: adsorção, aprisionamento (*entrapment*), ligação covalente e ligações cruzadas (*cross linking*). Entretanto, alguns autores como Sheldon e Van Pelt (2013) agrupam os métodos de imobilização em apenas três categorias: ligação ao suporte (nesta incluem os dois métodos que se baseiam em ligações químicas: adsorção e ligação covalente), aprisionamento e ligações cruzadas. Representações gerais dos princípios de funcionamento de cada método podem ser visualizadas na Figura 4.

Figura 4. Principais métodos utilizados para imobilização de enzimas. Fonte: O autor.



A adsorção é o mecanismo mais simples e mais antigo empregado para imobilizar enzimas, esta faz uso das interações físicas geradas entre o transportador e a enzima, que incluem forças de van der Waals, interações iônicas e pontes de hidrogênio. A ligação é bastante fraca e, o que é importante, pois normalmente não altera a estrutura nativa da enzima. Este tipo de imobilização é comumente agrupado em três subcategorias: adsorção física, ligações eletrostáticas e adsorção hidrofóbica.

A principal vantagem desse processo é a simplicidade de execução, uma vez que são necessários poucos passos para a ativação do suporte e não são necessários reagentes para esse

processo e a estrutura conformacional da enzima é pouco alterada. Basicamente a imobilização por adsorção consiste no contato entre o suporte sólido e a solução enzimática por um período fixo de tempo sob condições adequadas que mantenham a atividade enzimática. As enzimas não adsorvidas podem ser facilmente removidas da superfície do suporte através de lavagem com água ou solução tampão (JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014; NGUYEN; KIM, 2017). A grande desvantagem dessa técnica é a dessorção da enzima devido às variações de temperatura, pH e força iônica (AHMAD; SARDAR, 2015).

Com relação aos materiais que podem ser empregados como suporte para adsorção enzimática, podem-se afirmar que qualquer material pode ser utilizado para essa finalidade, todavia nem toda enzima pode ser imobilizada em todos os suportes. Isso se deve ao fato de que deve haver a presença de certos grupos ativos na superfície do suporte para que ocorram as interações enzima-suporte que mantenham o biocatalisador imobilizado. Em casos, em que essas interações sejam ausentes ou minimizadas, estas podem ser ajustadas pela aplicação de agentes intermediários que atuam na funcionalização do suporte (JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014).

Entre os principais materiais empregados como adsorventes incluem alumina, bentonita, carbonato de cálcio, fosfato de cálcio, carbono, celulose, carvão, argila, colágeno, terra de diatomáceas, vidro, resinas de troca iônica e sílica gel (EDET; NTEKPE; OMEREJI, 2013). Entre os poucos relatos na literatura envolvendo a imobilização de enzimas com atividade de transfrutosilação por adsorção, pode-se mencionar o estudo realizado por Aguiar-Oliveira e Maugeri (2010), o qual reporta a imobilização FTase de *Rhodotorula* sp. em minério de nióbio. Estes autores reportam a utilização da chamada imobilização orientada pela presença do substrato de modo a aumentar a efetividade da imobilização.

Outro método de imobilização que também se utiliza de ligações químicas é a formação de uma ligação covalente irreversível entre a superfície do suporte e os grupos funcionais presentes na enzima, desde que estes não sejam responsáveis pela atividade catalítica. Estas ligações podem ocorrer a partir das cadeias laterais de diferentes aminoácidos como lisina (grupo ε-amino), cisteína (grupo tiol) e ácidos aspártico e glutâmico (grupo carboxílico) (AHMAD; SARDAR, 2015). A imobilização por ligação covalente geralmente é escolhida quando se requer um produto livre da presença da enzima, o que é possível pela força da ligação covalente que não permite que a enzima seja desprendida e liberada na solução. Além disso, o biocatalisador imobilizado apresenta uma maior resistência quanto a variações de pH, temperatura e presença de solventes orgânicos. No entanto esse método apresenta como principal desvantagem a baixa recuperação de atividade, que é o resultado da alteração da

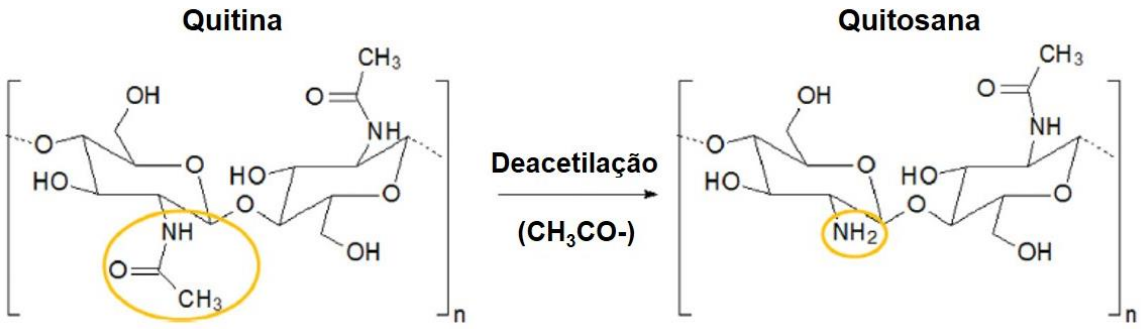
conformação ativa da enzima durante a reação de imobilização (ZHANG; YUWEN; PENG, 2013).

O processo de ligação entre a enzima e o suporte geralmente ocorre em duas etapas: ativação da superfície do suporte, geralmente pela presença de agentes de ligação, e acoplamento entre a enzima e o suporte ativado. O processo de ativação tem como objetivo gerar grupos eletrofílicos no suporte, para que na etapa de acoplamento reajam com os nuceófilos fortes nas proteínas. Neste processo, os reagentes bifuncionais como o glutaraldeído são amplamente utilizados, uma vez que reagem com vários grupos funcionais de proteínas e a ligação formada é excepcionalmente estável a temperaturas e pH extremos e facilita a ligação covalente multipontual (MIGNEAULT et al., 2004).

Já o acoplamento entre a enzima e o suporte pode ocorrer por ligação direta entre os componentes ou através de ligações intercaladas de comprimento diferente formado por espaçadores. A molécula espaçadora confere um maior grau de mobilidade a enzima acoplada, de modo que a sua atividade pode ser melhorada quando comparada com a enzima acoplada diretamente. Além disso, outro procedimento que pode melhorar o rendimento da atividade enzimática é a realização da reação de acoplamento na presença de análogos do substrato da referida enzima (AHMAD; SARDAR, 2015; BRENA; BATISTA-VIERA, 2013).

Entre as principais matrizes utilizadas como suporte para imobilização por ligação covalente, a quitosana apresenta-se como uma alternativa bastante viável principalmente devido a ser inerte, não tóxica, hidrofílica, biocompatível e por apresentar uma afinidade notável às proteínas. A quitosana consiste em um copolímero randômico obtido a partir da desacetilação da quitina, conforme ilustrado pela Figura 5, formada por unidades de d-glicosamina e N-acetil-d-glicosamina, ligadas por ligações glicosídicas β-1,4. Em termos de estrutura química, a quitosana possui uma estrutura molecular quimicamente similar à da celulose, diferenciando-se somente nos grupos funcionais. Quitina e quitosana são obtidas a um custo relativamente baixo sendo extraídas a partir de conchas de moluscos (principalmente caranguejos, camarões, lagostas e krills) e resíduos da indústria de processamento de frutos do mar (KRAJEWSKA, 2004; MENDES et al., 2011; MUXIKA et al., 2017).

Figura 5. Estrutura da quitina e quitosana com destaque para alteração no grupamento funcional após a desacetilação. Fonte: Adaptado de MUXIKA et al. (2017).



Para imobilização de enzimas, a quitosana é utilizada sob a forma de pós, flocos e géis de diferentes configurações geométricas, sendo a forma esférica preferencialmente usada. Devido a quitosana ser solúvel em soluções ácidas, as esferas de quitosana geralmente são produzidas pela coagulação desse polímero em soluções alcalinas. A imobilização por ligação covalente em esferas de quitosana já é largamente reportada na literatura envolvendo uma grande variedade de enzimas, todavia, existem poucos trabalhos envolvendo enzimas formadoras de FOS, podendo citar o estudo realizado por Lorenzoni et al. (2014) no qual foi imobilizada a FFase de *A. aculeatus* presente na preparação comercial Viscozyme L parcialmente purificada e que foi posteriormente aplicada na produção de FOS em reatores enzimáticos (LORENZONI et al., 2015) e de Smaali et al. (2012) que imobilizaram a FFase bruta obtida a partir de *A. awamori*.

Diferente dos métodos de imobilização por adsorção e ligação covalente, a imobilização por aprisionamento ou enclausuramento não ocorre por ligações químicas na superfície do suporte, mas consiste basicamente em uma restrição física da enzima dentro de um espaço ou rede confinada, não interagindo quimicamente com o polímero de aprisionamento. Esse tipo de imobilização apresenta as seguintes vantagens: propicia um microambiente que protege a enzima contra a desnaturação e contaminação externa, é rápido e necessita de condições amenas de processo para ser realizado. Todavia, apresenta como desvantagem a limitação de transferência de massa (NGUYEN; KIM, 2017; NISHA; KARTHICK; GOBI, 2012).

De modo geral, processos de imobilização que utilizam essa técnica são conduzidos através de duas etapas: mistura da enzima em uma solução de monômero e, em seguida, polimerização da solução de monômero por reação química ou alteração das condições experimentais. Esta segunda etapa pode ocorrer a partir de diferentes métodos a depender do suporte utilizado, tais como gelificação ionotrópica (ex. alginato), gelificação induzida por

temperatura (ex. agarose, gelatina), reação orgânica de polimerização (ex. poliacrilamida) e precipitação por solvente imiscível (ex. poliestireno) (HASSAN; TAMER; OMER, 2016).

Para esse tipo de imobilização é possível utilizar os seguintes polímeros como uma matriz: alginato, carragena, colágeno, poliacrilamida, gelatina, poliuretano e álcool polivinílico (MOHAMAD et al., 2015). Entre estes o alginato é a principal matriz devido principalmente a suas propriedades gelificantes e não toxicidade. Bedzo et al. (2019) realizaram um estudo comparativo envolvendo a imobilização da FFase recombinante de *A. japonicus* por adsorção em resinas de troca iônica (Amberlite) e por aprisionamento em esferas de alginato e foram observados melhores rendimentos de imobilização na imobilização via aprisionamento. Fernandez-Arrojo et al. (2013) imobilizaram a preparação comercial Pectinex Ultra SP-L em esferas de alginato que foi posteriormente submetida ao processo de liofilização.

Existem métodos de imobilização que dispensam a utilização de suporte e que utilizam ligações cruzadas entre moléculas de enzima para formar uma grande estrutura tridimensional que pode ser conseguida por métodos químicos e físicos. Uma vantagem primária desta técnica consiste no fato de a atividade enzimática não ser "dissolvida" por uma massa não catalítica, ou seja, o suporte. A ligação cruzada pode ser realizada através da enzima solúvel (*Cross-linked Enzymes*, CLEs), de enzimas cristalizadas (*Cross-linked Enzyme Crystals*, CLECs) ou por meio de agregados enzimáticos (*Cross-linked Enzyme Aggregates*, CLEAs), sendo que devido a difícil reprodutibilidade a utilização dos CLEs entrou em desuso (CAO; VAN LANGEN; SHELDON, 2003; ELNASHAR, 2010).

CLECs são produzidas por ligações cruzadas entre cristais de enzimas purificadas. Apresentam excelentes propriedades biotecnológicas tais como: alta estabilidade sobre condições adversas (altas temperaturas, valores extremos de pH e solventes orgânicos), resistência a autólise, no caso das proteases, e proteólise exógena. Todavia esse método apresenta como principal desvantagem o custo elevado para conferir alto grau de pureza a enzima que se pretende cristalizar, devido a isso poucos processos têm sido realizados com essa técnica. Já, os CLEAs são produzidos pela ligação cruzada de agregados de proteínas produzidos através de técnicas convencionais de precipitação, ao contrário dos CLECs essa técnica não requer enzimas purificadas. As principais vantagens dessa técnica são a possibilidade de co-imobilizar mais de uma enzima proporcionando complexos capazes de catalisar múltiplas reações de forma independente ou em sequência e em alguns casos o fenômeno de hiperativação, que consiste no aumento da atividade enzimática pós imobilização, e é atribuído à agregação da enzima em uma estrutura terciária pré-organizada que a tornou permanentemente insolúvel na formação da ligação cruzada. Todavia o principal problema na

preparação dos CLEAs ocorre na etapa de precipitação da enzima, onde frequentemente há perda substancial de atividade enzimática (CHAPMAN; ISMAIL; DINU, 2018; SHELDON, 2011).

Após apresentar os diferentes métodos de imobilização de enzimas, é importante mencionar a importância da nanotecnologia no campo da imobilização enzimática, sobretudo no desenvolvimento de nanomateriais que atuam como suporte. Estes possuem características ideais para equilibrar os principais fatores que determinam a eficiência dos biocatalizadores, incluindo alta área superficial, baixa limitação de transferência de massa, boa resistência à transferência de massa, carga enzimática efetiva e excelente mobilidade de partículas durante a reação. Diferentes tipos de nanomateriais encontram-se disponíveis para imobilização enzimática, por exemplo, nanopartículas, nanotubos de carbono, nanopartículas magnéticas, meios mesoporosos, nanofibras, nanocompósitos, *nanorods* e materiais sol-gel contendo partículas de tamanho nanométrico (AHMAD; SARDAR, 2015; LIU; WANG; JIANG, 2012).

As nanopartículas magnéticas de óxido de ferro (Fe_3O_4) (*magnetic nanoparticles*, MNPs) apresentam-se como um excelente nanomaterial. Pois, além das vantagens mencionadas acima ainda possuem a facilidade de separação do meio reacional pela aplicação de um campo magnético externo, biocompatibilidade, baixa toxicidade, maior estabilidade da enzima, possível modulação da especificidade catalítica, menor custo operacional e abundância de grupos hidroxila em sua superfície, o que facilita a modificação e possibilita a formação de ligações covalentes com a enzima (ANSARI; HUSAIN, 2012; ZDARTA et al., 2018). As MNPs podem ser sintetizadas através dos métodos de co-precipitação, microemulsão, decomposição térmica e síntese hidrotérmica. Entre estes, a co-precipitação apresenta-se como sendo uma metodologia conveniente e prática, uma vez que consiste basicamente na formação de precipitado a partir de soluções salinas aquosas na presença de uma base resultando em elevado rendimento e baixa distribuição de tamanho entre as nanopartículas. O rendimento final e tamanho das partículas depende diretamente das condições de síntese (temperatura e pH), tipo de sais utilizados e relação Fe^{2+}/Fe^{3+} entre outras (SEENUVASAN et al., 2014).

Sabe-se que MNPs de Fe_3O_4, são altamente sensíveis a condições ácidas e oxidativas e possuem a tendência de se agregar em meios líquido devido às fortes atrações dipolo-dipolo entre as partículas. Assim, a modificação ou funcionalização das MNPs é extremamente importante para manter a estabilidade das nanopartículas, para esta finalidade alguns polímeros biocompatíveis e biodegradáveis com grupos funcionais específicos tem sido utilizado como com estabilizadores (CHEN et al., 2018; PAN et al., 2009). Entre estes polímeros, a quitosana (CS) tem se destacado e sido amplamente utilizada como estabilizante em as nanopartículas

magnéticas (MNPs-CS) para imobilizar uma grande variedade de enzimas. Isso porque a presença de grupos amino na quitosana possibilita a ativação por glutaraldeído e formação de ligação covalente entre a enzima e o suporte possibilitando uma imobilização multipontual que promove uma maior rigidez da estrutura molecular da enzima, o que reduz as alterações conformacionais proporcionadas pelas condições do meio reacional. Relatos envolvendo a imobilização de enzimas produtoras de FOS em nanomateriais ainda são escassos, sendo o trabalho de Chen, Sheu e Duan (2014) envolvendo a imobilização da FFase de *A. japonicus* em MNPs-CS o único relato na literatura.

1.9. Reatores enzimáticos

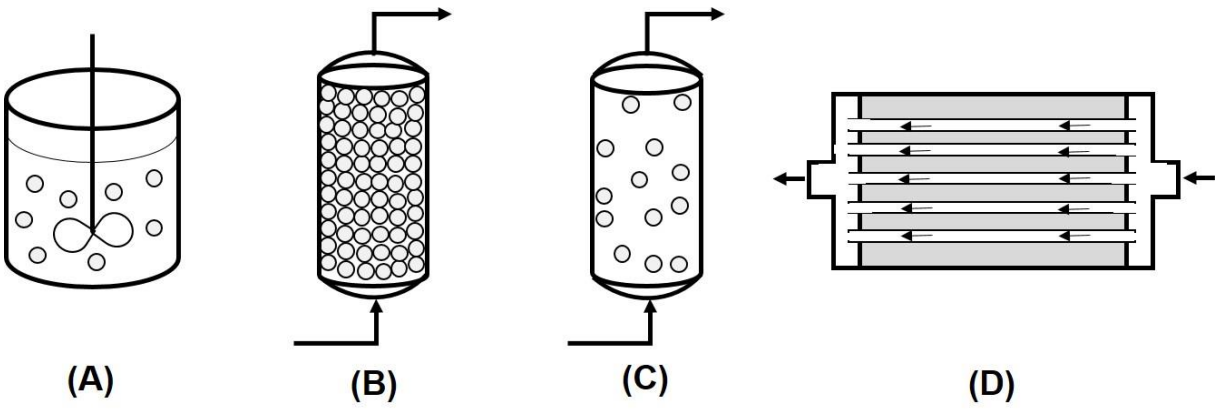
Reatores enzimáticos ou biorreatores são muito utilizados em processos industriais que envolvem catálise enzimática, sendo considerados o coração dos processos biotecnológicos e definidos como o equipamento onde substratos são convertidos a produtos desejados através da ação enzimática podendo operar através de processo contínuo ou descontínuo. Os processos de biocatálise podem ser realizados utilizando enzimas livres ou imobilizadas. Sendo estas últimas preferidas por serem em geral mais estáveis e possibilitar a reutilização do biocatalisador, além de permitirem uma variedade maior de configurações do reator e possibilitar operação por processos contínuos (ILLANES, 2008).

Os bioprocessos envolvendo reatores enzimáticos, podem ser operados através de processo em descontínuo ou em batelada e contínuo. Em geral, os custos operacionais totais dos reatores que operam em fluxo contínuo são geralmente significativamente menores que os processos equivalentes em batelada, uma vez que estes últimos precisam ser esvaziados e reabastecidos frequentemente em intervalos regulares. Esse procedimento não é apenas caro, mas também significa que há períodos consideráveis em que esses reatores não são produtivos (o chamado "tempo de inatividade"). Além disso, os processos em batelada geram demandas desiguais na mão de obra e nos serviços e podem apresentar variações pronunciadas a cada batelada, uma vez que as condições de reação mudam com o tempo, e podem ser difíceis de escalonar, devido à mudança nos requisitos de energia para uma mistura eficiente. Uma vez que apresentam maior eficiência geral, os processos contínuos usando enzimas imobilizadas podem ser realizados em instalações de produção que são cerca de 10 a 100 vezes menores do que as exigidas para processos descontínuos equivalentes usando enzimas solúveis. Portanto, os custos

de capital envolvidos na instalação da instalação também são consideravelmente menores (ROBINSON, 2015).

Além da definição sobre a condição do processo, a decisão sobre que tipo de reator a se utilizar é o primeiro passo no projeto, sendo que a seleção tipo de reator mais apropriado para um determinado bioprocesso depende das características da bioconversão e de condições reacionais, cinéticas e ligadas ao biocatalisador, que vão determinar o modo de operação e características do fluxo. Os principais reatores utilizados para enzimas imobilizadas são pertencentes aos seguintes tipos: tanque agitado (*Stirred Tank Reactor*, STR), leito empacotado (*Packed-bed Reactor*, PBR), leito fluidizado (*Fluidized Bed Reactor*, FBR) e de membrana. A Figura 6 apresenta uma representação esquemática do funcionamento destes reatores.

Figura 6. Representações esquemáticas do funcionamento das principais configurações de reatores utilizados em processos enzimáticos: (A) reator tipo tanque agitado, (B) leito empacotado, (C) leio fluidizado e (D) reator de membrana. Fonte: O autor.



Os reatores mais comuns utilizados são os do tipo tanque agitado também conhecidos como reatores de mistura, que por possuírem ampla utilização são definidos como padrão quando se deseja estudar a cinética de um processo fermentativo. Este tipo de reator consiste basicamente de um tanque cilíndrico e um agitador, sendo comumente equipado com chicanas que possuem a função de evitar a formação de vórtice durante a agitação do líquido e pode operar em diferentes tipos de processo (descontínuo e contínuo). A produção de FOS utilizando deste tipo de reator pode ser realizada tanto por enzimas imobilizadas quando por células microbianas, esta última será discutida na seção 1.8. Alvarado-Huallanco e Maugeri-Filho (2010) e Detofol et al. (2015), pertencentes ao mesmo grupo de pesquisa, realizaram estudos envolvendo a produção de FOS a partir da FTase proveniente de *Rhodoturula* sp. imobilizada em minério de nióbio e modelagem matemática da produção.

Os reatores de leito fixo ou empacotado constituem-se em uma alternativa bastante interessante para condução de bioprocessos envolvendo enzimas imobilizadas, 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 (HAMA et al., 2011).

Uma alternativa aos reatores de leito empacotado são os reatores de leito expandido ou fluidizado, no qual as partículas da enzima são retidas por um equilíbrio hidrodinâmico entre a gravidade e as forças de arrasto promovidas pela corrente de substrato ascendente. Estes reatores são caracterizados por suas forças de baixa fricção em comparação com reatores de tanque agitado e apresentam muitas vantagens, como boa mistura e condições isotérmicas, em comparação com os leitos empacotados. Estes reatores têm sido adaptados para utilização de campos magnéticos, de modo a possibilitar a utilização de enzimas imobilizadas em partículas magnéticas. Essa abordagem tem-se mostrado muito interessante uma vez que possibilita a estabilização do leito com ausência de forças de cisalhamento o que torna o leito um meio mais susceptível a catálise enzimática (AL-QODAH et al., 2017). A utilização de reatores de leito empacotado e fluidizado para produção de FOS já foi reportada por Lorenzoni et al. (2015), estes autores utilizaram a preparação comercial com atividade de transfrutosilação Viscozyme L. Vaňková et al. (2008) elaboraram um fluxograma de um processo industrial de produção de FOS utilizando reatores de leito empacotado na etapa de síntese de FOS propriamente dita.

Um reator baseado em membranas consiste basicamente em uma membrana porosa na qual enzimas ou células microbianas inteiras são imobilizadas nesta membrana (microfiltração ou ultrafiltração) que podem operar tanto em processos contínuos quanto descontínuos. Este tipo de reator pode ser utilizado de duas formas, na primeira os substratos e as enzimas são misturados num reator e posteriormente os produtos são permeados através das membranas. Neste tipo de processo o tamanho molecular da enzima e do produto devem ser levados em consideração para se selecionar o tamanho adequado dos poros das membranas. Na segunda abordagem, as enzimas são imobilizadas em uma membrana através da qual os substratos foram passados, e estes processados pelo catalisador imobilizado. Esta segunda abordagem possibilidade a reutilização do biocatalisador resultado em um processo de menor custo (KUMAR; DUBEY, 2019).

Os reatores de membrana já têm sido utilizados com sucesso em processos fermentativos, gestão de resíduos e remoção de poluentes da água. Entretanto, as pesquisas envolvendo a produção de FOS ainda é muito reportada. Rehman et al. (2016) realizaram a produção de FOS neste tipo de reator utilizando a preparação comercial Pectinex Ultra SP-L imobilizada no módulo de membrana e obtiveram um rendimento de 63% na produção de FOS após 4 h utilizando melaço de cana de açúcar como substrato.

1.10. Produção de FOS utilizando células microbianas

Além da produção de FOS utilizando as enzimas isoladas, também é possível realizar esse processo utilizando as células inteiras de um dado micro-organismo cultivado em meio de cultura otimizado, geralmente composto por sacarose e micronutrientes, para facilitar a síntese dos oligossacarídeos. Essa abordagem é vantajosa primeiramente do ponto de vista econômico, uma vez que os custos envolvendo as etapas de extração e purificação enzimática são inexistentes e por ocorrer em apenas uma etapa os custos totais do processo final são inferiores ao processo enzimático. Além disso, a utilização de células microbianas apresenta vantagens do ponto de vista produtivo, pois, em alguns casos o rendimento é ligeiramente superior ao rendimento máximo teórico obtido pela utilização de enzimas que é de 55–60% (GANAIE et al., 2014; NOBRE et al., 2019).

Diferentes linhagens microbianas têm sido utilizadas para esse tipo de processo, principalmente os fungos filamentosos, conforme pode ser visualizado na Tabela 3. Nesta também consta os respectivos rendimentos e produtividades na produção de FOS para cada linhagem microbiana. Estudos envolvendo a produção de FOS utilizando células são inicialmente realizados em frascos agitados a fim de obter o meio com condições otimizadas para posteriormente serem conduzidos em biorreatores que tradicionalmente são do tipo tanque agitado, mas também podem ser realizados em reatores não convencionais como o *air lift* (SÁNCHEZ et al., 2008). Além de utilizar uma única cultura, alguns autores têm realizado esse processo com um fungo filamento e uma co-cultura geralmente a levedura *Saccharomyces cerevisiae* devido a sua capacidade de reduzir a concentração de glicose presente no meio. Entretanto, foi observado que devido a competição entre esses micro-organismos, o rendimento final foi ligeiramente inferior ao obtido utilizando apenas uma linhagem (NOBRE et al., 2016).

As células microbianas também podem ser utilizadas em sua forma imobilizada, uma vez que esta possibilita uma melhor resistência térmica, química e ao cisalhamento. Além de permitir a reutilização das células repetidas vezes para produção de FOS, melhorando o impacto

econômico do processo global. Para a imobilização das células microbianas as mesmas técnicas mencionadas para imobilização de enzimas, tais como aprisionamento, microencapsulação, ligação covalente e ligações cruzadas (*cross linking*) já tem sido utilizadas para produção de FOS (CASTRO et al., 2019). Ganaie et al. (2014) reportaram a produção de FOS utilizando micélios de *A. flavus* imobilizados em alginato e quitosana e obtiveram 67,75 e 42,79% de conversão de FOS, respectivamente.

Além da produção de FOS utilizando células microbianas por fermentação submersa utilizando sacarose este processo também pode ser realizado via fermentação em estado sólido utilizando substratos alternativos. Este tipo de abordagem ainda é pouco utilizada entretanto tem-se mostrado promissora por possibilitar altos rendimentos de produção de FOS. Mussatto e Teixeira (2010) reportaram a produção de FOS por *A. japonicus* utilizando espigas de milho, tegumentos de grãos de café, sobreiro e fibras sintéticas obtendo rendimentos entre 61-70%. Posteriormente, estes autores realizaram um estudo de otimização da produção utilizando tegumentos de grãos de café como substrato e obtiveram 87% de rendimento (MUSSATTO et al., 2012).

Tabela 3. Produção de FOS por cultivo submerso de células microbianas utilizando sacarose como substrato.

Micro-organismos	**Tipo de cultivo**	**Tempo (h)**	**FOS (g/L)**	**Y[1] (%)**	**Q[2] (g.L.h)**	**Referência**
Aspergillus sp.	Reator *airlift*	26	-	69	-	(SÁNCHEZ et al., 2008)
Aspergillus ibericus	Reator STR	38	118,0	64	-	(NOBRE et al., 2018a)
A. ibericus e *S. cerevisiae*	Reator STR	45	133,7	70	-	(NOBRE et al., 2018b)
Aspergillus niger	Frascos agitados	40	314,6	51,1	-	(ZENG et al., 2016)
Aureobasidium pullulans	Frascos agitados	48	-	64,1	-	(DOMINGUEZ et al., 2012)
A. pullulans e S. cerevisiae	Reator STR	20	119,0	59	5,90	(CASTRO et al., 2019)
Bacillus subtilis	Frascos agitados	24	98,86	33	-	(BITTENCOUR; BORSATO; COLLIGOI, 2014)
Cladosporium cladosporioides	Frascos agitados	96	277,0	56	-	(ZAMBELLI et al., 2014)
Microbacterium paraoxydans	Frascos agitados	14	155,0	44	-	(OJHA; RANA; MISHRA, 2016)
Penicillium citreonigrum	Reator STR	48	126,3	65	2,28	(NOBRE et al., 2019)
Penicillium expansum	Frascos agitados	48	117,7	58	3,25	(PRATA et al., 2010)
Penicilium sizovae	Frascos agitados	96	184,0	31	-	(ZAMBELLI et al., 2014)
Sacharomyces cerevisiae	Frascos agitados	12	23,25	-	-	(DEFFERT et al., 2017)
*Xanthophyllomyces dendrorhous**	Reator STR	30	115,1	49	3,84	(SHEU et al., 2013)

[1]Rendimento.

[2]Produtividade.

*Produção de Neo-FOS.

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Production of β-fructofuranosidase with transfructosylating activity by *Aspergillus tamarii* URM4634 Solid-State Fermentation on agroindustrial by-products

Rodrigo Lira de Oliveira[1], Marcos Fellipe da Silva[2], Attilio Converti[3], Tatiana Souza Porto[2*].

[1]Northeast Biotechnology Network/RENORBIO, Federal Rural University of Pernambuco (UFRPE), Dom Manoel de Medeiros, Dois Irmãos, 52171-900 Recife, PE, Brazil.
[2]Academic Unit of Garanhuns/UAG, Federal Rural University of Pernambuco (UFRPE), Garanhuns, Av. Bom Pastor, Boa Vista, 55296-901 Garanhuns, PE, Brazil.
[3]Department of Civil, Chemical and Environmental Engineering, Pole of Chemical Engineering, Genoa University, via Opera Pia 15, 16145 Genoa, Italy

*Corresponding author
Tel.: +55 81 33206345.
Email address: portots@yahoo.com.br

ABSTRACT

Eight different *Aspergillus* strains were tested for their ability to produce β-fructofuranosidase (FFase) by Solid-State Fermentation. The *Aspergillus tamarii* URM4634 strain was selected as the most performant and tested on six different agroindustrial by-products. Soy, wheat and oat brans, which allowed for the highest hydrolytic (U_H) and transfructosylating (U_{TF}) activities, were tested individually or in mixtures according to a simplex-centroid mixture design in order to investigate their effects on FFase production at different times. The best results in terms of both enzyme activities were obtained with only soy bran. The influence of substrate, moisture and sucrose levels on FFase production was evaluated, and the highest U_H and U_{TF} activities were 229.43±4.88 and 66.93±3.02 U/mL, respectively. The obtained results indicate that *A. tamarii* FFase may be a biocatalyst with great potential for industrial applications such as sugar inversion and fructo-oligosaccharides production.

Keywords: β-fructofuranosidase; Fructo-oligosaccharides; Fermentation; Mixture design; Agroindustrial by-products.

1. Introduction

β-fructofuranosidase (FFase, EC 3.2.1.26), also named invertase, is an enzyme widely used in food industry that catalyzes the hydrolysis of sucrose β-2,1 glycosidic bonds, thereby producing invert sugar, an equimolar mixture of D-glucose and D-fructose [1]. FFases are included in the GH32 family of glycosyl hydrolases and classified in different isoforms, based on their pH of action, as acid, alkaline and neutral enzymes [2]. The main application of FFases is the production of sugar syrups such as high fructose syrup, high fructose corn syrup and high glucose syrup, which are extensively used in food industry to prepare creams, marshmallows, powder milk for infants, liquefied sugar-containing candies, chocolate covered cherries, digestive aid tablets and artificial honey, as well as in cosmetics as plasticizing agent [3,4]. The glucose obtained by sucrose hydrolysis can be used as natural osmolyte and several applications [5].

At high sucrose concentration, some FFases are able to catalyze the transfer of fructosyl residues to an acceptor compound to produce fructo-oligosaccharides (FOS) by two ways: reverse hydrolysis and transfructosylation [6]. FOS are prebiotic substances consisting of fructose units polymerized to different extent that display several biological and functional properties [7], among which 1-kestose, nystose and 1-fructofuranosyl-nystose are the best known. Some benefits associated with FOS ingestion are stimulation of probiotics growth in the intestinal tract, enhancement of calcium and magnesium absorption, reduction of total cholesterol and prevention of colonic carcinogenesis [8,9].

Most FFases with high transfructosylating activity have been found in fungi belonging the *Aureobasidium* [10], *Penicillium* [2,11] and *Aspergillus* [12] genera. They can be produced by either Submerged (SmF) or Solid-State (SSF) Fermentation. The latter is a process that uses a solid matrix, which is carried out in the absence or a very limited quantity of free water; however, the substrate must possess enough moisture to support microbial growth and metabolism [13]. SSF is considered the most appropriate process when using fungi, because solid substrates resemble their natural habitat, hence improving their growth and secretion of a wide range of extracellular enzymes [14]. An important advantage of SSF is associated with the use of agroindustrial by-products such as seeds, peels, husks and brans as substrates to produce valuable bioactive molecules, which provides economic feasibility and environmental friendliness to the entire process [15,16].

Enzyme production by SSF was extensively studied usually employing only one substrate and techniques of Design of Experiments (DOE) to optimize the influencing variables.

However, the use of statistical mixture designs to evaluate different substrate formulations for SSF is missing, as far as we are aware, when aiming at the production of FFases or other enzymes to synthesize FOS such as fructosyltransferases or inulinases. Mixture designs are a special class of response surface designs where the factors are the components of a mixture, and the responses are influenced by the proportions variation [17]. Such an approach to evaluate the interactions of different agroindustrial co-substrates in enzyme production by SSF was only reported for glutaminase [18], fructosyltransferase [19], L-asparaginase [20], protease, α-amylase [21] and a multi enzymatic complex composed by lipase, carboxymethyl cellulase, β-glucosidase and α-amylase [22].

Based on this background, the aim of this study was to study β-fructofuranosidase production by *Aspergillus tamarii* URM4634 in different agroindustrial co-substrates by SSF. A simplex centroid mixture design was performed to investigate the occurrence of synergistic or antagonistic effects among different substrates (wheat, soy and oat brans) on FFase production. Moreover, we explored the microbial growth as well as the variables mostly influencing the fermentation process, namely moisture, substrate amount and inducer concentration.

2. Materials and Methods

2.1. Microorganisms

Eight fungal strains belonging to the *Aspergillus* genus, provided by "Micoteca-URM" of Mycology Department, Centre of Biosciences of Federal University of Pernambuco (UFPE), Recife, PE, Brazil, were used to investigate FFase production with special focus on the enzyme transfructosylating activity. The strains tested, namely *A. aculeatus* URM4953, *A. heteromorphus* URM269, *A. japonicus* URM5620, *A. niveus* URM5870, *A. phoenicis* URM4924, *A. tamarii* URM4634, *A. terreus* URM4658 and *A. versicolor* URM5701, were preserved in mineral oil, maintained at room temperature (25±1 °C) in Czapek Dox Agar medium and grown in reactivation broth with the following composition (%, w/v): bacteriological peptone, 1.0; meat extract, 0.3; and glucose, 2.0. After 3 days they were inoculated in Potato Dextrose Agar medium for 7 days at 30 °C.

2.2. FFase production by Solid-State Fermentation

Screening of the best strain for FFase production was performed by Solid-State Fermentation (SSF) using wheat bran as a substrate. The fermentation was performed at 30°C for 72 h in 125 mL Erlenmeyer flasks containing 5 g of substrate, nutrition solution (10% sucrose and 0.5% yeast extract) and a spore suspension (10^7 spores/mL) corresponding to a 50% moisture content. The FFase crude extract was obtained by addition of 7.5 mL of 0.1 M acetate buffer (pH 5.0) per gram of fermented material and subsequent homogenization in orbital shaker for 90 min at 120 rpm. Solids were removed by centrifugation at 5000 rpm for 15 min at 4°C, and the crude extract was analyzed and stored at -22°C.

The *Aspergillus* strain that displayed the highest hydrolytic (U_H) and transfructosylating (U_{TF}) activities was submitted to a screening on different agroindustrial by-products as substrates, namely wheat bran, soy bran, oat bran, corn cobs, orange and lemon peels with granulometry between 0.5 and 2.0 mm. All the by-products were obtained in a local market in the city of Garanhuns, PE, Brazil. Fermentation conditions were the same as those used for strain screening. The activity results were expressed as arithmetic mean ± standard deviation, and the Tukey's test was used to check significant differences ($p < 0.05$) among samples.

2.3. Statistical mixture design and statistical analysis

To study the effect of fermentation medium composition on FFase production, we used a three-component simplex-centroid mixture design in which wheat, oat and soy brans were selected as the independent variables at four levels, i.e., different proportions of these components, namely 0 (0%), 1/3 (33%), 1/2 (50%) and 1 (100%). For this purpose, both U_H and U_{TF} were determined at different fermentation times (24, 48, 72 and 96 h) and expressed as arithmetic means ± standard deviations. The Tukey' test was used to check significant differences ($p < 0.05$) among different samples after a given fermentation time.

The following regression models were fitted to the experimental data of FFase activity:

$$Y_i = \sum_{i=1}^{q} \beta_i x_i + \sum \sum_{i<j}^{q} \beta_{ij} x_i x_j + \sum \sum_{i<j<k}^{q} \sum \beta_{ijk} x_i x_j x_k \quad (1)$$

where Y_i is the predicted response (enzyme activity); q is the number of components of the system (3); x_i, x_j and x_k are the coded levels of the independent variables, i.e., wheat, oat and soy bran, respectively; β_i are the coefficients of linear terms, and β_{ij} and β_{ijk} those of binary and ternary interaction terms, respectively.

The statistical analysis of the experimental design was performed using the Statistica 7.0 software package (Statsoft Inc., Tulsa, OK, USA), while the quality of fit of the above models was checked by the analysis of variance (ANOVA), the *F* test and the coefficient of determination (R^2), being considered acceptable $R^2 > 0.90$,

2.4. Full factorial design of experiments of FFase production by SSF

After choosing the best strain and substrate, FFase production experiments were performed according to a 2^3-full factorial design plus three central points, where the independent variables were substrate mass (3, 5 and 7 g), moisture content (40, 50 and 60 %) and sucrose concentration (5, 10 and 15% w/v). The statistical analysis of results obtained according to this experimental design was performed using the same software package mentioned above.

2.5. Hydrolytic and transfructosylating FFase activities

Both U_H and U_{TF} were determined at 55 °C for 1 h after addition of 0.25 mL of enzyme solution to 0.75 mL of 60 % (w/v) sucrose (Sigma-Aldrich, St. Louis, MO, USA) solution in 0.1 M acetate buffer (pH 5.0), according to the method described by Sangheetha et al. [23] with some modifications. Briefly, the concentrations of released glucose (*G*) and reducing sugar (*RS*) were determined in medium samples collected at the end of reaction by a commercial glucose oxidase colorimetric kit (Liquiform, Labtest, Lagoa Santa, MG, Brazil) and the 3'5' dinitrosalicylic acid method [24], respectively, from which U_H and U_{TF} were assessed. In particular, the following equations first proposed by Chen and Liu (1996) were used to calculate the concentration of transferred fructose (*F'*) and then U_{TF}:

$$F = RS - G \quad (2)$$

$$F' = G - F = 2G - RS \quad (3)$$

where *F*, *RS* and *G* is the concentrations of fructose, glucose and total reducing sugars in the reaction medium, respectively.

One unit of hydrolytic activity was defined as the amount of enzyme required to hydrolyze 1 μmol of sucrose per minute, while one unit of transfructosylating activity as that to transfer 1 μmol of fructose per minute.

2.6. Qualitative aflatoxin detection

Possible aflatoxin production by the three strains that gave the best results in terms of production of FFase with transfructosylating activity was qualitatively checked either in the Coconut Milk Agar Medium according to Lin and Dianese [26] or by the Ammonia Vapor Test [27]. Mycotoxin production was expected to be highlighted, in the former method, by the presence of a fluorescence ring in the agar surrounding colonies under UV-A 365 nm radiation, while, in the latter, by the change from pink to red color in the underside of strain colonies after the addition of 2.0 mL ammonium hydroxide solution (35 %). In both cases, after inoculation in Petri-dishes, the strains were grown in triplicate for seven days at 30°C.

2.7. Physical-chemical characterization of agroindustrial by-products

The composition of dry agroindustrial by-products in terms of protein, lipid, moisture and ash contents was determined according to the methods of the Association of Official Analytical Chemists (AOAC) [28], with some adaptations, and the carbohydrate content by difference between the total mass and those of the other components as reported by de Castro et al. [14].

The water activity (a_w) was determined by an Aqualab Pre Water Activity Analyzer (Decagon Devices Inc., Pullman, WA, USA), while the Water Absorption Index (WAI) and Critical Humidity Point (CHP) were determined according to Flores-Maltos et al. [29], with some adaptations. Briefly, WAI was determined suspending 1.25 g of co-product in 15 mL of distilled water in 50-mL centrifuge tubes, mixing for 1 min at room temperature (25 ± 1 °C), centrifuging at 8000 *g* for 15 min, discarding the supernatant and weighing the centrifuged pellet. WAI was then expressed as g pellet/g dry weight. To determine CHP, 1.0 g of sample impregnated with water at saturation was placed at 120°C for 60 min, and then the residual moisture was assessed.

2.8. Determination of fungal biomass in solid state fermentation

The fugal biomass profile was followed under the best conditions for FFase production. Due the impossibility to separate the fungus from substrate in solid-state fermentation, its growth was determined indirectly from the concentration of N-acetyl glucosamine released by the acid hydrolysis of chitin present in fungal cell wall, as described by de Castro et al. [14]. Measurements were done after acid hydrolysis of biomass contained in dried fermented samples using concentrated sulfuric acid at 121 ºC for 1 h, conditions under which the released glucosamine undergoes successive reductions leading to chromogen that reacts with Ehrlich's reagent (2.67 g of *p*-dimethylaminobenzaldehyde in 1:1 (v/v) mixture of analytical reagent grade ethanol and concentrated hydrochloric acid). After that, the absorbance of the reaction mixture was read at 530 nm. Glucosamine concentration was measured at time intervals of 12-24 h of fermentation using glucosamine (Sigma-Aldrich) as a standard. The results were expressed as mg of glucosamine per gram of dry substrate.

3. Results and discussion

3.1. Preliminary screening of fungus and substrate for FFase production

FFase production by Solid-State Fermentation (SSF) was assessed on wheat bran as a substrate using eight different strains belonging to the *Aspergillus* genus. The results of Table 1 show that *A. tamarii* URM 4634, which was already successful in the productions of protease [15] and xylanase [30], ensured the highest hydrolytic (U_H = 55.47 ± 0.18 U/mL) and transfructosylating (U_{TF} = 26.24 ± 3.42 U/mL) activities compared to the other strains. The three best performing strains in terms of both activities, i.e., *A. tamarii* URM4634, *A. aculeatus* URM4953 and *A. terreus* URM4658, were evaluated for their ability to produce aflatoxin in Coconut Milk Agar (CMA) medium and by the Ammonium Vapor Test (AVT). The absence of any fluorescent halo around the colonies of growing mycelia in CMA medium and of any pinkish pigmentation on the reverse of colonies by the AVT indicated no aflatoxin production by all the three strains. These results are consistent with the observations made by da Silva et al. [15] and Yazdani et al. [31] on different strains of *A. tamarii*. Therefore, based on its higher FFase activities as well as the absence of any aflatoxin production, *A. tamarii* URM4634 was selected for further runs to overproduce FFase with high U_{TF}.

Table 1. FFase activities from *Aspergillus* strains produced by solid-state fermentation using wheat bran as substrate after 72 h of fermentation.

Microorganism	U_H[1] (U/mL)	U_{TF}[2] (U/mL)
A. aculeatus URM4953	38.37 ± 2.04[b]	18.58 ± 1.81[b]
A. heteromorphus URM0269	14.40 ± 1.14[e]	0.00 ± 0.00 [d]
A. japonicus URM5620	19.32 ± 1.04[d]	10.92 ± 0.74[c]
A. niveus URM5870	10.45 ± 1.91[e]	0.00 ± 0.00[d]
A. phoenicis URM4924	19.84 ± 0.54[d]	11.72 ± 0.29[c]
A. tamarii URM4634	55.47 ± 0.18[a]	26.24 ± 3.42[a]
A. terreus URM4658	25.67 ± 0.52[c]	7.81 ± 1.39[c]
A. versicolor URM5701	14.61 ± 0.04[e]	0.00 ± 0.00 [d]

Different letters are significantly different from each other ($p < 0.05$).

[1]Hydrolytic activity.

[2]Transfructosylating activity.

Different agroindustrial by-products were then tested as substrates to improve FFase production by SSF, whose results are listed in Table 2. Soy bran ensured the highest values of U_H (135.29 ± 4.26 U/mL) and U_{TF} (42.35 ± 5.89 U/mL), whereas wheat and oat brans allowed for about one-fourth to one-half of the above U_H and about one-fifth to two-thirds of U_{TF}, respectively. On the other hand, lemon and orange peels as well as corn cobs had disappointing performances. Surprisingly, Rustiguel et al. [32] observed a 20 % increase in FFase production by *Aspergillus phoenicis*, evaluated only as U_H, when a mixture of soy and wheat brans was used instead of soy bran alone. A mixture of these substrates was successfully used also for the production of FFase with transfructosylating activity by *Penicillium oxalicum* in submerged cultivation [11].

Table 2. FFase activities from *Aspergillus tamarii* URM4634 in solid-state fermentation using different agroindustrial substrates after 72 h of fermentation.

Substrates	U_H[1] (U/mL)	U_{TF}[2] (U/mL)
Corn cobs	4.90 ± 0.71^{d}	2.00 ± 0.87^{bc}
Lemon peel	0.27 ± 0.03^{e}	0.19 ± 0.05^{c}
Oat bran	34.11 ± 0.38^{c}	8.66 ± 0.73^{bc}
Orange peel	0.19 ± 0.03^{e}	0.19 ± 0.05^{c}
Soy bran	135.29 ± 4.26^{a}	42.35 ± 5.89^{a}
Wheat bran	62.82 ± 1.34^{b}	26.58 ± 1.60^{b}

Different letters are significantly different from each other ($p < 0.05$).

[1]Hydrolytic activity.

[2]Transfructosylating activity.

The centesimal composition and physicochemical parameters of agroindustrial by-products used for FFase production are listed in Table 3. As known, the C:N ratio is one of the most important factors to balance biomass and produce biomolecules of interest, which means that the substrate must have a value of this ratio suitable for the fermentation. FFase production was the highest in substrates with the lowest C:N values (Table 2), i.e., soy (0.77 g/g) and wheat bran (2.60 g/g). These values are close to those detected by de Castro et al. [14] for wheat bran (4.27 g/g), soybean (0.64 g/g) and cottonseed meal (2.15 g/g) used as substrates for protease production by *A. niger*.

Table 3. Centesimal composition and physical-chemical parameters of dry agroindustrial substrates used for FFase with transfructosylating activity from *Aspergillus tamarii* URM4634 by solid state fermentation (SSF).

Parameter	Corn cobs	Lemon peel	Oat bran	Orange peel	Soy bran	Wheat bran
Moisture (%)	6.24 ± 0.02^{c}	8.88 ± 0.35^{a}	8.35 ± 0.02^{a}	9.09 ± 0.08^{a}	7.93 ± 0.11^{ab}	6.95 ± 0.74^{bc}
Ash (%)	1.36 ± 0.09^{e}	4.25 ± 0.14^{b}	2.14 ± 0.01^{d}	3.47 ± 0.03^{c}	6.34 ± 0.01^{a}	6.19 ± 0.08^{a}
Protein (%)	1.82 ± 0.29^{e}	6.47 ± 0.00^{d}	15.78 ± 0.29^{c}	7.08 ± 0.00^{d}	56.24 ± 0.00^{a}	18.31 ± 0.43^{b}
Lipids (%)	$2.54 \pm 0.34^{c,d}$	$2.14 \pm 0.23^{d,e}$	6.84 ± 0.13^{a}	1.41 ± 0.24^{e}	3.23 ± 0.30^{c}	4.71 ± 0.57^{b}
Carbohydrates (%)	88.04	78.26	66.89	78.95	26.26	63.84
C:N	21.39	6.78	3.16	6.06	0.77	2.60
a_w	0.487 ± 0.006^{d}	0.573 ± 0.001^{a}	0.525 ± 0.001^{c}	0.551 ± 0.001^{b}	0.551 ± 0.001^{b}	0.471 ± 0.003^{e}
WAI[1] (g of water/g of dried substrate)	6.95 ± 0.48^{a}	$6.38 \pm 0.05^{a,b}$	2.03 ± 0.13^{e}	$5.72 \pm 0.05^{b,c}$	4.18 ± 0.04^{d}	$5.08 \pm 0.33^{c,d}$
CHP[2] (%)	$14.04 \pm 0.60^{c,d}$	12.58 ± 0.14^{e}	32.85 ± 2.23^{a}	$13.88 \pm 0.15^{c,d}$	18.23 ± 0.14^{b}	$16.70 \pm 0.16^{b,c}$

Different letters for a same parameter are significantly different from each other ($p < 0.05$).

[1]Water Absorption Index.

[2]Critical Humidity Point.

Others important parameters are the Water Absorption Index (WAI) and Critical Humidity Point (CHP), which indicate the amounts of water absorbed and intrinsically contained in the support, respectively, the latter not being available to the microorganism for its metabolic functions [29]. Substrates with high WAI and low CHP values are usually preferred in SSF since their moisture content tends to decrease during fermentation. However, despite its intermediate WAI and CHP values compared to the other substrates, soy bran allowed for the highest FFase production, which suggests that these physicochemical parameters did not play a crucial role in FFase production by *Aspergillus tamarii* URM4634.

3.2. FFase production by Solid-State Fermentation on different agroindustrial substrates

A simplex centroid mixture design was used to investigate the influence of different proportions of the three best substrates (soy, wheat and oat brans) on U_H and U_{TF} at different fermentation times (Table 4). The formulation used in run 1, made up only on soy bran, resulted in a maximum hydrolytic FFase activity as high as 136.26 ± 2.32 U/mL after 48 h of fermentation, 87% of which was preserved even after 96 h. This substrate ensured the highest U_{TF} as well (56.48 ± 1.66 U/mL), but it took twice as long (96 h). Contrary to what expected from the literature, where mixtures of agroindustrial by-products are often pointed out as the best substrates for SSF [32], these results indicate that soy bran alone would be the most suite medium among the tested ones seeking FFase production.

Table 4. Matrix and results of the simplex centroid mixture design for FFase production by *A. tamarii* URM4634 under solid-state fermentation, evaluated in terms of hydrolytic and transfructosylating activities, at different fermentation times.

Run	Independent variables			Hydrolytic activity (U/mL)			
	Soy bran (x_1)	Wheat bran (x_2)	Oat bran (x_3)	24 h	48 h	72 h	96 h
1	1	0	0	13.31 ± 0.20^{d,e}	136.26 ± 2.32^{a}	132.11 ± 3.301^{a}	118.62 ± 1.35^{a}
2	0	1	0	9.58 ± 0.77^{e}	66.05 ± 0.85^{d}	62.82 ± 1.34^{d}	61.23 ± 0.61^{d}
3	0	0	1	11.93 ± 0.04^{d,e}	15.03 ± 0.61^{f}	15.91 ± 0.32^{f}	15.41 ± 0.85^{f}
4	1/2	1/2	0	31.81 ± 1.92^{c}	93.12 ± 1.83^{b}	108.22 ± 0.12^{b}	84.09 ± 2.69^{b}
5	1/2	0	1/2	60.10 ± 0.77^{a}	81.62 ± 0.12^{c}	81.58 ± 1.95^{c}	75.58 ± 0.24^{c}
6	0	1/2	1/2	15.43 ± 1.18^{d}	54.06 ± 1.47^{e}	30.44 ± 3.42^{e}	33.23 ± 0.24^{e}
7	1/3	1/3	1/3	42.42 ± 1.71^{b}	68.42 ± 1.83^{d}	103.31 ± 1.59^{b}	75.48 ± 3.79^{c}
				Transfructosylating activity (U/mL)			
1	1	0	0	10.63 ± 0.04^{d}	40.21 ± 0.80^{a}	51.17 ± 0.07^{a}	56.48 ± 1.66^{a}
2	0	1	0	7.19 ± 1.55^{d}	38.80 ± 0.62^{a}	26.58 ± 1.60^{c}	13.83 ± 0.67^{d}
3	0	0	1	8.72 ± 0.10^{d}	10.27 ± 0.41^{c}	22.11 ± 0.28^{d}	12.07 ± 2.80^{d}
4	1/2	1/2	0	20.17 ± 0.34^{c}	21.71 ± 0.68^{b}	47.64 ± 1.39^{a}	30.94 ± 0.48^{b}
5	1/2	0	1/2	37.77 ± 0.27^{a}	26.91 ± 1.87^{b}	26.05 ± 1.91^{c,d}	20.47 ± 0.60^{c}
6	0	1/2	1/2	11.03 ± 1.27^{d}	26.77 ± 0.21^{b}	12.56 ± 3.03^{e}	14.85 ± 1.14^{c,d}
7	1/3	1/3	1/3	28.47 ± 2.70^{b}	36.54 ± 2.87^{a}	39.45 ± 1.72^{b}	35.35 ± 2.76^{b}

Different letters are significantly different from each other ($p < 0.05$).

The influence of different formulations on both FFase hydrolytic and transfructosylating activities after 24, 48, 72 and 96 h is illustrated in two-dimensional ternary contour plots (Figure 1). After 24 h of fermentation, maximum activity zones are located at the side of triangle having mixtures of soy (x_1) and oat (x_3) brans as the vertices, indicating a synergistic effect. Such a binary formulation (run 5) did in fact show about 4.5-5.0-fold increases in U_H and 3.5-4.5-fold increases in U_{TF} compared with the individual substrates; similar synergistic effect was also observed for both enzyme activities with the other binary (runs 4 and 6) and ternary (run 7) mixtures. U_H achieved a maximum value (132.11-136.26 U/mL) after 48-72 h in the medium containing only soy bran as the substrate (run 1), and then decreased by about 10-13% after 96 h. On the other hand, all binary mixtures showed antagonist effects in the same time interval, except the ones made up of soy bran and wheat bran (x_2) (run 4) after 48 and 96 h and of wheat bran and oat bran (run 6) after 96 h.

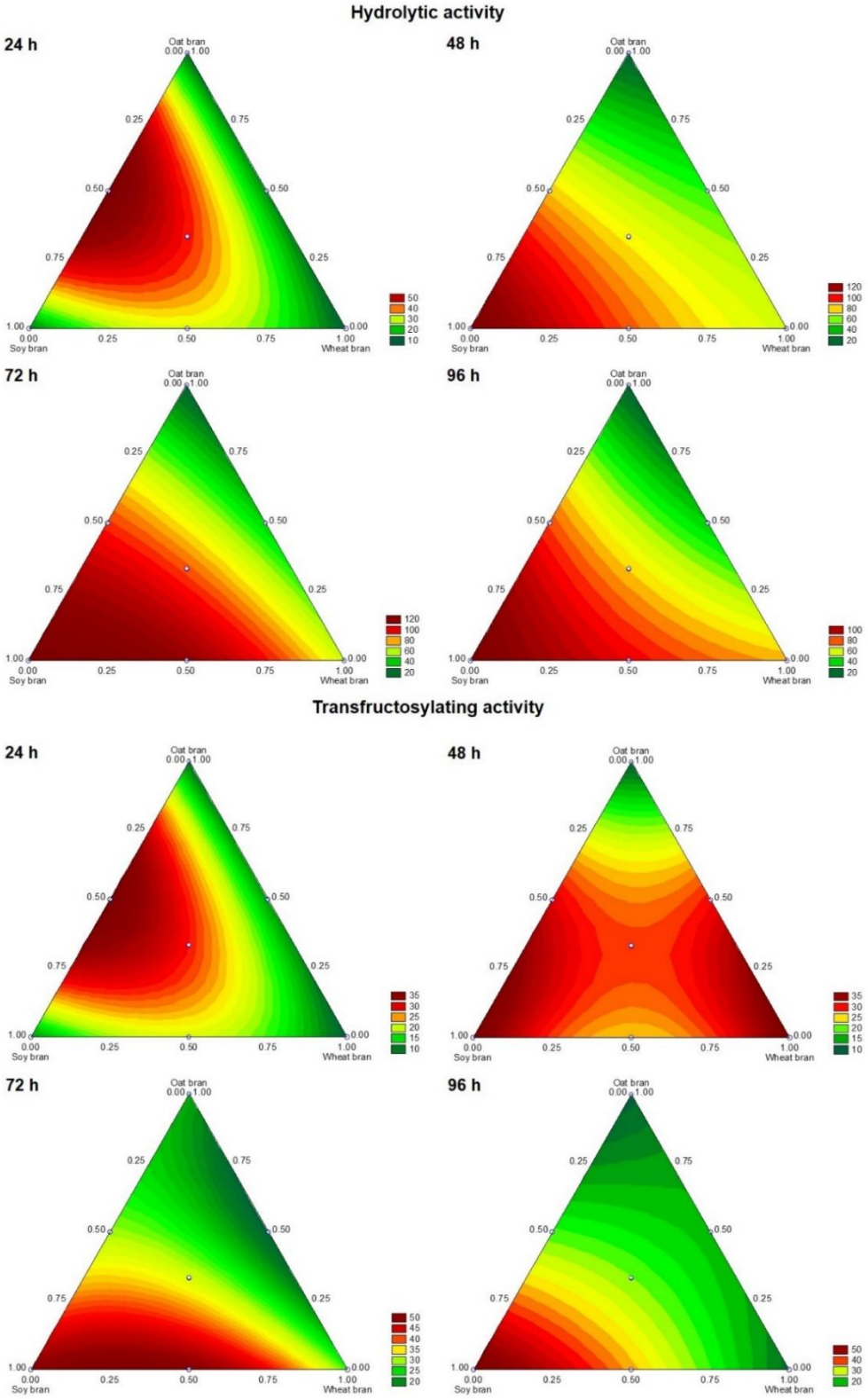

Figure 1. Mixture contour plots for hydrolytic and transfructosylating activities of *A. tamarii* URM4634 FFase produced by Solid-State Fermentation after 24, 48, 72 and 96 h.

As regards U_{TF}, the contour plot after 48 h showed the highest value of this response (38.80-40.21 U/mL) in the vertices referring to soy bran (run 1) and wheat bran (run 2) alone,

with no statistically significant difference between them ($p > 0.05$). After 72 h, the highest activity (47.64-51.17 U/mL) was observed in the presence only of soy bran (run 1) and the mixture composed of soy bran and wheat bran (run 4), with no statistically significant difference ($p > 0.05$). However, after 96 h this activity achieved the maximum value obtained in this study (56.48 ± 1.66 U/mL) in run 1, while all mixtures of substrates showed antagonist effects.

Different models were used to predict the optimum composition of medium for FFase production, taking into consideration either enzyme activity. Among them, the quadratic models based on Eq. (1) allowed by far the best fit ($R^2 > 0.90$) to the experimental data (Table 5), thus proving useful for predictive purposes. In each model, negative and positive terms do refer to antagonistic and synergistic effects, respectively, that reflect the influence of binary and ternary mixtures on enzyme production [21], while their absolute values indicate how strong are these effects. Based on these criteria, the most significant effects on U_H were that of the binary mixture composed by soy bran (x_1) and oat bran (x_3) and that of soy bran alone for long fermentation times (≥ 48 h). Apart from only one exception after 48 h of fermentation, a similar behavior was also observed for U_{TF}.

Table 5. Models of mixture and their respective statistical parameters for FFase production from *A. tamarii* URM4634 by Solid-state Fermentation calculated for hydrolytic and transfructosylating activities.

U_H[1] (U/mL)	F calculated	F tabulated	R^2	Equations
24 h	361.75	2.90	0.998	$Y = 13.40x_1 + 9.67x_2 + 12.03x_3 + 79.53x_1x_2 + 187.99x_1x_3 + 16.76x_2x_3$
48 h	32.21	2.90	0.994	$Y = 136.88x_1 + 66.67x_2 + 15.65x_3 - 44.56x_1x_2 + 11.47x_1x_3 + 41.67x_2x_3$
72 h	4.18	2.90	0.954	$Y = 130.19x_1 + 61.68x_2 + 13.99x_3 + 79.82x_1x_2 + 68.66x_1x_3 + 8.37x_2x_3$
96 h	14.08	2.90	0.986	$Y = 117.76x_1 + 70.50x_2 + 14.55x_3 - 26.47x_1x_2 + 51.39x_1x_3 - 23.48x_2x_3$
U_{TF}[2] (U/mL)	F calculated	F tabulated	R^2	Equations
24 h	449.14	2.90	0.999	$Y = 10.57x_1 + 7.14x_2 + 8.67x_3 + 46.09x_1x_2 + 113.42x_1x_3 + 13.34x_2x_3$
48 h	1.85	2.90	0.902	$Y = 38.95x_1 + 38.55x_2 + 9.87x_3 - 53.23x_1x_2 + 26.26x_1x_3 + 21.82x_2x_3$
72 h	2.27	2.90	0.919	$Y = 50.30x_1 + 27.28x_2 + 21.26x_3 - 49.21x_1x_2 - 25.09x_1x_3 - 35.42x_2x_3$
96 h	2.01	2.90	0.910	$Y = 55.46x_1 + 12.81x_2 + 11.04x_3 + 3.66x_1x_2 - 34.69x_1x_3 + 28.11x_2x_3$

For all models obtained, p-value < 0.001.

[1]Hydrolytic activity.

[2]Transfrucosylating activity.

3.3. Optimization of FFase production by Solid-State Fermentation using soy bran as substrate

After selection of soy bran alone as the best substrate for both enzyme activities, the influence of substrate mass (3, 5 and 7 g), moisture content (40, 50 and 60%) and sucrose concentration (5, 10 and 15% w/v) on FFase production was investigated using a 2^3-full factorial design, whose conditions and results after 72 h of fermentation are listed in Table 6 in terms of both U_H and U_{TF}. One can see that the maximum U_H (209.11-209.99 U/mL) was obtained in runs 7 and 8 and the maximum U_{TF} (51.08-53.90 U/mL) in runs 4 and 7; therefore, the run 7 carried out using 3 g soy bran, 15% sucrose and 60% moisture was selected as the best compromise to simultaneously optimize them. The former activity was more than thrice that reported for *Aspergillus japonicus* FFase produced on coffee silverskin (65.82 ± 1.46 U/mL) [12], and the latter more than twice those of *A. oryzae* [23] and *Rhizopus stolonifer* [33] FTases using rice bran and cassava waste as substrates, respectively.

Table 6. Experimental conditions and results for FFase with transfructosylating activity production by *A. tamarii* URM4634 under solid state fermentation using soy bran as substrate performed according to the 2^3-full factorial design.

Run	Mass of substrate (g)	Sucrose (%)	Moisture (%)	U_H[1] (U/mL)	U_{TF}[2] (U/mL)
1	3	5	40	130.20	23.24
2	7	5	40	130.85	36.86
3	3	15	40	119.13	22.34
4	7	15	40	155.23	51.08
5	3	5	60	175.17	34.06
6	7	5	60	191.67	29.96
7	3	15	60	209.11	53.90
8	7	15	60	209.99	47.67
9 (C)	5	10	50	139.17	32.47
10 (C)	5	10	50	151.09	35.48
11 (C)	5	10	50	159.39	28.99

[1]Hydrolytic activity.

[2]Transfrucosylating activity.

The analysis of effects summarized in Table 7 shows that the moisture content exerted a statistically significant positive effect on U_H, i.e., such a response was greatly enhanced by an increase in that independent variable. As known, moisture, which has in general a great importance in any microbial process, is crucial in enzyme production by SSF, because it not only promotes the diffusion of solutes and gases, but also mitigates the osmotic changes brought about by excess metabolites in the vicinity of cells [34]. Moisture contents in the range selected for this study are considered suitable for fungal development; higher values may result in decreased substrate porosity, oxygen limitation and bacterial contamination, whereas lower values may cause poor accessibility of nutrients and then poor microbial growth [13].

Table 7. Calculated effect of the responses in the 2^3-full factorial design for FFase with transfructosylating activity production under solid state fermentation using soy bran as substrate.

Variable or interaction	U_H[1]	U_{TF}[2]
(1) Substrate	2.86	3.48
(2) Sucrose	3.46	5.54*
(3) Moisture	13.24*	3.49
1 x 2	1.05	1.41
1 x 3	-1.02	-5.73*
2 x 3	2.06	2.64
1 x 2 x 3	-2.70	-1.87

*Statistically significant at 95% confidence level ($p < 0.05$).

[1]Hydrolytic activity.

[2]Transfrucosylating activity.

On the other hand, U_{TF} was positively influenced only by sucrose concentration. This result is consistent not only with the fact that sucrose is the best carbon source for the production of enzymes with transfructosylating activity [35], but also with the importance of this sugar in the formation of cell constituents as well as its role as an inducer of FFase synthesis. Nonetheless, explaining the statistically significant antagonistic effect of the interaction between substrate concentration and moisture content on this response is a challenge.

As a final effort, we investigated fungal growth during run 7 that ensured the most satisfactory results in terms of both activities (Table 6). For this purpose, due the practical impossibility to discern biomass and substrate masses in SSF, *A. tamarii* URM4634 growth was

followed indirectly as the increase in concentration of glucosamine released by cell wall hydrolysis. These results are illustrated in Figure 2A together with those of FFase production. The glucosamine level progressively increased along the time and reached a maximum value (132.0 ± 2.05 mg/g) after 36 h of cultivation, whereupon a decrease in biomass level took place, likely due to substrate limitation. Doing just a few comparisons, da Silva et al. [15] reported a maximum glucosamine concentration of 119.33 ± 4.8 mg/g after 96 h of SSF using the same *A. tamarii* strain and wheat bran as a substrate, while de Castro et al. [14], cultivating *A. niger* in different inexpensive agroindustrial substrates, achieved a maximum glucosamine level of 83.35 mg/g in soybean meal. FFase production showed peaks of U_H (229.43 ± 4.88 U/mL) and U_{TF} (66.93 ± 3.02 U/mL) after 120 and 96 h, respectively. As far as the FFase productivity is concerned, the best results in terms of hydrolytic (3.48 ± 0.03 U mL^{-1} h^{-1}) and transfructosylating (0.70 ± 0.03 U mL^{-1} h^{-1}) activities were obtained after 48 and 96 h (Figure 2B), respectively.

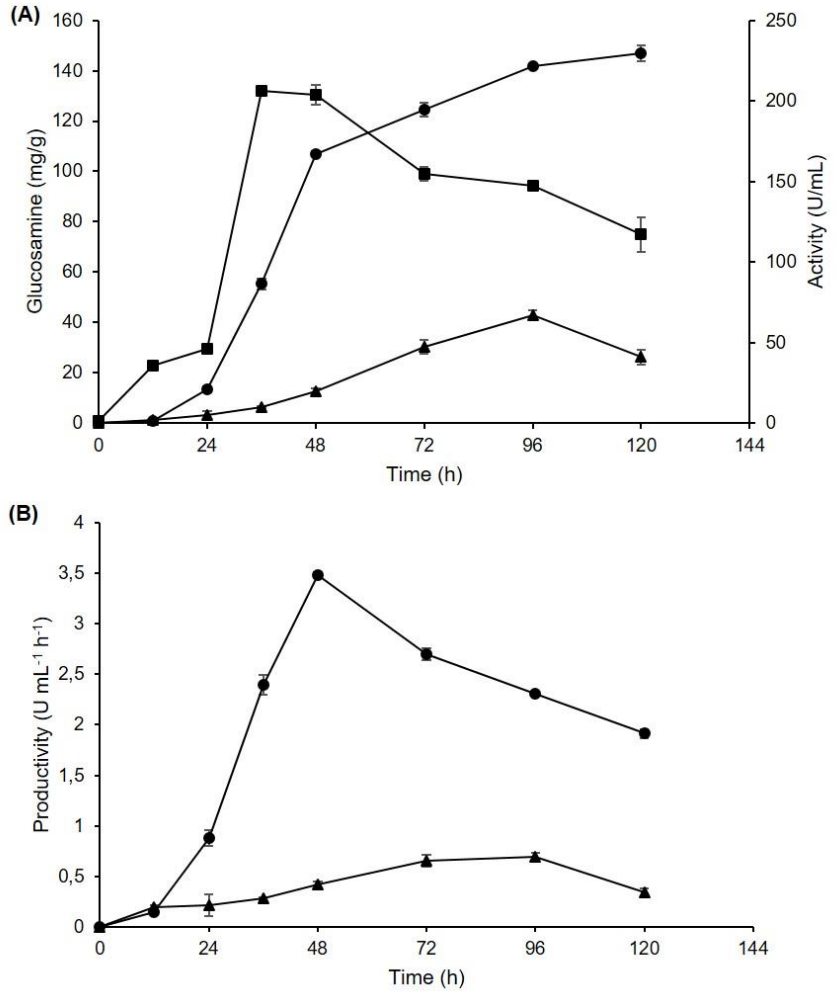

Figure 2. *A. tamarii* URM4634 Solid-State Fermentation using soy bran as substrate. (A) Biomass growth expressed as glucosamine concentration (■); FFase production expressed as FFase hydrolytic (●) and transfructosylating (▲) activities. (B) FFase productivity in terms of hydrolytic (●) and transfructosylating (▲) activities.

4. Conclusions

Aspergillus tamarii URM4634, selected among different fungal strains as the best FFase producer in Solid-State Fermentation, was tested in different substrates. Among them, soy bran, wheat bran and oat bran showed the high FFase activities; therefore, the influence of each substrate at four levels was evaluated using a three-component simplex-centroid mixture design. The maximum enzyme production in terms of both enzyme activities was obtained using only soy bran as the substrate. The above experimental design provided satisfactory statistical models to predict enzyme production at different fermentation times, thus confirming to be a powerful statistical approach to optimize the performance and find the optimum

formulations for SSF. Finally, to maximize FFase production, additional SSF runs were performed according to a 2^3-full factorial design where moisture content, soy bran mass and sucrose concentration were selected as the independent variables. Based on the information obtained from this statistical design, a longer fermentation was carried out under optimal conditions (3 g of substrate, a 15 % sucrose concentration and a 60 % moisture content), in which the highest hydrolytic (229.43 ± 4.88 U/mL) and transfructosylating (66.93 ± 3.02 U/mL) activities were obtained after 120 and 96 h, respectively. The results obtained in this study, although preliminary, provide a projection of the potential of *A. tamarii* URM4634 FFase in industrial applications such as sugar inversion and FOS production.

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Biochemical characterization and kinetic/thermodynamic study of *Aspergillus tamarii* URM4634 β-fructofuranosidase with transfructosylating activity

Rodrigo Lira de Oliveira[1], Marcos Fellipe da Silva[2], Attilio Converti[3], Tatiana Souza Porto[2*].

[1]Northeast Biotechnology Network/RENORBIO, Federal Rural University of Pernambuco (UFRPE), Dom Manoel de Medeiros, Dois Irmãos, 52171-900 Recife, PE, Brazil.
[2]Academic Unit of Garanhuns/UAG, Federal Rural University of Pernambuco (UFRPE), Garanhuns, Av. Bom Pastor, Boa Vista, 55296-901 Garanhuns, PE, Brazil.
[3]Department of Civil, Chemical and Environmental Engineering, Pole of Chemical Engineering, Genoa University, via Opera Pia 15, 16145 Genoa, Italy.

*Corresponding author
Tel.: +55 81 33206345.
Email address: portots@yahoo.com.br

ABSTRACT

This study reports on the biochemical characterization as well as the kinetic and thermodynamic study of *Aspergillus tamarii* URM4634 β-fructofuranosidase (FFase) with transfructosylating activity. Conditions for FFase activity were optimized by means of a Central Composite Rotational Design using pH and temperature as the independent variables, while residual activity tests carried out in the temperature range of 45-65°C enabled us to investigate FFase thermostability and estimate the kinetic and thermodynamic parameters of enzyme denaturation. Optimal conditions for sucrose hydrolysis and fructosyl transfer catalyzed by crude FFase were 50°C, and pH 6.0 and 7.4, respectively. The thermodynamic properties of irreversible enzyme inactivation were found to be an activation energy of 293.1 $kJ \cdot mol^{-1}$, and activation enthalpy, entropy and Gibbs free energy in the ranges 290.3-290.4 $kJ \cdot mol^{-1}$, 568.7-571.0 $J \cdot mol^{-1} \cdot K^{-1}$ and 97.9-108.8 $kJ \cdot mol^{-1}$, respectively. The results obtained in this study point out satisfactory enzyme activity and thermostability at temperatures commonly used for industrial fructo-oligosaccharide (FOS) synthesis; therefore, this novel FFase appears to be a promising biocatalyst with great potential for long-term FOS synthesis and invert sugar production. To the best of our knowledge, this is the first report on kinetic and thermodynamic parameters of an *A. tamarii* FFase.

Keywords: β-fructofuranosidase; fructo-oligosaccharides; *Aspergillus*; solid state fermentation; kinetic and thermodynamic parameters.

1. Introduction

Fructo-oligosaccharides (FOS) is the common name of fructose oligomers composed of one glucose moiety followed by a number of fructose units ranging from 2 to 60 and linked by β-(2, 1) or β-(2, 6) glycosidic bonds. They are usually represented by the formula GF_n, being 1-kestose (GF_2), 1-nystose (GF_3) and 1-fructofuranosyl-nystose (GF_4) the best known of these compounds.[1,2] FOS is an important food ingredient, which is mainly used as an artificial sweetener and is rapidly becoming popular because of beneficial effects on human health. It has in fact been shown that it is able to promote a good balance of intestinal microbiota, decrease gastrointestinal infections, regulate blood glucose and cholesterol levels, induce growth of probiotic microorganisms in the intestinal tract, thus contributing to the prevention of colonic cancer, stimulate the absorption of Ca^{2+}, Mg^{2+}, Fe^{2+} and other ions and exert immunomodulatory effects.[3–5]

FOS can be produced enzymatically by controlled inulin hydrolysis by inulinases or sucrose transfructosylation by fructosyltransferases (FTases, EC 2.4.1.9) or β-fructofuranosidases (FFases, EC 3.2.1.26). Even though FFases can catalyze either hydrolysis or transfructosylation, the latter reaction requires higher sucrose concentrations. On the other hand, FTases act on sucrose by cleaving the β-(1,2) linkage and transferring the fructosyl group to an acceptor molecule such as another sucrose molecule; as a result, FOS is synthesized and glucose released.[4,6,7] It is noteworthy that definition and nomenclature of these enzymes, which can be either extracted by several vegetables or produced by microorganisms, remain a matter of debate. The most objective way of specifying the nomenclature of these enzymes would be their biochemical characterization in terms of reaction affinity, in that, FFase has higher affinity for hydrolysis reaction, while FTase for transfructosylation.

The yield of sucrose conversion into FOS is highly species-dependent, and, in general, the enzymes produced by fungi belonging to the genus *Aspergillus* are responsible for the highest industrial yields, around 60%.[8] This genus is deeply explored to produce industrial enzymes due to the ability of its members to grow on solid or liquid substrates and to largely produce extracellular enzymes. Moreover, some *Aspergillus* strains are generally recognized as safe (GRAS) microorganisms, according to the Food and Drug Administration, and used for human and animal nutrition or production of various enzymes employed in the food industry.[9]

Commercial preparations of enzymes from strains of *Aspergillus aculeatus* (Pectinex Ultra SP-L[10] and Viscozyme L[11]) and *Aspergillus niger* (Rohapect CM[12]), with high transfructosylating activity, have been successfully used to synthesize FOS. Moreover, studies

involving the production of FFases were already reported using *Aspergillus japonicus*,[13] *A. niger*[14] and other filamentous fungi such as *Penicillium oxalicum*,[15] *Penicillium citreonigrum*,[16] *Penicillium sizovae* and *Cladosporium cladosporioides*,[17] the last two standing out in the production of neo-FOS[18] and the last one in that of FOS using seawater.[19] Nonetheless, there is a strong need to further explore the *Aspergillus* genus diversity in order to identify new strains able to overproduce these enzymes.

FTases and FFases can be produced by submerged (SmF) or solid-state (SSF) fermentation. The main advantages of SSF over SmF are a) higher growth rate, b) ability of filamentous fungi to colonize the substrate surface and matrix in the search for nutrients, and c) secretion of higher levels of metabolites and enzymes.[20] Furthermore, other characteristics make SSF more attractive than SmF such as lower risk of contamination, higher productivity, use of inexpensive substrates, simplicity of downstream processing, lower energy requirements and lower wastewater production.[21]

After production of enzymes, an important step to evaluate their performance and predict their successful use in specific applications is biochemical characterization in terms of optimum pH, temperature and stability profiles, as well as kinetic and thermodynamic parameters.[20,22] In particular, kinetic and thermodynamic parameters of thermal inactivation can provide valuable information about the thermostability of enzymes at a given operating temperature.[23]

Based on this background, aims of the present study were the production of *Aspergillus tamarii* URM4634 FFase by SSF, its biochemical characterization, the kinetic investigation of reactions catalyzed by it and the estimation of the thermodynamic parameters of its irreversible thermal inactivation.

2. Materials and Methods

2.1. Microorganism

The *Aspergillus tamarii* URM4634 strain was provided by "Micoteca-URM" of Mycology Department, Centre of Biosciences of Federal University of Pernambuco (UFPE), Recife-PE, Brazil, preserved in mineral oil and maintained at 28°C in Czapek Dox Agar medium.

2.2. FFase production by Solid State Fermentation

Soy bran used as substrate for β-fructofuranosidase (FFase) production by solid state fermentation (SSF) was purchased from the local market of Garanhuns, PE, Brazil, and standardized to a particle size between 0.5-2.0 mm. Before the fermentation, the *A. tamarii* URM4634 strain was inoculated in Potato Dextrose Agar medium and grown for 7 days at 30°C. After this time, the fermentation was performed under previously established conditions (30°C for 96 h) in 125-mL Erlenmeyer flasks containing 3 g of substrate, nutrition solution [15% (w/v) sucrose and 0.5% (w/v) yeast extract] and a spore suspension (10^7 spores/mL) corresponding to 60% moisture. The crude extract was obtained by addition of 7.5 mL of 0.1 M acetate buffer (pH 5.0) per gram of fermented material and homogenized in orbital shaker for 90 min and 130 rpm. Solids were removed by centrifugation at 5000 rpm for 15 min, and the crude extract was analyzed and stored at -22°C.

2.3. Analytical determinations

The reaction medium used to determine the enzyme activities, consisting of 0.25 mL of enzyme solution and 0.75 mL of 60% (w/v) sucrose in 0.1 M acetate buffer (pH 5.0), was incubated at 55°C for 1 h as described by Sangeetha et al.[24] with some modifications. After reaction, samples were collected to determine concentrations of glucose (G) by a commercial glucose oxidase colorimetric kit (Liquiform, Labtest, Lagoa Santa, MG, Brazil), and reducing sugar (RS) by the 3,5-dinitrosalicylic acid method.[25] The hydrolytic and transfructosylating activities were assessed from the concentrations of G and RS released into the reaction medium. One unit of hydrolytic (U_H) or transfructosylating (U_{TF}) activity was defined as the amount of enzyme required to hydrolyze 1 μmol sucrose per minute or to transfer 1 μmol of fructose per minute, respectively. To calculate the concentration of transferred fructose for the latter purpose, we used the following equations proposed by Chen and Liu:[26]

$$F = RS - G \quad (1)$$

$$F' = G - F = 2G - RS \quad (2)$$

where *F* and *F'* are the concentrations of free and transferred fructose in the reaction medium.

The total protein content was determined according to Bradford[27] using Comassie Brilliant Blue G-250 as the dye and Bovine Serum Albumin as the standard protein. The results

obtained for protein concentration were used to calculate de turnover number (k_{cat}) as described in section 2.6.

2.4. Biochemical characterization of FFase with transfructosylating activity

2.4.1. Effect of pH and temperature on activity and stability of FFase

The effect of temperature on hydrolytic and transfructosylating activities of FFase was investigated by activities tests carried out at different temperatures (30–80°C) and constant pH (5.0), while that of pH by activities tests using the substrate diluted in different 0.1 M buffers, namely citrate (3.0–4.0), citrate-phosphate (4.0–6.0), phosphate (6.0–8.0) and Tris-HCl (8.0–9.0), at constant temperature (55°C). After that, we studied the effects and interactions of these two variables on the above activities selected as responses using a Central Composite Rotational Design (CCRD) plus 3 central points, in which their levels were selected based on the optimum pH and temperature determined by the traditional (one-way factor) approach (Table 1). The experimental data of both responses were fitted to the polynomial second-order equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (3)$$

where Y = FFase hydrolytic or transfructosylating activity, expressed in U/mL of crude extract, X_1 and X_2 are the coded levels of the independent variables (temperature and pH, respectively), β_1 and β_2 the regression coefficients of linear terms, β_{11} and β_{22} those of quadratic terms, β_{12} the interaction coefficient and β_0 the intercept term.

Table 1. Experimental conditions of pH and temperature and results of hydrolytic (U_H) and transfructosylating (U_{TF}) activities of FFase from *A. tamarii* URM4634 according to the Central Composite Rotational Design.

Run	pH	Temperature (°C)	U_H (U/mL)	U_{TF} (U/mL)
1	5.00	40.0	110.34	32.04
2	5.00	60.0	158.00	17.91
3	7.00	40.0	83.05	49.79
4	7.00	60.0	94.23	43.72
5	4.59	50.0	154.47	44.75
6	7.41	50.0	117.98	63.16
7	6.00	35.9	83.38	33.54
8	6.00	64.1	158.58	16.75
9 (C)	6.00	50.0	159.81	55.07
10 (C)	6.00	50.0	160.63	53.12
11 (C)	6.00	50.0	153.89	43.24

The coefficient of determination (R^2) and the F-test of the analysis of variance (ANOVA) were used to check the quality of fit of the second-order model equation. The statistical analysis for this experimental design was performed using the software package Statistica 7.0.

The effect of temperature on FFase stability was investigated by keeping the enzyme extract at temperatures from 30 to 65˚C for different time intervals (0–180 min). Aliquots were withdrawn every 60 min, except at temperature of 60°C (0, 15, 30, 45 and 60 min) and 65°C (0, 5, 10 and 15 min), in order to determine the residual activity, and then rapidly cooled to room temperature (about 25°C) to refold the reversibly inactivated enzyme molecules.

The effect of pH on FFase stability was checked by a previous incubation of the enzyme crude extract with the above buffers at 5˚C. Aliquots were analyzed to determine residual activity at time intervals of 0, 4, 8 and 24 h.

2.4.2. Effect of metal ions on FFase activities

The effect of metal ions as inhibitors or activators of hydrolytic and transfructosylating activities of the enzyme preparation was evaluated at a concentration of 10 mM in 0.1 M acetate

buffer (pH 5.0). For this purpose, the following salts were tested: $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $CaCl_2$, $HgCl_2 \cdot 4H_2O$, KCl and NaCl.

2.5. Kinetic and thermodynamic parameters of FFase thermal inactivation

Enzyme thermal inactivation is generally described by a reversible unfolding of its native form to give an intermediate less stable unfolded form, which is then subject to irreversible denaturation to an inactivated form.[28] This process can be described as a temperature-dependent first-order reaction, with k_d as the rate constant, which can be kinetically depicted by the equation:

$$\ln \psi = - k_d t \quad (4)$$

where ψ is the residual activity coefficient (dimensionless) defined as the ratio of residual enzyme activity to that at the beginning of thermal treatment ($\psi = A/A_o$). Only the results of the hydrolytic activity were used to calculate the kinetic and thermodynamic parameters of thermal denaturation.

The k_d values were estimated in the temperature (T) range of 45–65°C from the slopes of the straight lines obtained by plotting the experimental data of $\ln\psi$ vs. time. The values of $\ln k_d$ were then plotted according to Arrhenius vs. $1/T$, and the activation energy of irreversible enzyme inactivation (E^*_d) was estimated from the slope of the resulting straight line. The other thermodynamic parameters, namely activation enthalpy (ΔH^*_d), Gibbs free energy (ΔG^*_d) and entropy (ΔS^*_d), were calculated according to the following equations:

$$\Delta H^*_d = E^*_d - RT \quad (5)$$

$$\Delta G^*_d = -RT \ln\left(\frac{k_d h}{k_b T}\right) \quad (6)$$

$$\Delta S^*_d = \frac{\Delta H^*_d - \Delta G^*_d}{T} \quad (7)$$

where h (6.626×10^{-34} J.s) is the Planck constant, k_b (1.381×10^{-23} J.K^{-1}) the Boltzmann constant and R the gas constant (8.314 J.mol^{-1} K^{-1}).

Additional kinetic parameters such as half-life ($t_{1/2}$), decimal reduction time (D-value) and thermal resistance constant (Z-value) can be usefully exploited to better understand the

influence of temperature on the denaturation process. $t_{1/2}$ is defined as the time after which the enzyme activity is reduced to one-half of the initial value, while the *D*-value corresponds to the time needed for a 10-fold reduction of the initial activity at a given temperature. These parameters were calculated using the equations:

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{8}$$

$$D = \frac{\ln 10}{k_d} \tag{9}$$

On the other hand, the *Z*-value is the temperature increase needed to achieve a 10-fold reduction in the *D*-value and was calculated from the slope of the Thermal Death Time plot of log*D* versus *T* (°C).

2.6. Kinetic and thermodynamic parameters of FFase-catalyzed reactions

The maximum reaction rates (V_{max}) for hydrolysis and fructosyl transfer reactions catalyzed by FFase as well as the related apparent Michaelis-Menten constants (K_m) were estimated through Lineweaver-Burk double reciprocal plots, while k_{cat} was calculated as the ratio of V_{max} to protein concentration. Enzyme activities used for these estimations were determined at different sucrose concentrations ($25 \leq S_0 \leq 700$ mg/mL) as described in section 2.3.

The activation energies (E^*_a) of both FFase-catalyzed hydrolysis and fructosyl transfer were estimated from the slopes of typical Arrhenius type-plots of $\ln A_0$ versus $1/T$, being A_0 the starting activity. The other thermodynamic parameters such as activation enthalpy (ΔH^*), Gibbs free energy (ΔG^*) and entropy (ΔS^*) were calculated using the equations:

$$\Delta H^* = E_a^* - RT \tag{10}$$

$$\Delta G^* = -RT \ln\left(\frac{k_{cat} h}{k_b T}\right) \tag{11}$$

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T} \tag{12}$$

The effect of temperature on the reaction rate was also investigated in terms of quotient of temperature (Q_{10}), which is the factor by which the enzyme activity increases due to a 10°C

temperature increase and was calculated by the following equation initially proposed by Dixon and Webb:[29]

$$Q_{10} = \text{anti log}\left(\frac{E_a^* \text{x } 10}{RT^2}\right) \quad (13)$$

3. Results and discussion

3.1. Effect of temperature on FFase activities and stability

The profiles of FFase activities at different temperatures show that the optimum temperature for both hydrolytic and transfructosylating activities was 50°C (Figure 1A). Guo et al.[30] reported the same optimum temperature for transfructosylating activity of recombinant FTase from *Aspergillus oryzae* overexpressed in *Pichia pastoris*. The optimum pH was 6.0 for both activities (Figure 1B), a value coincident with that reported for an *A. oryzae* FTase.[31] However, the hydrolytic activity was slightly more active under acidic conditions ($4.0 \leq pH \leq 5.0$), whereas the transfructosylating one under neutral to alkaline conditions ($7.0 \leq pH \leq 9.0$).

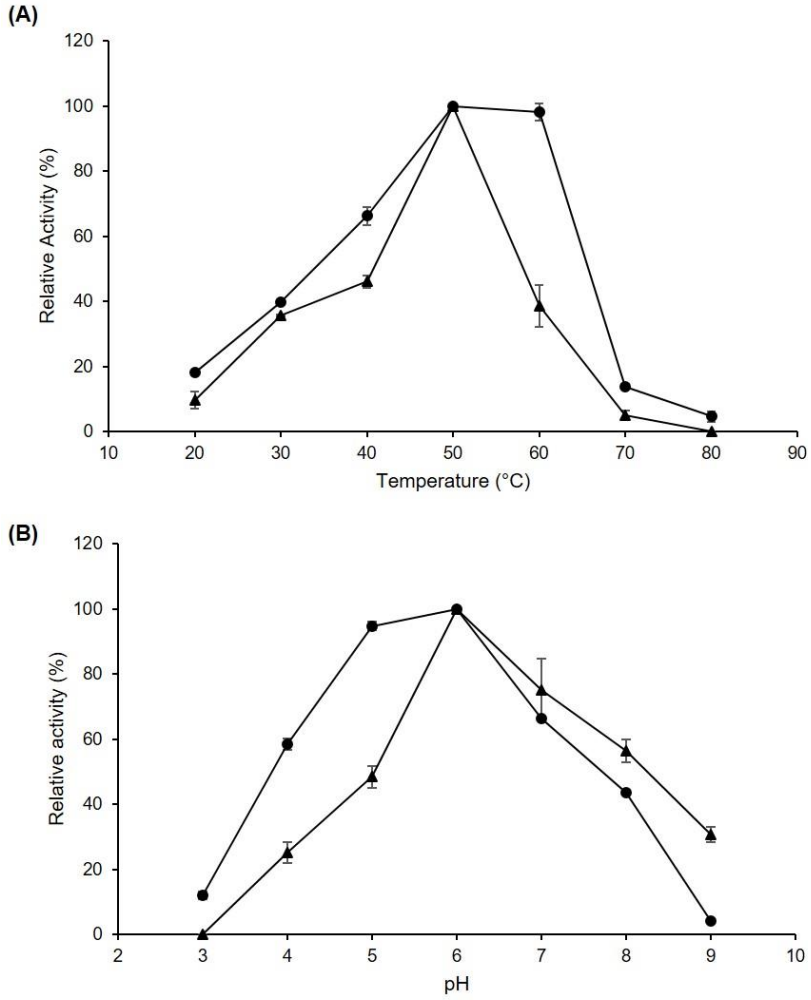

Figure 1. Effect of temperature (A) and pH (B) on the hydrolytic (●) and transfructosylating (▲) activities of FFase from *Aspergillus tamarii* URM4634.

To investigate the possible effect of temperature and pH interaction on FFase activities and identify the optimum conditions for both, we performed a set of cultivations according to a Central Composite Rotational Design (CCRD), where the levels of these two independent variables were selected based on the above individually determined optimum values. Such an approach, which is still poorly reported in the literature for biochemical characterization, provides valuable information for a better understanding of how operating variables influence the catalytic process and was already used to investigate the biochemical profile of enzymes of industrial interest such as proteases,[32] cellulases,[33] pectin lyases,[34] and β-fructofuranosidases.[35]

From the matrix of independent variables and responses used in CCRD (Table 1), one can see that the highest hydrolytic (159.81-160.63 U/mL) and transfructosylating (63.16 U/mL) activities were obtained in the central points (runs 9 and 10 carried out at 50°C and pH 6.00)

and in run 6 (50°C and pH 7.41), respectively, conditions under which the response surfaces showed optimum regions for both (Figure 2).

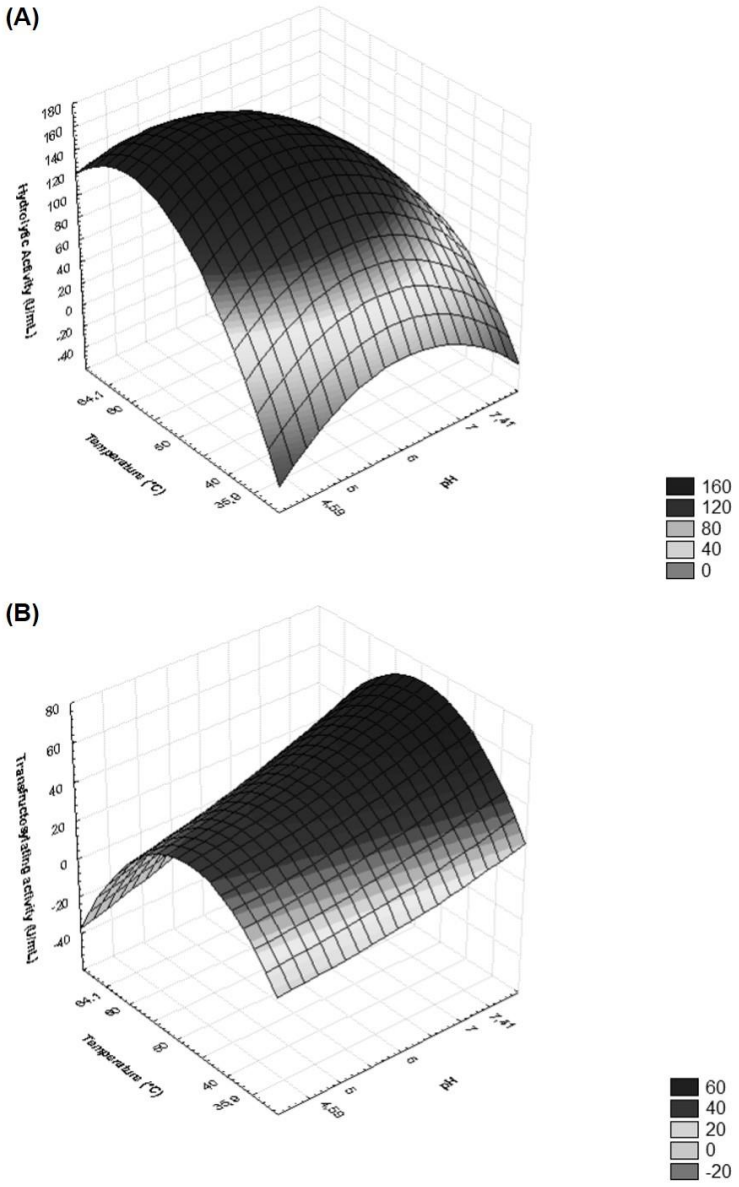

Figure 2. Response surfaces of the hydrolytic (A) and transfructosylating (B) activities of FFase from *A. tamarii* URM4634.

The analysis of effects (Table 2) shows that both variables exerted complex statistically significant effects on the hydrolytic activity, in that it was strongly affected by decreases in both pH (either linear or quadratic effect) and temperature (quadratic effect) or even by their interaction. The trend of hydrolytic activity increase resulting from a pH decrease can be observed in the one-way factor approach, since at pH 5.0 the relative activity was very close to

100%. On the other hand, only temperature exerted a negative statistically significant quadratic effect on the transfructosylating one, which means that it was strongly enhanced by a temperature decrease.

Table 2. Estimated effects of pH and temperature on the hydrolytic (U_H) and transfructosylating (U_{TF}) activities of FFase from *A. tamarii* URM4634 according to the CCRD.

Variable or interaction	Estimates	
	U_H	U_{TF}
(1) pH (L)	-13.72*	3.88
(2) Temperature (L)	15.88*	-2.45
pH (Q)	-9.85*	0.31
Temperature (Q)	-14.78*	-5.09*
1 (L) x 2 (L)	-4.96*	0.63

*Statistically significant estimates at 95% confidence level ($p < 0.05$).

The analysis of the second-order response surface model yielded the following empirical equation to predict the optimal transfructosylating activity (*Y*) of FFase:

$$Y = 50.47 + 8.70X_1 - 5.49X_2 - 13.58{X_1}^2 + 0.8195{X_2}^2 + 2.02X_1X_2 \quad (14)$$

where X_1 and X_2 are the coded levels of temperature and pH, respectively.

The statistical analysis applied to this response indicated the statistical model was adequate, exhibiting no significant lack of fit and a high value of the determination coefficient ($R^2 = 0.93$), whereas that for the hydrolytic activity was unsatisfactory (results not shown).

As far as the thermal stability of FFase is concerned, it was observed that the enzyme was highly stable in the temperature range of 30-55°C and retained as much as 82.7% of starting hydrolytic activity after 180 min of incubation at 50°C (Figure 3A), i.e., the temperature usually employed for industrial FOS synthesis using immobilized FTase.[36] Similar temperature sensibility profile was reported by Gonçalves et al.[37] for FFase from *Fusarium graminearum* and Xu et al.[15] for a novel β-fructofuranosidase from *Penicillium oxalicum* with transfructosylating activity.

Regarding sensitivity to pH variations (Figure 3B), FFase was highly stable within the pH range from 4.0 to 5.0 for 24 h, retaining 93.54 and 87.25% of initial hydrolytic activity,

respectively, but such an activity decreased to 64.95-72.37% at pH > 6.0. This behavior was probably due to deprotonation of important amino acids in the active site and protein surface, and/or to impact on the essential interaction required for stabilization of the enzyme conformational structure.[37,38]

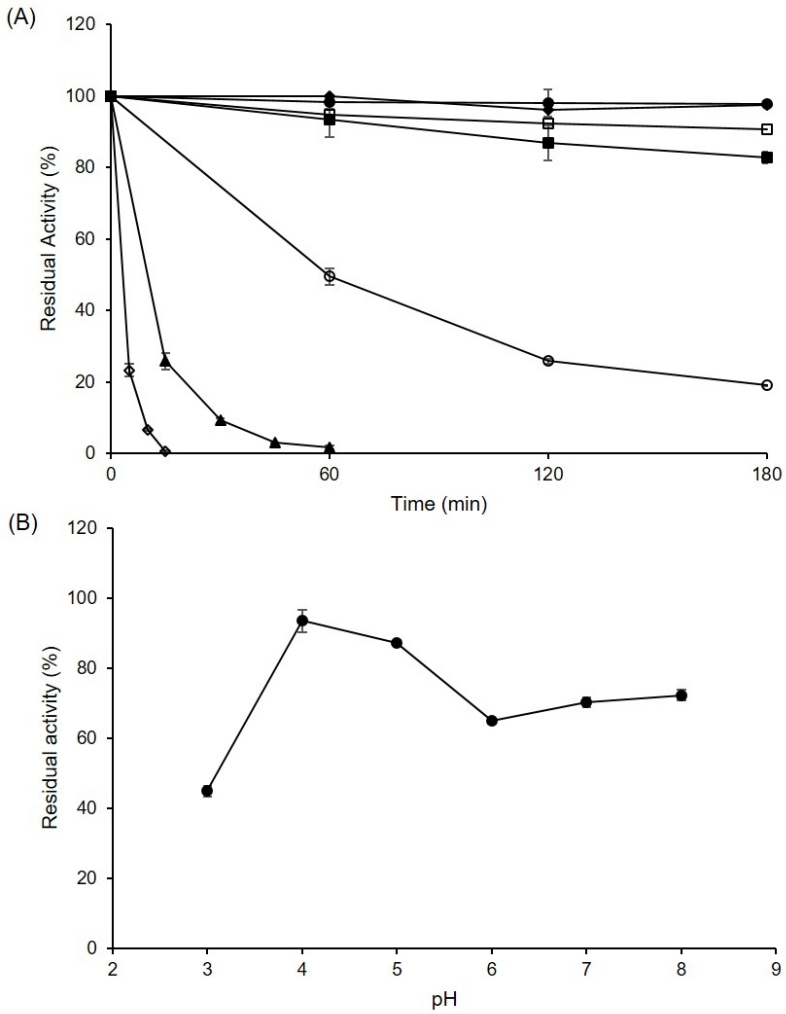

Figure 3. Effects of (A) temperature (°C): 30 (●), 40 (♦), 45 (□), 50 (■), 55 (○), 60 (▲), 65 (◊) and (B) pH on the stability of FFase from *Aspergillus tamarii* URM4634. Residual activity does refer only to hydrolytic activity after an incubation time of 24 h.

3.2. Effect of metal ions on FFase activities

The hydrolytic and transfructosylating activities were also studied in terms of influence of different ions, whose results are given in Table 3. The hydrolytic activity was slightly stimulated by Mn^{2+}, whereas the transfructosylating one was totally inhibited by its presence. In general, the transfructosylating activity was highly sensitive to the presence of metal ions, in

that it was inhibited by all tested ions except Na^+, which suggests that this ion is required for proper catalytic action. A similar effect on transfructosylating activity was reported by Hernalsteens and Maugeri[39] for a FTase from *Rhodotorula* sp., which was stimulated only by Cu^{2+} and inhibited by almost all the remaining ions. Both FFase activities were totally inhibited by Hg^{2+}, which can be explained by the ability of this ion to react with thiol groups, converting them into mercaptides, as well as with histidine and tryptophan residues.[40]

Table 3. Effect of metal ions at 10-mM concentration on hydrolytic and transfructosylating activities of FFase from *A. tamarii* URM4634.

Metal ions	Hydrolytic residual activity (%)	Transfructosylating residual activity (%)
K^+	96.9 ± 0.5^{c}	54.1 ± 3.7^{e}
Ca^{2+}	94.1 ± 0.4^{d}	$63.7 \pm 0.1^{c,d}$
Zn^{2+}	73.2 ± 0.2^{f}	38.2 ± 0.2^{f}
Mg^{2+}	100.1 ± 0.8^{b}	94.2 ± 5.0^{b}
Na^+	100.6 ± 1.4^{b}	118.7 ± 5.8^{a}
Mn^{2+}	102.5 ± 0.0^{a}	0.0 ± 0.0^{g}
Fe^{2+}	91.8 ± 0.6^{e}	65.8 ± 0.9^{c}
Hg^{2+}	0.1 ± 0.0^{h}	0.0 ± 0.0^{g}
Cu^{2+}	42.7 ± 0.5^{g}	61.0 ± 1.0^{d}

Different letters in the same column indicate statistically significant difference between values ($p<0.05$).

3.3. *Kinetic and thermodynamic parameters of FFase thermal inactivation*

During industrial application, enzymes are subject to denaturation upon variation of temperature and prolonged exposure. Therefore, a long-term operational stability of an enzyme preparation is a common prerequisite of successful operation of an enzyme-based bioprocess.[41] Then, to evaluate FFase thermostability, long-term residual activity tests were carried out in the temperature range of 45–65°C, whose results in terms of residual activity coefficient (ψ) are illustrated in Figure 4. The slope of the extrapolated straight lines was used to estimate with satisfactory correlation ($0.92 \leq R^2 \leq 0.99$) the first-order rate constant of inactivation (k_d), whose values at different temperatures are summarized in Table 4 together with those of other kinetic and thermodynamic parameters of enzyme thermal inactivation. In the above tested temperature

range, FFase showed the typical decay of classic first-order denaturation pattern observed by Onderková et al.[41] for FTase from *Aureobasidium pullulans*. One can see that k_d progressively increased with temperature, which means that the inactivation became progressively more significant, likely due to breaking of strong electrostatic bonds.[28]

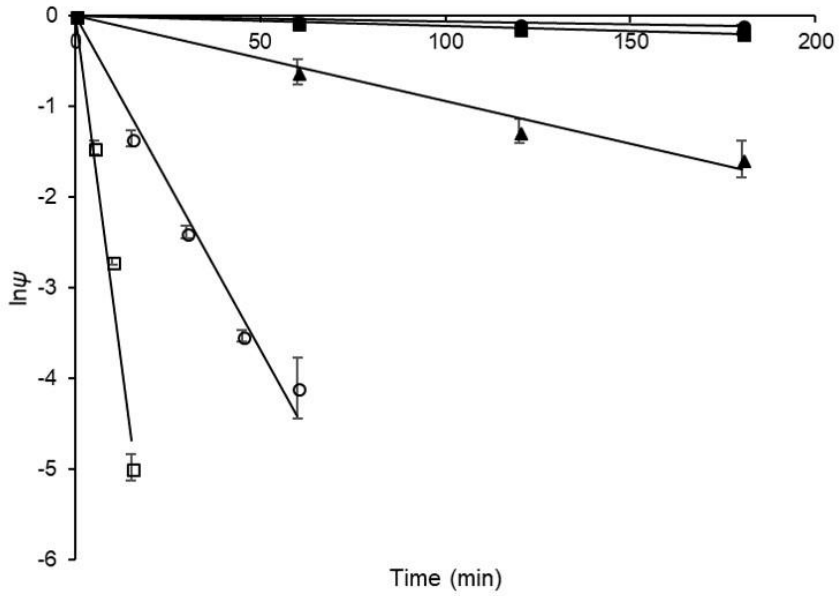

Figure 4. Semi-log plots of the coefficient of residual hydrolytic activity (ψ) of FFase from *A. tamarii* URM4634 vs. time. Temperature (°C): 45 (●), 50 (■), 55 (▲), 60 (○), 65 (□).

Table 4. Kinetic and thermodynamic parameters for thermal denaturation of FFase from *Aspergillus tamarii* URM4634.

Temperature (°C)	k_d (min^{-1})	R^2	$t_{1/2}$ (min)	*D*-value (min)	Z (°C)	E^*_d (kJ mol^{-1})	ΔG^*_d (kJ mol^{-1})	ΔH^*_d (kJ mol^{-1})	ΔS^*_d (J mol^{-1} K^{-1})
45	0.0006	0.92	1270.77	3837.64			108.78	290.43	570.95
50	0.0011	0.99	651.68	2558.43			108.70	290.39	562.22
55	0.0094	0.97	74.28	225.74	6.81	293.08	104.54	290.35	566.22
60	0.0737	0.98	9.39	30.46			100.45	290.30	569.86
65	0.3121	0.98	2.22	7.31			97.95	290.26	568.71

The higher the half-life ($t_{1/2}$) and the *D*-value of an enzyme at a given operating temperature, the longer the time required to reduce its activity by 50 and 90% compared to that at the beginning; therefore, the higher its thermostability.[23] At 50°C, the temperature commonly used for industrial FOS synthesis with immobilized enzymes,[36] the crude *A. tamarii* URM4634 FFase showed very long $t_{1/2}$ (651.7 min) and high *D*-value (2558 min), indicating appreciable thermostability that would enable its possible use in industrial processes (Table 4).

For better understanding of FFase thermosensitivity, its thermal resistance constant, also known as *Z*-value, was estimated with very good correlation (R^2 = 0.98) from the slope of Thermal Death Time curve, i.e. the semi-log plot of *D*-values vs. temperature. Since, in general, low *Z*-values mean more sensitivity to increase in temperature than to duration of heat treatment,[42] the quite low value estimated here (6.81°C) (Table 4) constitutes further proof of thermal stability of the enzyme under investigation. Unfortunately, however, no comparison with literature data is possible due to the lack of specific studies that refer to this parameter.

The activation energy of *A. tamarii* FFase denaturation (E^*_d) was estimated according to Arrhenius, with very good correlation (R^2 = 0.98), from the slope of the straight line obtained by plotting $\ln k_d$ vs. the reciprocal absolute temperature (1/*T*) (Figure 5). Remembering that the higher the value of E^*_d, the greater the energy required to denature an enzyme exposed to thermal treatment, the particularly high value of this parameter (293.1 kJ.mol^{-1}) is consistent with the high thermostability already highlighted by $t_{1/2}$, *D*- and *Z*- values. Moreover, this value is very close to that estimated for a free fructosyltransferase from *Rhodotorula sp*. (294.35 kJ.mol^{-1}),[43] but significantly higher than those of pre-purified (136 kJ.mol^{-1}) and purified (98 kJ.mol^{-1}) levansucrase from *Aspergillus awamori*.[44]

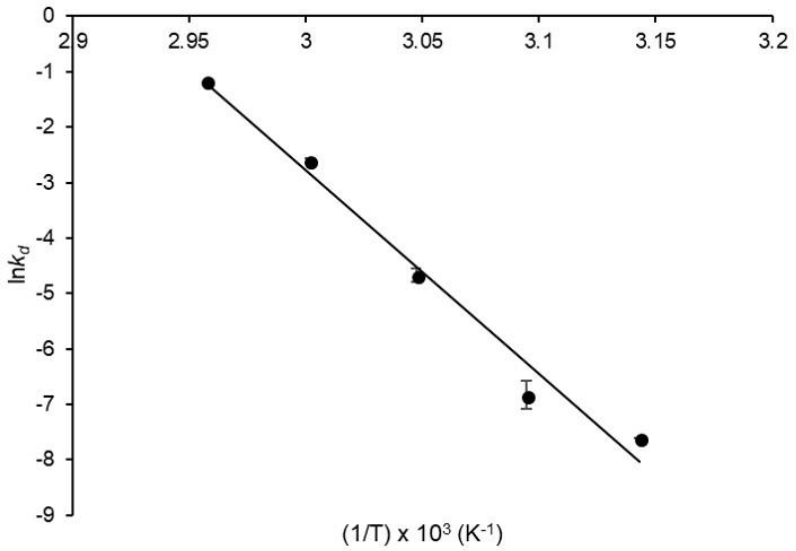

Figure 5. Arrhenius plot to estimate the thermodynamic parameters of irreversible thermal inactivation of FFase from *A. tamarii* URM4634, using the hydrolytic activity.

As is known, E^*_d is directly related to the activation enthalpy of thermal denaturation (ΔH^*_d), which is the total amount of thermal energy required to denature the enzyme and is related to the number of non-covalent bonds broken during the denaturation process.[45–47] The positive and large values of ΔH^*_d estimated for *A. tamarii* FFase (290.4-290.3 kJ.mol^{-1}) (Table 4) are consistent with the fact that enzyme denaturation is an endothermic process and with its high thermostability. It has been proposed that the absorbed thermal energy increases the structural fluctuation in the enzyme structure and thus weakens or disrupts non-covalent bonds that hold it together; therefore, higher values of E^*_d and ΔH^*_d indicate stronger intramolecular stabilizing forces and a less extended conformation.[48] Since the energy required to remove a –CH_2 moiety from a hydrophobic bond is approximately 5.4 kJ mol^{-1},[49] it is possible to estimate that the formation of the transition state preceding FFase denaturation implied the disruption of no less than 53.8 noncovalent bonds. This number is significantly higher compared to other enzymes from the *Aspergillus* genus such as crude and purified protease from *A. tamarii* (8.7 and 4.8, respectively)[43] and pectinase from *A. aculeatus* either free or entrapped in calcium alginate beads (15.3 and 18.3, respectively).[48]

The opening up of the enzyme structure resulting from thermal denaturation is usually accompanied by an increase in the degree of disorder and randomness, i.e. positive values of the activation entropy (ΔS^*_d)[50,51] like those estimated in this study (568.7-570.9 J mol^{-1} K^{-1}). The Gibbs free energy of thermal denaturation (ΔG^*_d) is another important thermodynamic property incorporating both the enthalpic and entropic contributions, therefore it should be

preferred for this kind of comparisons. Negative values of such a parameter are associated with a more spontaneous process, that is to say, the enzyme becomes less stable and more easily undergoes denaturation; contrariwise, positive values of ΔG^*_d like those estimated here (97.9-108.8 kJ mol^{-1}) denote increased resistance to denaturation, i.e. increased thermostability.[47]

3.4. Kinetic and thermodynamic parameters of reactions catalyzed by FFase

The kinetic parameters of both reactions catalyzed by FFase were described by the Michaelis–Menten model and calculated by typical Lineweaver-Burk plots with satisfactory correlation ($0.96 \leq R^2 \leq 0.99$) (results not shown). One can see in Table 5, where these values are gathered together with those of the related thermodynamic parameters, that K_m and V_{max} for hydrolytic and transfructosylating reactions were 54.72 and 369.7 mM and 227.3 and 50.76 mM.min^{-1}, respectively. Such high K_m values may have been due the presence of other enzymes and/or contaminants in the crude extract responsible for a negative impact mainly on FFase transfructosylating activity. As a result, sucrose hydrolysis proceeded more quickly (V_{max} and k_{cat} values more than 4-fold higher) and with higher affinity (K_m values more than 6-fold lower) than fructosyl transfer, confirming that the enzyme under study is a FFase. Similar behavior was reported by Xu et al.[15] for the same kinetic parameters of FFase from *Penicillium oxalicum*, whereas Huang et al.[52] observed for an *A. aculeatus* FTase a V_{max} of transfructosylating activity approximately 42-fold higher than that of hydrolytic activity.

Table 5. Kinetic and thermodynamic parameters of reactions catalyzed by FFase from *A. tamarii* URM4634 using sucrose as a substrate.

Parameter	Sucrose hydrolysis	Fructosyl transfer
K_m (mM)	54.72	369.7
V_{max} (mM.min^{-1})	227.3	50.76
k_{cat} (min^{-1})	31.50	7.03
E^*_a (kJ.mol^{-1})	44.52	45.16
ΔH^* (kJ.mol^{-1})	41.79	42.43
ΔG^* (kJ.mol^{-1})	82.39	86.48
ΔS^* (J mol^{-1} K^{-1})	-123.7	-134.2

The activation energy (E^*_a) of both sucrose hydrolysis and fructosyl transfer catalyzed by FFase from *A. tamarii* URM4634 were estimated through Arrhenius-type plots of lnA_0 vs. 1/T with satisfactory correlation ($0.95 \leq R^2 \leq 0.99$) (Figure 6). This parameter, which indicates the amount of energy required to form the activated complex, was 44.52 kJ.mol^{-1} (R^2 = 0.99) and 45.16 kJ.mol^{-1} (R^2 = 0.95) for sucrose hydrolysis and fructosyl transfer reactions, respectively. While the former value is almost 20% lower than that reported for β-fructofuranosidase from *Saccharum officinarum* L. (sugarcane),[53] highlighting a slightly better thermodynamic condition, no study was found in the literature to compare that of transfructosylation reaction.

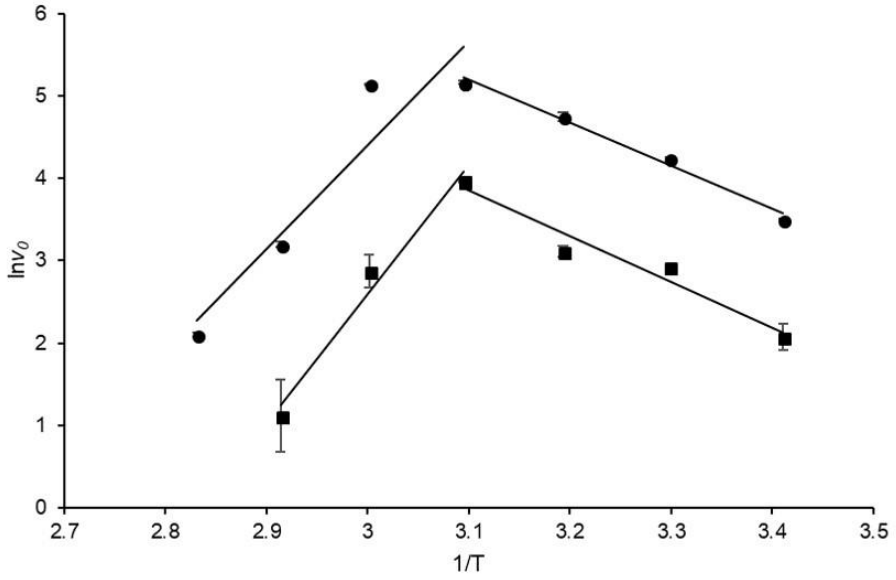

Figure 6. Arrhenius-type plots for determination of the activation energy of the reactions catalyzed by FFase from *A. tamarii* URM4634: hydrolytic (●) and transfructosylating (■) activities.

The temperature coefficient (Q_{10}), which indicates the rate increase resulting from a 10°C increase, is often used to know if a catalytic reaction is controlled by temperature or not. Q_{10} values calculated for sucrose hydrolysis and fructosyl transfer reactions in the temperature range of 20-50°C were coincident, varying both from 1.20 to 1.18 (results not shown). It has been reported that deviations of Q_{10} values from the typical range of enzyme reactions (1 to 2) are indicative of the involvement of factors other than temperature in the control of the reaction rate.[54] Therefore, we can infer that both reactions catalyzed by *A. tamarii* FFase were kinetically controlled by temperature within the entire temperature range investigated. Similar results were reported for a partially purified (1.33) and a purified levansucrase (1.24) from *A. awamori*.[44]

Values of the activation enthalpy (ΔH^*), entropy (ΔS^*) and Gibbs free energy (ΔG^*) of the reactions catalyzed by FFase from *A. tamarii* are listed in Table 5. The relatively low ΔH^* values estimated for sucrose hydrolysis (41.79 kJ.mol^{-1}) and fructosyl transfer (42.43 kJ.mol^{-1}) indicate that the formation of the transition state or activated enzyme–substrate complex occurred effectively. Moreover, the negative values of ΔS^* of both reactions indicate that the structure of enzyme–substrate at transition state was more ordered than the separate reactants and characterized by fewer degrees of freedom.[28,55] Finally, the ΔG^* values of both sucrose hydrolysis (82.39 kJ.mol^{-1}) and fructosyl transfer (86.48 kJ.mol^{-1}) were positive and relatively high, likely because the unfavorable entropic contribution could have almost completely counterbalanced by the favorable enthalpic one,[56] hence confirming the endergonic character of either reaction.

4. Conclusions

A novel β-fructofuranosidase (FFase) from *Aspergillus tamarii* URM4634 displaying both hydrolytic and transfructosylating activities was biochemically characterized. Kinetic results were then used to estimate the thermodynamic parameters of both reactions and those of enzyme thermal denaturation. Optimum pHs of sucrose hydrolysis and fructosyl transfer catalyzed by crude FFase were 6.0 and 7.4, respectively, while optimum temperature was 50°C for both. As demonstrated by very high values of half-life and *D*-value, the enzyme was highly stable in the pH range of 4.0-5.0 and showed high thermostability at 50°C, i.e. the temperature commonly employed to synthesize fructo-oligosaccharides. This conclusion was corroborated by the values of the thermodynamic parameters of thermal denaturation. The results obtained in present study clearly indicate that the novel FFase is a promising efficient biocatalyst for potential long-term use in industrial FOS and invert sugar productions.

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CAPÍTULO IV

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Immobilization of a commercial *Aspergillus aculeatus* enzyme preparation with fructosyltransferase activity in chitosan beads: a kinetic/thermodynamic study and fructo-oligosaccharides production in packed bed reactor

Rodrigo Lira de Oliveira[1], Marcos Fellipe da Silva[2], Suzana Pedroza da Silva[2], Jorge Vinícius Fernandes Lima Cavalcanti[3] Attilio Converti[4], Tatiana Souza Porto[2*].

[1]Northeast Biotechnology Network/RENORBIO, Federal Rural University of Pernambuco (UFRPE), Dom Manoel de Medeiros, Dois Irmãos, 52171-900 Recife, PE,
Brazil.
[2]Academic Unit of Garanhuns/UAG, Federal Rural University of Pernambuco (UFRPE), Garanhuns, Av. Bom Pastor, Boa Vista, 55296-901 Garanhuns, PE, Brazil.
[3]Department of Chemical Engineering, Federal University of Pernambuco (UFPE), Av. Prof. Moraes Rêgo, 1235, Cidade Universitária, 50670-901, Recife, PE, Brazil.
[4]Department of Civil, Chemical and Environmental Engineering, Pole of Chemical Engineering, Genoa University, via Opera Pia 15, 16145 Genoa, Italy

*Corresponding author
Tel.: +55 81 33206345.
Email address: portots@yahoo.com.br

ABSTRACT

Pectinex Ultra SP-L, an enzymatic preparation from *Aspergillus aculeatus* with high fructosyltransferase activity, was covalently immobilized on chitosan beads. The highest immobilization yield (95.9%) was obtained using 4.0% glutaraldehyde for 60 min and 80 rpm at 25±1°C. The immobilized biocatalyst showed good operational stability, being able to retain, after the third cycle of reuse, no less than 100.0 and 73.9% of starting hydrolytic and transfructosylating activities, respectively. The results of residual activity tests enabled us to estimate, for irreversible thermal inactivation of both free and immobilized enzyme, the activation energy (E^*_d = 234.3 and 242.2 kJ·mol^{-1}), enthalpy (234.4≤ΔH^*_d≤234.2 kJ·mol^{-1} and 239.4≤ΔH^*_d≤239.3 kJ·mol^{-1}), entropy (381.2≤ΔS^*_d≤379.8 J·mol^{-1}·K^{-1} and 390.4≤ΔS^*_d≤389.7 J·mol^{-1}·K^{-1}) and Gibbs free energy (109.3≤ΔG^*_d≤103.9 kJ·mol^{-1} and 111.3≤ΔG^*_d≤103.6 kJ·mol^{-1}). The use of the immobilized enzyme in a packed bed reactor allowed obtaining, after 100 min, a fructo-oligosaccharide mixture containing more than 25 g/L 1-kestose.

Keywords: Fructo-oligosaccharides; Fructosyltransferase; Chitosan beads; Kinetic and thermodynamic parameters; Pectinex.

1. Introduction

People have become increasingly interested in health care and safe diet; therefore, nutraceuticals and functional foods have received special attention in the development of new food products during the last few decades (Bali et al., 2015). Fructo-oligosaccharides (FOS) have been the subject of special attention because of their important health benefits, among which are bifidogenic and non-cariogenic properties, stimulation of calcium and magnesium absorption, consumption by diabetic patients, prevention of colon cancer and reduction of total cholesterol, phospholipids and triglycerides in serum (Dominguez et al., 2013; Ganaie et al., 2014).

FOS, which are mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1F-fructofuranosylnystose (GF_3), can be synthesized by the action of two classes of enzymes, namely fructosyltransferases (FTases; E.C. 2.4.1.9) and β-fructofuranosidases (FFases; E.C. 3.2.1.26), commonly called invertases, with the former class possessing higher transferring activity than the latter (Antošová and Polakovič, 2001). Although these enzyme are present either in vegetable or microbial sources, most of commercial enzyme preparations with high FTase activity used in the food industry are obtained from filamentous fungi (Vega-Paulino and Zúniga-Hansen, 2012). Among them, Pectinex Ultra SP-L and other preparations obtained from *Aspergillus aculeatus* have been used in several studies to produce fructo-oligosaccharides (Fernandez-Arrojo et al., 2013; Nemukula et al., 2009) and lactulose-based oligosaccharides (Nguyen et al., 2018, 2019). These preparations contain many enzymes including pectinase, cellulase, β-galactosidase and FTase (Tanriseven and Aslan, 2005).

The use of native enzymes in industrial applications such as FOS synthesis is often hampered by the lack of long-term operational stability and difficulty of recovering and reusing the enzyme. These drawbacks are usually overcome by immobilizing the enzymes (Sheldon and van Pelt, 2013), i.e. confining or attaching free or soluble enzymes in a defined region of space with retention of their catalytic activities (Edet et al., 2013).

Among the large number of supports used for immobilization, chitosan is considered an excellent and inexpensive support due to its inert, hydrophilic, non-toxic and biocompatible characteristics, in addition to its immunological compatibility (Wahba, 2017). Chitosan is as versatile biopolymer made up of linear, high molecular weight polyglucosamine chains with reactive amino and hydroxyl groups, which can be crosslinked with different compounds. The choice of chitosan as an immobilization support is also justified by its economic attractiveness,

being prepared by deacetylation of chitin, the second most abundant natural polymer after cellulose (Muxika et al., 2017). It is usually obtained at a relatively low cost from the shells of shellfish (mainly crabs, shrimps, lobsters and krill) and wastes from the seafood processing industry. Finally, its recycling as immobilization material reduces the environmental impact caused by chitin accumulation in places where it is generated or stored (Bakshi et al., 2019; Krajewska, 2004).

Immobilization by covalent attachment is usually carried out using bifunctional reagents such as glutaraldehyde, one of whose two terminal aldehyde groups reacts with an amine group of chitosan and the other with the enzyme amino terminal group (Klein et al., 2012; Mohamed et al., 2013). Despite its toxicity, it is used under conditions close to physiological pH, ionic strength and temperature, which makes the process easier compared with other reactants. In addition, this crosslinker agent is characterized by an impressive multifunctionality, being able to establish three different kinds of interactions with proteins, namely hydrophobic, anionic exchange and covalent interactions (Barbosa et al., 2014). There are no studies reporting the immobilization of FTase contained in Pectinex Ultra SP-L commercial preparation on chitosan, the only reports dealing with its immobilization on epoxy-activated Sepabeads (Ghazi et al., 2005), alginate (Fernandez-Arrojo et al., 2013), Eupergit (Tanriseven and Aslan, 2005) and anion exchange resin (Csanadi and Sisak, 2006).

Thermodynamic data of enzyme-catalyzed reactions are essential to predict the extent of reaction of any process in which these reactions occur. Enzyme thermostability encompasses thermodynamic and kinetic stability. Whereas the activity of an enzyme is measured by the biochemical reaction catalyzed by it, its stability is related to its residual activity (Gohel and Naseby, 2007). Thermodynamic and kinetic parameters such as activation energy, Gibbs free energy, entropy and enthalpy, as well as half-life ($t_{1/2}$) can provide valuable information on enzyme activity and thermostability at given operating temperatures, thus allowing for better understanding and prediction of free and immobilized enzyme behavior in industrial applications.

Usually, these parameters are improved by immobilization, indicating a stabilization of protein structure with respect to thermal denaturation. High stability is usually considered an economic advantage because it reduces enzyme consumption. Therefore, before proceeding with the development of enzyme formulations, it is necessary to acquire information on enzyme stability under different conditions (Souza et al., 2015). Due the importance of enzyme kinetic/thermodynamic parameters, several studies were performed on different important

enzyme classes for the food industry such as pectinases (Silva et al., 2018a), proteases (Silva et al., 2018b), amylases (Karam et al., 2017), β-galactosidase (Ji et al., 2019) and lipases (Ferreira et al., 2018). However, to the best of our knowledge, no systematic thermodynamic investigation has been performed to date on the activity and thermostability of free or immobilized FTase present in the commercial enzyme preparation used in this study.

Based on this background, the objectives of this study were to covalently bind the commercial Pectinex Ultra SP-L enzyme preparation from *A. aculeatus*, with high FTase activity, to chitosan beads using glutaraldehyde as a crosslinking agent, to undertake a kinetic and thermodynamic study on free and immobilized enzyme and to produce FOS in a packed bed reactor.

2. Materials and Methods

2.1. Materials

Pectinex® Ultra SP-L (P2611), a commercial enzyme preparation from *Aspergillus aculeatus* with fructosyltransferase (FTase) activity, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sucrose (Sigma-Aldrich), chitosan from shrimp shells with deacetylation degree ≥ 75% (Sigma-Aldrich), glutaraldehyde (Dinâmica Química Contemporânea, Diadema, SP, Brazil) and all the other reagents used in this study were of analytical grade.

2.2. Immobilization of enzyme preparation by covalent binding to chitosan beads

Enzyme immobilization was performed according to Klein et al. (2012), with some adaptations. Briefly, chitosan beads were prepared by dropwise addition of 2% (w/v) chitosan solution in 0.35 M acetic acid to a coagulant solution containing 26% (v/v) ethanol in 1.0 M NaOH. The beads were thoroughly washed with distilled water and activated by incubation for 3 h at room temperature (25±1°C) and 150 rpm with glutaraldehyde solutions at different concentrations, keeping a 1:10 (w/v) ratio between beads and glutaraldehyde solution. After activation, the beads were removed by filtration and thoroughly washed to remove unbound glutaraldehyde, whose presence was checked by absorbance measurements at 245 nm as described by Pal and Khanum (2011). Coupling of activated chitosan beads with enzyme preparation was ensured by incubating them in a 1:1 (w/v) ratio at room temperature for

different contact times and using different agitation speeds. After coupling, they were filtered and washed with distilled water until no enzyme activity was detected.

Immobilization experiments were performed according to a 2^3-full factorial design with three additional repetitions of the central point, where glutaraldehyde concentration (2.0, 3.0 and 4.0 % v/v), agitation rate (80, 100 and 120 rpm) and contact time (60, 120 and 180 min) were selected as the independent variables and the immobilization yield (*Y*) as the response. *Y* was defined as:

$$Y\ (\%) = \left(\frac{A}{A_0}\right) \text{x } 100 \quad (1)$$

where *A* is the activity of the immobilized enzyme and A_0 the initial activity of the free enzyme before immobilization. Enzyme activities were determined as described in the following section.

The statistical analysis of the experimental results was performed using the Statistica 7.0 software package (Statsoft Inc., Tulsa, OK, USA) and $p < 0.05$ was considered as statistically significant level. The quality of fit of the linear model was checked by the analysis of variance (ANOVA), the F test and the coefficient of determination (R^2), being $R^2 \geq 0.90$ considered acceptable.

2.3. Analytical determinations

FTase activity was determined according to Sangeetha et al. (2004) with some modifications. Briefly, 0.25 mL of enzyme solution and 0.75 mL of 60% (w/v) sucrose in 0.1 M acetate buffer, pH 5.0, were incubated at 55°C for 1 h. After reaction, samples were collected to determine the concentrations of glucose (*G*) by a commercial glucose oxidase colorimetric kit (Liquiform, Labtest, Lagoa Santa, MG, Brazil) and reducing sugars (*RS*) by the 3'5'-dinitrosalicylic acid method (Miller, 1959), from which the hydrolytic and transfructosylating activities were assessed. For determination of immobilized enzyme activity, 0.25 g of chitosan beads were added to 0.75 mL of sucrose solution. One unit of hydrolytic activity (U_H) was defined as the amount of enzyme required to hydrolyze 1 μmol of sucrose per minute, while one unit of transfructosylating activity (U_{TF}) as that required to transfer 1 μmol of fructose per minute. To calculate the concentration of transferred fructose and estimate the transfructosylating activity, we used the equations initially proposed by Chen and Liu (1996):

$$F = RS - G \quad (2)$$

$$F' = G - F = 2G - RS \quad (3)$$

where *F* and *F'* are the concentrations of free and transferred fructose in the reaction medium, respectively.

Total protein content was determined according to Bradford (1976) using Comassie Brilliant Blue G-250 as the dye and Bovine Serum Albumin as the standard protein. Values of protein concentration were used to calculate de turnover number (k_{cat}) for either free or immobilized enzyme (see section 2.5). Total protein content of the immobilized system was determined as the difference between the initial protein concentration offered to immobilization and that of protein not bound to the support.

2.4. Effect of temperature and pH on the activities of free and immobilized enzyme preparations

Optimum temperatures for enzyme activities were assessed by activity tests carried out at temperatures ranging from 20 to 80°C. Thermal stability of both free and immobilized enzyme preparations was assessed by keeping them in 0.1 M acetate buffer, pH 5.0, at different temperatures for different time intervals (up to 180 min), rapidly cooling aliquots to room temperature in order to refold the reversibly-inactivated enzyme molecules, and then withdrawing samples every 60 min to determine residual activity. The effect of pH on hydrolytic and transfructosylating activities of free and immobilized enzyme was investigated in different buffers, namely 0.1 M sodium citrate ($3.0 \leq pH \leq 4.5$), citrate-phosphate ($4.5 \leq pH \leq 6.0$), sodium phosphate ($6.0 \leq pH \leq 7.5$) and Tris-HCl ($7.5 \leq pH \leq 9.0$).

2.5. Kinetic parameters of reactions catalyzed by free and immobilized enzyme preparations

The apparent Michaelis constant (K_m), maximum reaction rate (V_{max}) and k_{cat} for hydrolysis and fructosyl transfer reactions catalyzed by free and immobilized preparations were estimated using Lineweaver-Burk double reciprocal plots. For this purpose, the hydrolytic and transfructosylating activities were measured at different sucrose concentrations ($73.0 \leq S_0 \leq 2045.3$ mM) as described in section 2.3.

2.6. Storage stability and reusability of immobilized enzyme preparation

Storage stability of immobilized enzyme preparation was assessed by determining hydrolytic and transfructosylating activities over 45 days. For this purpose, the enzyme preparations were kept completely immersed at 4°C in 0.1 M acetate buffer, pH 5.0. The reusability of the immobilized enzyme was checked by conducting repeated cycles of either hydrolysis or transfructosylation reaction. One cycle of use was considered as a batch bioconversion of sucrose to glucose (hydrolytic activity) or fructosyl group transfer (transfructosylating activity) using the immobilized enzyme preparation at pH 5.0 and 55 °C for 1 h. For either storage stability or reusability studies, the residual activity (%) was calculated by considering the enzyme activities of the first day or the first cycle equal to 100%.

2.7. Kinetic and thermodynamic study of free and immobilized enzyme preparations

Enzyme thermostability was studied in the temperature ranges of 55-70°C and 55-75°C for free and immobilized enzyme, respectively, over an incubation time of 180 min. The selection of a larger temperature range for immobilized enzyme was based on its higher thermostability observed during experiments compared to the free enzyme. Since FTase exhibits two interdependent enzyme activities, only the results of its hydrolytic activity were used to calculate the kinetic and thermodynamic parameters of thermal denaturation. Assuming that the long-term thermal inactivation of an enzyme can be described by a first-order irreversible reaction, its first-order rate constant (k_d) was estimated from the slopes of the straight lines obtained by plotting the experimental data of $\ln\psi$ vs time at each temperature (T), being ψ the activity coefficient defined as the ratio of residual enzyme activity to that at the beginning of thermal treatment ($\psi = A/A_o$). The statistical analysis of first-order rate constant (k_d) was only based on standard deviation and correlation coefficient (R^2) of semi-log plots.

The activation energy of the irreversible enzyme inactivation (E^*_d) was determined, according to the logarithmic form of Arrhenius equation, from the slope of the straight line obtained by plotting $\ln k_d$ versus $1/T$:

$$\ln k_d = -E_d^*/RT + \ln A \qquad (4)$$

where R is the ideal gas constant (8.314 J.mol^{-1} K^{-1}).

The activation enthalpy (ΔH_d^*), Gibbs free energy (ΔG_d^*) and entropy (ΔS_d^*) were estimated by the equations:

$$\Delta H_d^* = E_d^* - RT \quad (5)$$

$$\Delta G_d^* = -RT \ln\left(\frac{k_d h}{k_b T}\right) \quad (6)$$

$$\Delta S_d^* = \frac{\Delta H_d^* - \Delta G_d^*}{T} \quad (7)$$

where h (6.626 x 10^{-34} J.s) is the Planck constant and k_b (1.381 x 10^{-23} $J.K^{-1}$) the Boltzmann constant.

The enzyme half-life ($t_{1/2}$) is a parameter often used to predict the viability of long-term use of immobilized-enzyme preparations in continuous processes. It is defined as the time after which the enzyme activity is reduced to one-half of the initial value and can be calculated by the equation (Melikoglu et al., 2013; Souza et al., 2015):

$$t_{1/2} = \frac{\ln 2}{k_d} \quad (8)$$

The *D*-value is defined as the decimal reduction time or the time required to pre-incubate the enzyme at a given temperature to maintain 10% residual activity, while the thermal resistance constant (*Z*-value) the temperature rise required to reduce the *D*-value by one logarithmic unit, i.e. by 90%. The former was calculated, according to Heldman and Hartel (1997), by the equation:

$$D = \frac{\ln 10}{k_d} \quad (9)$$

while the latter from the slope of the graph of log*D* versus *T* (°C), as described by Pal and Khanum (2011).

Due to denaturation, the enzyme activity becomes a function of the operating time. Therefore, the integral activity, *P* (mM), was estimated for both free and immobilized enzyme preparations by integrating the product of the initial hydrolytic activity and the activity coefficient as described by Porto et al. (2006) according to the equation:

$$P(t) = \int A_0\psi \ dt = \int A_0 exp(-k_d t)dt = \frac{A_0}{k_d}[1 - exp(-k_d t)] \quad (10)$$

According to this equation, the integral activity up to the enzyme half-life ($P_{1/2}$) was estimated as:

$$P_{1/2} = \frac{A_0}{2k_d} \quad (11)$$

The activation energies (E^*_a) of both hydrolysis and fructosyl transfer reactions catalyzed by free and immobilized preparations were estimated from the slopes of Arrhenius type-plots of lnA_0 versus 1/T. The other thermodynamic parameters, i.e., activation Gibbs free energy (ΔG^*), enthalpy (ΔH^*) and entropy (ΔS^*), were calculated by equations 5-7 with some adaptations: E^*_d was replaced by E^*_a and k_d by k_{cat}. All these parameters were estimated at a reference temperature of 55°C, i.e., the same temperature used to determine enzyme activities.

The temperature quotient (Q_{10}), which is the factor by which the enzyme activity increases due to a 10 °C temperature increase, was estimated by the equation proposed by Dixon and Webb (1979):

$$Q_{10} = \text{anti log}\left(\frac{E_a^* \text{x } 10}{RT^2}\right) \quad (12)$$

2.8. Enzymatic FOS synthesis

FOS production using immobilized enzyme preparation was performed in a jacketed packed bed reactor (13 cm in length and inner diameter of 1.3 cm) with recirculation to maintain temperature at 50°C. Sucrose solution (600 g/L in 0.1 M acetate buffer, pH 5.0) was used as substrate and was pumped at a flow rate of 1.0 mL/min for 100 min. Samples collected from the reactor were analyzed with a high-performance liquid chromatography (HPLC) device equipped with a pump, model CG 480-E (Instrumentos Científicos C.G. Ltda, São Paulo, SP, Brazil), a refractive index detector (HP 1047A, Hewlett Packard, Valley Forge, PA, USA) and a Luna C-18 (250 mm × 4.6 mm) column (Phenomenex, Torrence, CA, USA). 5.0 mM H_2SO_4 in ultrapure water was used as eluting solvent at a flow rate of 1.1 mL/min and 25 ± 1°C. FOS

standards for the chromatographic analysis (1-kestose and nystose) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

3. Results and discussion

3.1. Enzyme immobilization by covalent binding to chitosan beads

Hydrolytic (378.0 U/mL) and transfructosylating (301.7 U/mL) activities of commercial Pectinex Ultra SP-L preparation were initially reported by Hang and Woodams (1995). The effectiveness of its covalent immobilization was investigated in this study through a set of runs carried out according to a 2^3-full factorial design where the glutaraldehyde concentration, contact time between enzyme and chitosan beads and agitation rate were selected as the independent variables, while the immobilization yield as the response (Table 1). The highest immobilization yield (Y = 95.9%) was obtained at glutaraldehyde concentration of 4.0%, contact time of 60 min and agitation rate of 80 rpm. Similar yields were reported for covalent immobilization on the same type of support of partially purified β-fructofuranosidase from a different commercial preparation (90.0%) (Lorenzoni et al., 2014) and a serine protease (87.5%) (Singh et al., 2011), but using longer contact time (3 and 24 h, respectively). The analysis of variable effects and interactions (Table 2) showed that glutaraldehyde concentration and contact time had statistically significant effects ($p < 0.05$).

Table 1. Experimental conditions and results of immobilization of Pectinex Ultra SP-L commercial enzyme preparation by covalent bonding to chitosan beads performed according to 2^3-full factorial design.

Run	Glutaraldehyde concentration (%)	Coupling time (min)	Agitation rate (rpm)	Immobilization yield (%)
1	2.0	60	80	75.1
2	4.0	60	80	95.9
3	2.0	180	80	69.6
4	4.0	180	80	92.1
5	2.0	60	120	33.2
6	4.0	60	120	93.0
7	2.0	180	120	53.8
8	4.0	180	120	86.4
9	3.0	120	100	73.9
10	3.0	120	100	75.4
11	3.0	120	100	83.8
12	3.0	120	100	68.0

Table 2. Estimated effects and interactions of the independent variables used to immobilize Pectinex Ultra SP-L commercial enzyme preparation by covalent bonding to chitosan beads according to the 2^3-full factorial design shown in Table 1.

Variable or interaction	Estimates
(1) Glutaraldehyde concentration	7.35*
(2) Coupling time	0.26
(3) Agitation rate	-3.59*
1 x 2	-1.38
1 x 3	2.66
2 x 3	1.25
1 x 2 x 3	-1.57

*Statistically significant at 95% confidence level ($p < 0.05$).

Particularly, glutaraldehyde concentration exerted a positive effect, which means that its increase enhanced the immobilization yield. This result is in agreement with the ability of

the two terminal aldehyde groups of glutaraldehyde to react with the amino groups of D-glucosamine units, resulting in the irreversible formation of a Schiff's base among chitosan chains able to stabilize the beads (Singh et al., 2011). Consequently, the higher the concentration of glutaraldehyde, the greater the number of activated amino groups and the higher the immobilization yield. Contrariwise, the negative effect of contact time may be ascribed to excess crosslinking at too long exposure, with consequent distortion of enzyme active site configuration and reduction of its activity used to determine *Y* (Rehman et al., 2014). According to ANOVA, the linear model obtained for this experimental design did not have lack of fit, and the immobilization yield was very high (95.9%). However, since this is only a mathematical statistical and not a biochemical evaluation, the model was validated experimentally. For this purpose, additional experiments were performed to confirm the predicted immobilization yield and verify process reproducibility. Quite similar results were obtained from two repetitions performed under the best condition, which provided a mean *Y* value of 94.59 ± 1.81%.

3.2. Effect of pH on the activities of free and immobilized enzyme preparations

The effect of pH on hydrolytic and transfructosylating activities of both enzyme preparations is depicted in Figure 1. There were no changes in the optimum pH of immobilized and free enzyme, in that the highest relative activities were observed for both at pH values in the range 4.5–6.0 and of 5.0, respectively. Smaali et al. (2012) reported a similar optimum pH for *Aspergillus oryzae* β-fructofuranosidase immobilized on chitosan beads. The immobilized enzyme was slightly more active than the free one under alkaline conditions ($7.0 \leq pH \leq 8.5$) likely due to positive conformational changes in the enzyme tertiary structure induced by covalent binding to chitosan beads (Rehman et al., 2014).

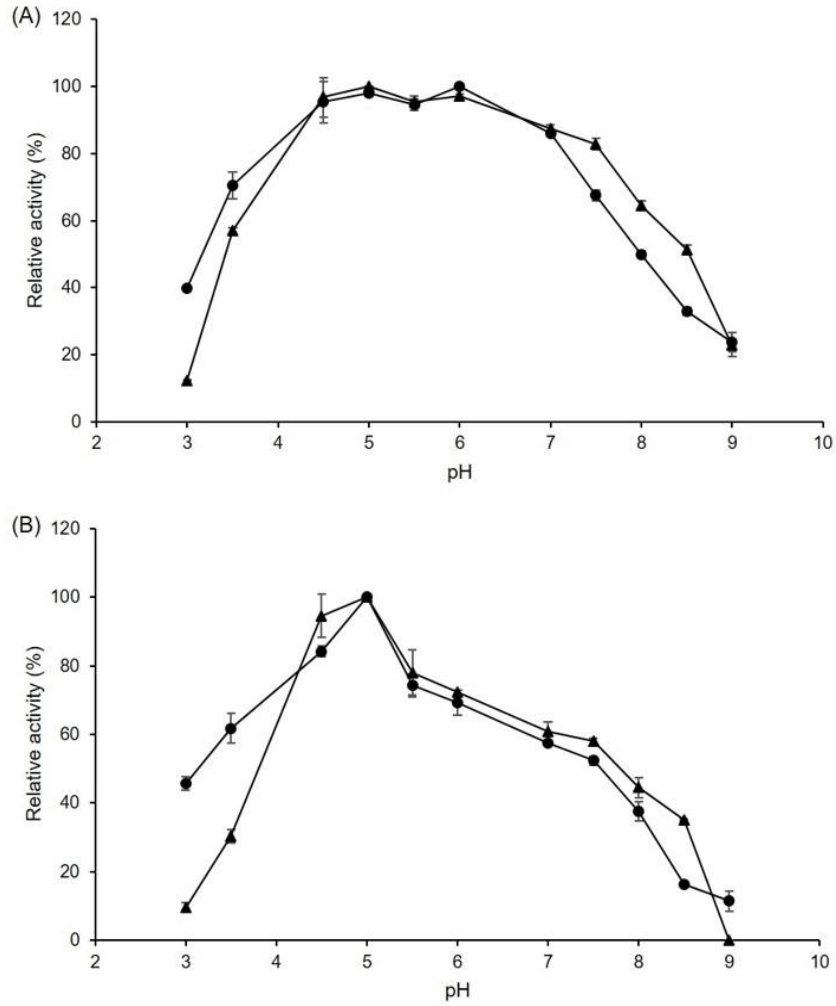

Figure 1. Effect of pH on hydrolytic (A) and transfructosylating (B) activities of *Aspergillus aculeatus* commercial enzyme preparation, either free (●) or immobilized in chitosan beads (▲).

3.3. Effect of temperature on the activity and stability of free and immobilized enzyme preparations

The profiles of hydrolytic and transfructosylating activities at different temperatures are illustrated in Figure 2, panels A and B, respectively. The optimum temperature of the former activity was 60°C using either enzyme preparation, while that of the latter was reduced from 60 to 50°C by immobilization. On the other hand, Figure 2C shows that the immobilized enzyme was more thermostable, being able to retain, in the temperature range 40-60ºC, no less than 85.2-74.5% of starting hydrolytic activity after 180 min of incubation, compared to only 75.1-43.4% of the free one. Such an improved thermostability may have been due to a more rigid shape acquired by the enzyme bound to chitosan beads that preserved its active site

conformation (Rehman et al., 2014). Indeed, an increased enzyme thermostability is a desirable property for any industrial application, since high temperatures are often used to enhance reaction rate, conversion degree and solubility of some reactants. The thermostability of both forms of the enzyme will be discussed in more detail in Section 3.7 as far as the kinetics and thermodynamics of thermoinactivation are concerned.

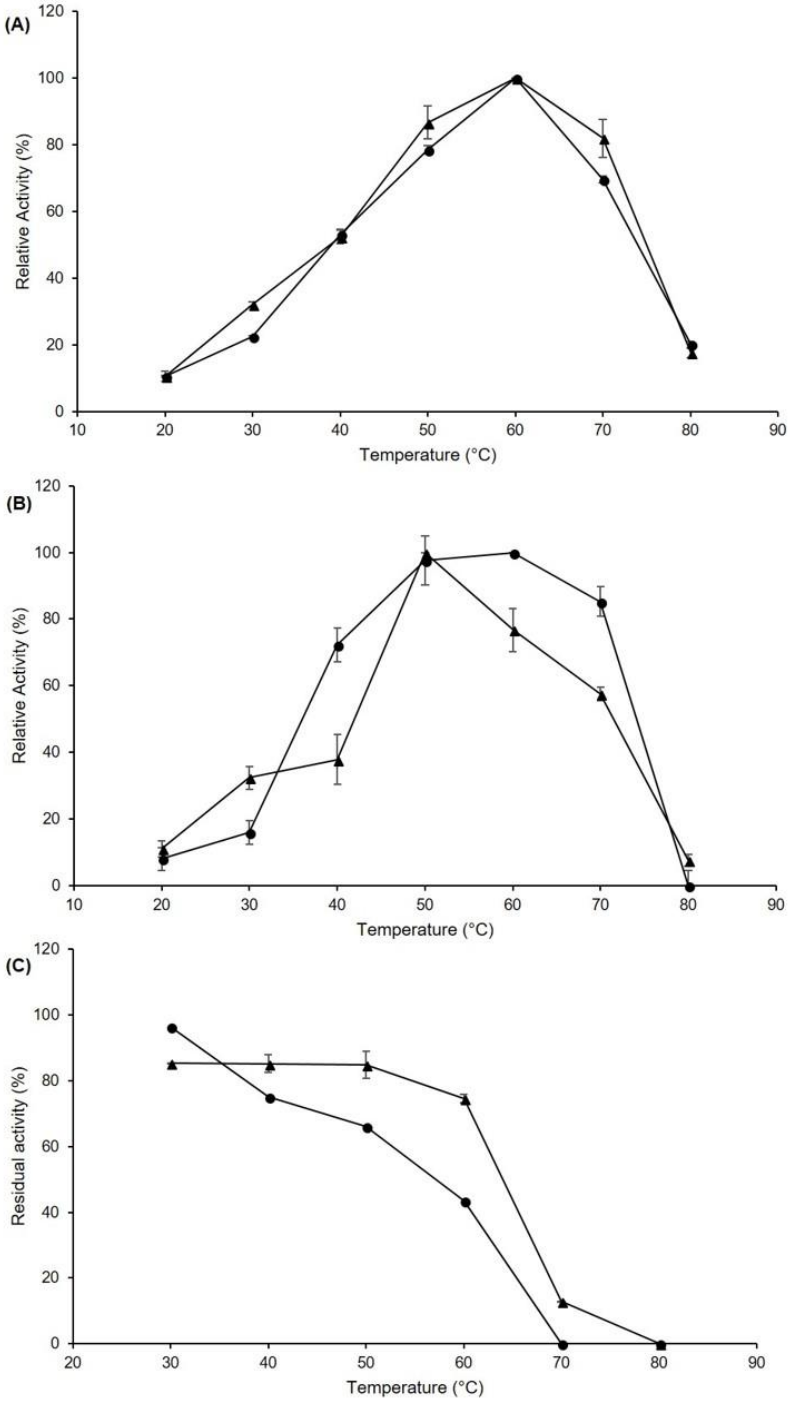

Figure 2. Effect of temperature on hydrolytic (A) and transfructosylating (B) activities, and thermal stability towards hydrolytic activity (C) of *Aspergillus aculeatus* commercial enzyme preparation, either free (●) or immobilized in chitosan beads (▲).

3.4. Reusability and storage stability of the immobilized enzyme preparation

The main advantage of enzyme immobilization is that it allows reusing an enzyme preparation in subsequent cycles. This possibility is often a key factor for the financial feasibility of undertaking a bioprocess with immobilized enzymes (Rehman et al., 2013). The enzyme preparation immobilized on chitosan beads retained 100.0 and 73.9% of starting hydrolytic and transfructosylating activities after three reutilization cycles, and even 74.0 and 42.4% after eight cycles, respectively. Similar retention of hydrolytic activity after eight cycles (70%) was reported by Wahba (2017) for β-galactosidase immobilized on chitosan beads treated with sodium carbonate. It has been proposed that the decrease in immobilized enzyme activity after reutilization may be the result of frequent collisions of substrate molecules with the active site that may distort its regular three-dimensional arrangement (Kishore and Kayastha, 2012), or of the strong covalent linkage of the enzyme to the support that reduces the enzyme 'leaching' during the catalysis (Pal and Khanum, 2011).

Storage stability is another important feature of industrial enzymes because they are susceptible to long-time activity loss even at the low temperatures used for storage. In general, free enzymes are less stable during storage than the immobilized ones, and the improved stability induced by immobilization, especially by covalent binding to chitosan beads, was reported for different industrial enzymes such as amylases (Nwagu et al., 2013), lipases (Chiou and Wu, 2004) and invertases (Koli and Gaikar, 2017). Our immobilized construct retained no less than 100.0 and 80.7% of starting hydrolytic and transfructosylating activities after 45-day storage at 4°C, respectively, which denotes an excellent long-time storage stability that may be exploited for industrial use. Comparable activity retentions were reported for levansucrase immobilized on chitosan beads after 7 weeks of prolonged storage (90%) (Sangmanee et al., 2016) and for amylase immobilized on the same carrier using multipoint covalent binding on polyglutaraldehyde (100%) after 30 days (Nwagu et al., 2013).

3.5. Kinetics of reactions catalyzed by free and immobilized enzyme preparations

Table 3 lists the kinetic parameters of reactions catalyzed by both enzyme preparations, which were estimated from Lineweaver-Burk plots (Supplementary Material, Fig. S1) with satisfactory correlation ($0.945 \leq R^2 \leq 0.994$). Both forms of fructosyltransferase (FTase) followed Michaelis-Menten kinetics, as already observed for other FTases from different

sources such as free FTase from *Aspergillus aculeatus* (Huang et al., 2016) and immobilized FTase from *Rhodotorula* sp. (Alvarado-Huallanco and Maugeri-Filho, 2010), considering both hydrolytic and transfructosylating activities. Michaelis-Menten kinetics were also reported for the FTase contained in Pectinex Ultra SP-L considering only the hydrolytic activity (Tanriseven and Aslam, 2005) and for a recombinant FTase from *Aspergillus niger* considering only the transfructosylating one (Guo et al., 2016).

Table 3. Kinetic parameters of reactions catalyzed by free and immobilized *Aspergillus aculeatus* commercial enzyme preparations using sucrose as a substrate.

Free enzyme				
Reaction	K_m (mM)	V_{max} (mM.min^{-1})	k_{cat} (min^{-1})	R^2
Hydrolysis	191.0	400.0	7.4	0.945
Fructosyl transfer	278.2	370.4	6.9	0.994
Immobilized enzyme				
Reaction	K_m (mM)	V_{max} (mM.min^{-1})	k_{cat} (min^{-1})	R^2
Hydrolysis	232.4	384.6	10.2	0.987
Fructosyl transfer	265.7	357.1	9.5	0.963

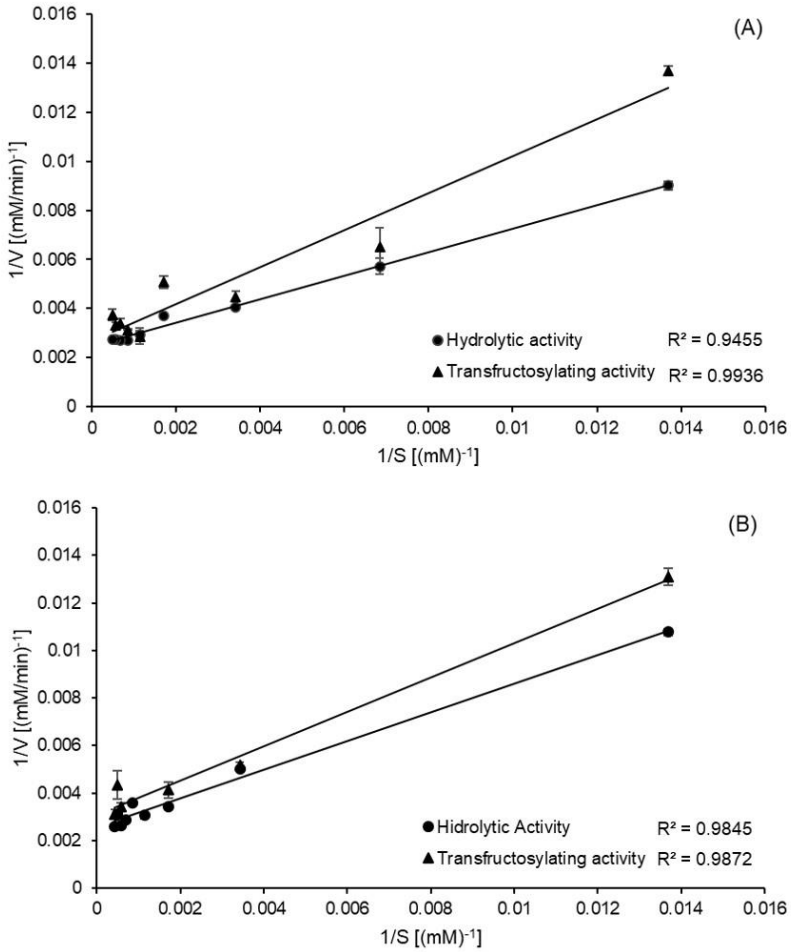

Figure S1. Lineweaver-Burk plots for determination of kinetic parameters of free (A) and immobilized (B) *Aspergillus aculeatus* commercial enzyme preparation.

The Michaelis constant (K_m) of the hydrolysis reaction was 191.0 and 232.4 mM for the free and immobilized enzyme, respectively. The increase in K_m resulting from enzyme immobilization indicates that higher substrate concentration is required to saturate the immobilized enzyme than that normally required to saturate the enzyme in its freely soluble form, thus leading to a decrease in its affinity for the substrate. Another possible reason is that inter- and intramolecular crosslinking may have brought about reduction in surface area, substrate inaccessibility to the active site and limitations in mass transfer (Nwagu et al., 2013). Similar behavior was reported for different enzymes when they were covalently immobilized on chitosan beads such as peroxidase (Mohamed et al., 2013), β-galactosidase (Wahba, 2017) and invertase (Koli and Gaikar, 2017).

A for the fructosyl transfer reaction, a K_m decrease from 278.2 to 265.7 mM was observed after immobilization, which means that, contrary to the hydrolysis, the immobilization favored the fructo-oligosaccharide (FOS) synthesis. Ghazi et al. (2007) reported K_m values of 27 ± 3 and 535 ± 45 mM for hydrolysis and fructosyl transfer reactions, respectively, using a purified FTase from a similar commercial preparation. Such a comparison suggests that the presence of other enzymes in the new raw preparation used in this study may have affected the former activity and enhanced the latter.

The immobilization led to a decrease in V_{max} from 400.0 to 384.6 mM/min and from 370.4 to 357.1 mM/min for the hydrolysis and fructosyl transfer reactions, respectively, which can be ascribed to a limited accessibility of substrate molecules to the active site as well as an interaction of enzyme molecules with functional groups on the support surface (de Oliveira et al., 2018). The apparent turnover numbers (k_{cat}) of both reactions catalyzed by the immobilized enzyme were higher than those obtained with the free enzyme. Such an increase is not common for immobilized enzymes. A possible explanation for this behavior is that immobilization led to a more active enzyme, as it may happen with multimeric biocatalysts, which are endowed with a more flexible active site than the monomeric counterparts (Ionata et al., 2018).

3.6. Thermodynamic parameters of free and immobilized enzyme activities

As is well known, the activation energy (E^*_a) of a reaction is the energy required to proceed from the reactant state to the transition state. The values of E^*_a of sucrose hydrolysis and fructosyl transfer catalyzed by free and immobilized enzyme preparations were estimated with satisfactory correlation from Arrhenius plots of $\ln A_0$ vs. the reciprocal absolute temperature ($1/T$) (Figure 3). For sucrose hydrolysis we estimated very close E^*_a values using free (44.94 kJ.mol^{-1}; $R^2 = 0.933$) and immobilized (45.51 kJ.mol^{-1}; $R^2 = 0.966$) enzyme. On the other hand, for fructosyl transfer E^*_a was little lower with the former (50.14 kJ.mol^{-1}; $R^2 = 0.923$) than with the latter (53.03 kJ.mol^{-1}; $R^2 = 0.938$) biocatalyst. These results suggest for fructosyl transfer a greater difficulty of the immobilized enzyme to achieve the optimum conformational structure needed to form the enzyme–substrate complex, whereas no appreciable variation took place for sucrose hydrolysis, contrary to what observed for covalent immobilization of invertase (Cadena et al., 2010).

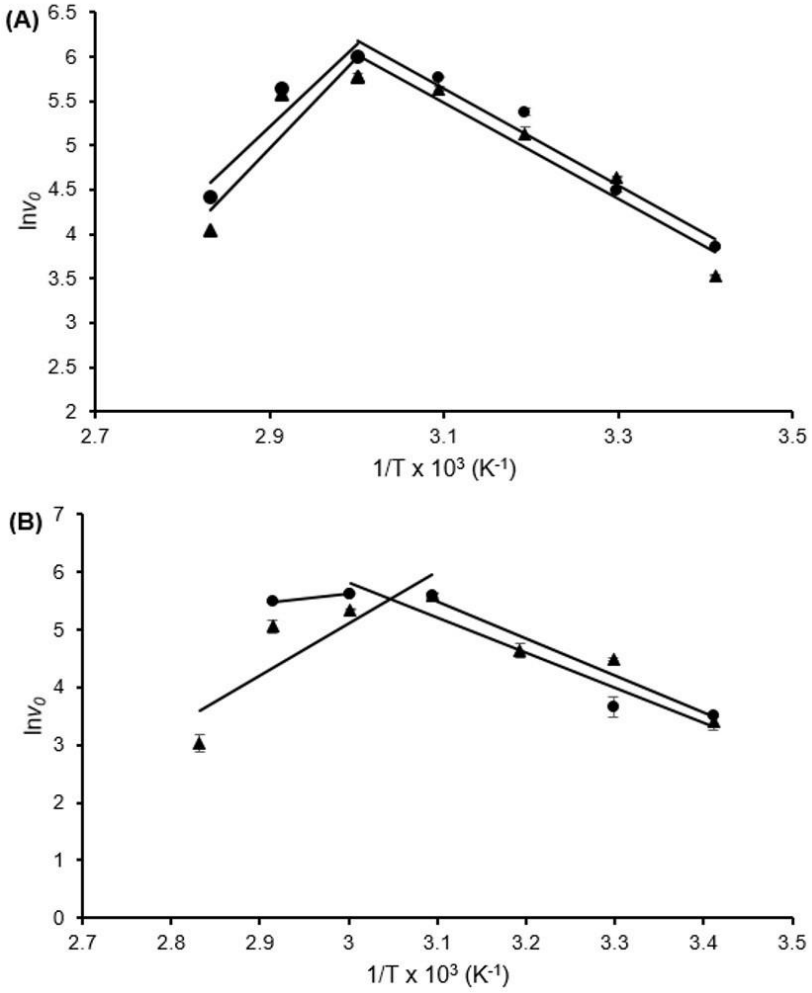

Figure 3. Arrhenius-type plots used to estimate the activation energy of sucrose hydrolysis (A) and fructosyl transfer (B) catalyzed by *Aspergillus aculeatus* commercial enzyme preparation, either in free form (●) or immobilized in chitosan beads (▲), using sucrose as a substrate.

The other thermodynamic parameters, namely activation enthalpy (ΔH^*), Gibbs free energy (ΔG^*) and entropy (ΔS^*), of the reactions catalyzed either by free or immobilized enzyme are given in Table 4. ΔH^* and ΔS^* values of both reactions catalyzed by the immobilized enzyme were higher than those obtained with the free one. Compared with other enzyme systems (Porto et al., 2006; Silva et al., 2018b; Wehaidy et al., 2018), the relatively low ΔH^* values of both sucrose hydrolysis and fructosyl transfer using either free (42.21 and 47.41 kJ.mol^{-1}) or immobilized (42.68 and 50.30 kJ.mol^{-1}) forms of the enzyme indicate that the formation of transition state or activated enzyme–substrate complex occurred effectively. ΔS^* is known to be correlated to the order (rigidity) degree of the enzyme-substrate activated complex. The ΔS^* values of both reactions catalyzed by either enzyme preparation were negative, which suggests that the structure of enzyme–substrate at transition state was more ordered than that of enzyme–

substrate complex (Silva et al., 2018b; Xiong et al., 2005). Moreover, the lower ΔS^* values obtained with the free enzyme may have been due to more ordered structures of transition states compared with the immobilized one. It is likely that the lack of specificity of the immobilization reaction led to a lot of immobilized enzyme stable forms, i.e., a much greater degree of freedom. Oppositely, there was only one most stable form of the free enzyme.

Table 4. Thermodynamic parameters of sucrose hydrolysis and fructosyl transfer reactions catalyzed by free and immobilized *Aspergillus aculeatus* commercial enzyme preparations.

Parameter	**Sucrose hydrolysis**		**Fructosyl transfer**	
	Free	Immobilized	Free	Immobilized
ΔH^* (kJ.mol^{-1})	42.21	42.68	47.41	50.30
ΔG^* (kJ.mol^{-1})	86.32	85.46	86.54	85.66
ΔS^* (J mol^{-1}.K^{-1})	-134.44	-130.38	-119.23	-107.77

ΔG^* is the true thermodynamic parameter able to shed light on the feasibility and extent of a chemical reaction. Thus, the relative low ΔG^* values obtained with both enzyme preparations for either sucrose hydrolysis or fructosyl transfer reactions indicate that the conversion of the enzyme-substrate complex to product should occur very quickly (Riaz et al., 2007; Wehaidy et al., 2018).

The temperature coefficient (Q_{10}), which indicates the rate increase due to a 10°C temperature rise, is a kinetic parameter sometimes used to infer whether a catalytic reaction is controlled by temperature or not. Generally, enzyme reactions are characterized by Q_{10} values between 1 and 2, and deviations from this range are indicative of the involvement of factors other than temperature in the control of the reaction rate (Wehaidy et al., 2018). The Q_{10} values estimated in the temperature range of 20-60°C for sucrose hydrolysis reaction were practically coincident using free and immobilized enzyme, varying from 1.88 to 1.63 and from 1.89 to 1.64, respectively, while those for fructosyl transfer reaction were appreciably lower with the free (2.02-1.72 at 20-60°C) rather than the immobilized (2.10-1.78 at 20-50°C) form. The slightly higher Q_{10} values for transfructosylating reaction catalyzed by the immobilized enzyme indicate that its rate increased with temperature more than using the free one. On the other hand, the two biocatalysts behaved almost coincidently with respect to the hydrolysis reaction. Nonetheless, both reactions were kinetically controlled by temperature within almost the entire temperature ranges investigated.

3.7. Kinetic and thermodynamic parameters of thermal inactivation of free and immobilized enzyme preparations

Irreversible thermal enzyme inactivation is one of the major constraints in long-term enzyme processes. Therefore, knowledge of its kinetic and thermodynamic parameters is of great importance, and enzyme resistance to thermal inactivation has become a desirable property in many industrial applications (Mateo et al., 2007). As far as the FTase thermostability is concerned, either free or immobilized enzyme preparation was subjected to residual activity tests as function of time in the temperature ranges of 55-70°C or 55-75°C, respectively, whose results in terms of residual activity coefficient (ψ) are illustrated in the semi-log plots of Figures 4A and B. In both cases the activity followed the typical decay of the classic first-order denaturation pattern also observed by Onderková et al. (2010) for free FTase from *Aureobasidium pullulans*.

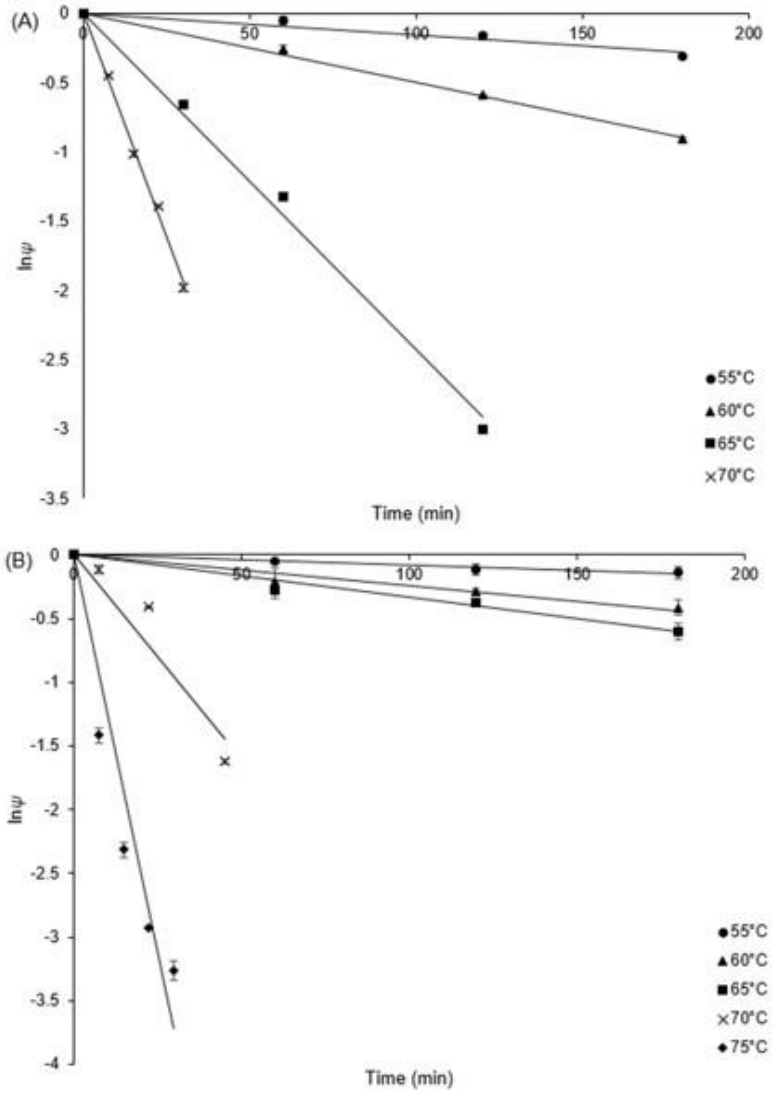

Figure 4. Semi-log plots of irreversible thermal inactivation of free (A) and immobilized (B) *Aspergillus aculeatus* commercial enzyme preparation, using hydrolytic activity.

The main kinetic parameters of thermal inactivation of both forms of the enzyme were estimated with satisfactory correlation from the straight lines of Figure 4 and gathered in Table 5. It was observed, for both enzyme preparations, that the first-order rate constant (k_d) progressively increased with temperature, whereas the half-life ($t_{1/2}$) decreased, which means that the inactivation was progressively accelerated and the thermostability reduced. The values of k_d obtained at 60°C for free and immobilized FTase were 0.0050 and 0.0024 min^{-1}, and the corresponding ones of $t_{1/2}$ 140.04 and 293.40 min. At the same temperature, both preparations showed higher thermostability than free β-fructofuranosidase present in *A. aculeatus* commercial Viscozyme-L preparation also exhibiting FTase activity (k_d = 0.222 min^{-1}; $t_{1/2}$ =

192 min) (Lorenzoni et al., 2014). The thermostability at 55-65°C of immobilized FTase was better than that of free FTase at 55-60°C, as proven by longer decimal reduction times (*D*) (3070-668.7 min vs. 1397-465.2 min) and $t_{1/2}$ (924.2-201.3 min vs. 420.5-140.0 min). Such an enzyme protection against heat inactivation induced by immobilization can be ascribed to the formation of multiple covalent bonds between enzyme and chitosan beads, which may have reduced conformational flexibility, thermal vibrations, protein unfolding and denaturation (Karam et al., 2017), and/or to restricted interaction among enzyme molecules (Pal and Khanum, 2011). Such a stability increase was confirmed by the so-called stabilization factor, defined as the ratio between the half-lives of immobilized and free enzyme, that varied from 2.19 and 2.01 in the temperature range 55-70°C, consistently with what reported by other authors for covalent immobilization of different enzymes using as a bifunctional reagent (Mateo et al., 2007).

Table 5. Kinetic and thermodynamic parameters of irreversible thermal inactivation of free and immobilized *Aspergillus aculeatus* commercial preparations with FTase activity.

Temperature (°C)	k_d (min^{-1})	R^2	$t_{1/2}$ (min)	*D*-value (min)	*Z*-value (°C)	E^*_d (kJ mol^{-1})	ΔG^*_d (kJ mol^{-1})	ΔH^*_d (kJ mol^{-1})	ΔS^*_d (J mol^{-1} K^{-1})	$P_{1/2}$ (mM)
Free enzyme										
55	0.0017	0.942	420.5	1397	9.09	237.0	109.3	234.4	381.2	53083
60	0.0050	0.996	140.0	465.2			107.9	234.3	379.4	17911
65	0.0258	0.993	26.92	89.42			105.0	234.3	382.4	1880
70	0.0648	0.996	10.71	35.56			103.9	234.2	379.8	928.4
Immobilized enzyme										
55	0.0008	0.976	924.2	3070	9.0	242.2	111.3	239.4	390.4	118873
60	0.0024	0.948	293.4	974.6			110.0	239.4	388.5	38964
65	0.0035	0.968	201.3	668.7			110.6	239.4	380.7	22983
70	0.0321	0.913	21.56	71.62			105.9	239.3	388.7	1649
75	0.1240	0.902	5.59	18.57			103.6	239.3	389.7	159.0

Semi-log plots of *D*-values vs. temperature allowed obtaining Thermal Death Time curves, from whose slopes we estimated almost coincident thermal resistance constants (*Z*-values) for free (9.1°C; R^2 = 0.989) and immobilized (9.0°C; R^2 = 0.953) preparations, thus highlighting similar sensitivity to temperature increases (Tayefi-Nasrabadi and Asadpour, 2008). Therefore, the slightly lower *Z*-value of immobilized enzyme compared to the free one suggests that immobilization made the enzyme slightly more sensitive to temperature increases than to the duration of heat treatment. It is likely that covalent immobilization, because of its lack of specificity, involved several different lateral amino groups of the enzyme molecules or, better still, each enzyme molecule may have reacted through different amino groups. Therefore, the resulting thermostability may have been an average result, better or worse than that of the free enzyme depending on the amino groups mainly involved in the attachment. Contrariwise, a decrease in *Z*-values induced by immobilization was reported for xylanase covalently bound to alginate beads (Pal and Khanum, 2011) and pectinase bound to alginate-agar gel beads (Wahab et al., 2018).

The semi-log plot of $\ln k_d$ vs. $1/T$ (Figure 5) enabled us to estimate activation energies of the irreversible thermal inactivation (denaturation) (E^*_d) of 237.0 kJ/mol (R^2 = 0.989) for free and 242.2 kJ/mol (R^2 = 0.998) for immobilized FTase. These values can be considered particularly high compared with those of most enzyme systems (Porto et al., 2006), confirming the high thermostability of both enzyme preparations early evidenced by the other parameters. Moreover, the higher E^*_d value observed for the immobilized enzyme indicates that a larger temperature increase was needed to inactivate it, i.e. it was more thermostable, compact and resistant to heat than the free one (Pal and Khanum, 2011). The high positive values of activation enthalpy of denaturation (ΔH^*_d) estimated using free and immobilized enzyme mean that high total energy was required to denature it in both preparations (Souza et al., 2015).

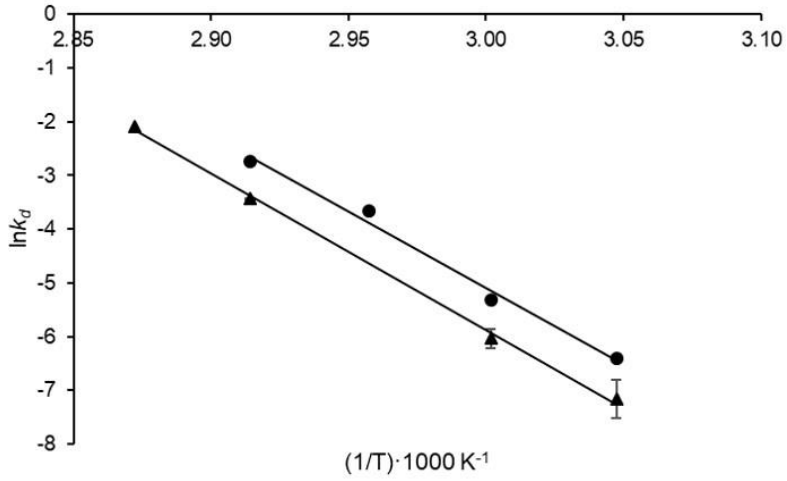

Figure 5. Arrhenius-type plots used to estimate the thermodynamic parameters of thermal inactivation of *Aspergillus aculeatus* commercial enzyme preparation, either in free form (●) or immobilized in chitosan beads (▲), using hydrolytic activity.

Moreover, in the range of temperature investigated, the immobilized enzyme showed little higher ΔH^*_d values (239.4–239.3 kJ.mol^{-1}) than the free one (234.4–234.2 kJ.mol^{-1}), suggesting that immobilization slightly increased the total energy required to denature the enzyme through conformational changes in the structure induced by covalent binding, hence stabilizing it (Wahab et al., 2018). The results listed in Table 5 also show that an increase in temperature slightly reduced ΔH^*_d of both enzyme preparations, making the enzyme more sensitive to denaturation, as the likely result of disruption of non-covalent bonds, including hydrophobic interactions (Melikoglu et al., 2013). It has been reported that the energy required to remove a -CH_2- group from a hydrophobic bond is approximately 5.4 kJ.mol^{-1} (Pace, 1992). Therefore, we can estimate that the transition state formation preceding denaturation of free and immobilized FTase required the disruption, on average, of 43.4 and 44.3 non-covalent bonds, respectively. In other words, one can infer that FTase covalent immobilization was able to prevent the disruption of one of these bonds.

Disruption of enzyme structure is usually accompanied by an increase in the degree of disorder or randomness, which is expressed by the activation entropy of denaturation (ΔS^*_d), i.e., the amount of energy per temperature degree involved in the transition from a native to a denatured state (Marangoni, 2003). The positive values of this parameter for both enzyme preparations (Table 5) are consistent with an increase in such degree (Saqib et al., 2010)

qualitatively similar to that reported for free and immobilized *Rhodutorula* sp. FTase (Aguiar-Oliveira and Maugeri, 2011).

The activation Gibbs free energy (ΔG^*_d) of thermal denaturation is the most accurate and reliable parameter for predicting and evaluating enzyme thermostability because it incorporates the enthalpic and entropic contributions. A smaller or negative value of such a parameter is associated with a more spontaneous process, i.e., the enzyme becomes less stable and more easily undergoes denaturation, whereas an increase in ΔG^*_d is an index of better resistance to denaturation (Souza et al., 2015). ΔG^*_d values for free and immobilized FTase were quite high, varying in the ranges 109.3-103.9 kJ.mol^{-1} and 111.3-103.6 kJ.mol^{-1}, respectively, with slightly higher values for the immobilized one. The observed increase in ΔG^*_d and the related increase in thermostability induced by covalent immobilization was already reported for different enzymes (Karam et al., 2017; Pal and Khanum, 2011; Silva et al., 2018a). These results taken together confirm the excellent thermostability of both preparations as well as the enhanced intrinsic ability of enzyme polypeptide chains to face thermal unfolding at high temperatures due to immobilization.

3.8. Integral activity of free and immobilized enzyme preparations

As discussed in other sections, free and immobilized enzyme preparations showed desirable operational characteristics that would enable them to be industrially applied in continuous FOS synthesis (Aguiar-Oliveira and Maugeri, 2011; Vaňková et al., 2008). In view of this possibility, we investigated the time behavior of enzyme integral hydrolytic activity, $P(t)$, as defined in Eq. (10), which represents the amount of substrate (sucrose) hydrolyzed by 1.0 g of biocatalyst under continuous feed conditions. Such a parameter was estimated at temperature ranges of 55-65°C and 55-70°C for free and immobilized enzyme preparations, respectively, and plotted versus time in bi-log plots (Figure 6). All the curves grew linearly with nearly the same starting rate, which appreciably decreased when temperature was increased because of quicker long-term enzyme denaturation. The only exception in this trend was the run performed at 75°C with the immobilized enzyme, which displayed remarkably lower initial activity than the others. A possible explanation for this phenomenon is a temperature-dependent starting activity decay, which could not be evidenced in runs carried out at 55–70°C with both forms of the enzyme. The same phenomenon was observed by Silva et al. (2018b) in their study on thermostability of purified *Aspergillus tamarii* protease.

Since an improved enzyme thermostability is often counterbalanced by a long-term activity decrease, an optimum compromise between these opposite tendencies should be looked for to select the best conditions to perform an industrial process (Porto et al., 2006). For this purpose, the integral activities up to the half-life ($P_{1/2}$) for both enzyme preparations were calculated, whose values are also listed in Table 5. At all the temperatures evaluated, $P_{1/2}$ of the immobilized enzyme was higher than that of the free one, confirming its preference to perform a continuous process. Simulation of integral hydrolytic activity is especially useful for FOS synthesis because sucrose hydrolysis is responsible for glucose and fructose release, and fructosyl groups are transferred later forming oligosaccharides. Such a simulation indicates a satisfactory thermostability of both enzyme preparations at temperatures higher than those commonly used in industrial FOS synthesis (50°C) by a continuous enzyme process (Vaňková et al., 2008). However, the successful implementation of free and immobilized enzymes would require more extensive stability studies correlating residual enzyme activity with formulation variations under different reaction conditions.

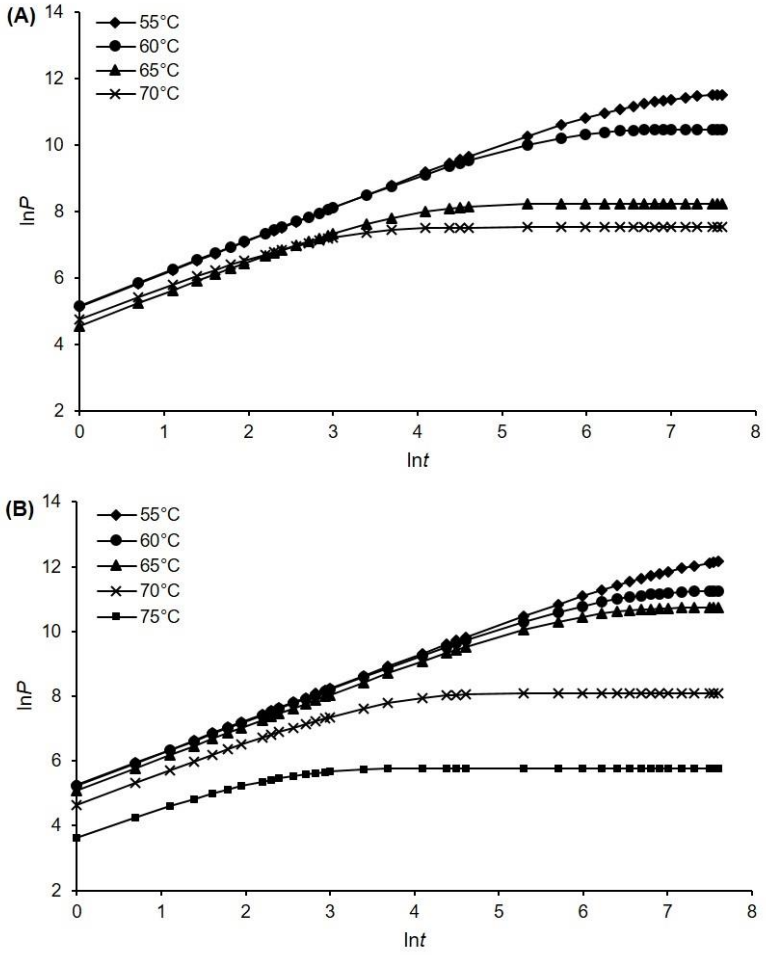

Figure 6. Plot of the logarithm of integral hydrolytic activity (ln*P*) versus the logarithm of time (ln*t*) of *Aspergillus aculeatus* free FTase (A) and FTase immobilized in chitosan beads (B) at different temperatures.

3.9. FOS synthesis in packed bed reactor

The synthesis of FOS, catalyzed by the commercial Pectinex Ultra SP-L enzyme preparation immobilized on chitosan beads, was studied in a packed-bed reactor for 100 min under continuous operation. The final FOS mixture contained 25.73 g/L of FOS and was mainly composed of 1-kestose (23.77 g/L) and nystose (1.96 g/L), whereas no production of 1-fructofuranosylnystose was detected. These results indicate that the time of reaction was insufficient for 1-kestose to have been used as a fructosyl residue donor to complete glycosidic chain elongation, which suggests that only sucrose may have acted as fructosyl donor and acceptor for FOS formation (Antošová et al., 2008). Nascimento et al. (2016) reported a similar

behavior for a crude β-fructofuranosidase from *Penicillium citreonigrum*, but at lower sucrose concentration (20% w/v).

4. Conclusions

The Pectinex Ultra SP-L commercial preparation with transfructosylating activity was successfully immobilized (yield > 95%) by covalent binding to chitosan beads using glutaraldehyde as a crosslink agent. The hydrolytic and transfructosylating activities of the enzyme were maintained at high levels after immobilization. The immobilized fructosyltransferase exhibited many industrially interesting properties such as greater thermal stability than the free enzyme as well as high reusability. Kinetic and thermodynamic parameters of free and immobilized enzyme indicated that both preparations were highly thermostable, especially the latter. The immobilized enzyme was able to produce FOS efficiently. These results may be exploited to develop a continuous bioconversion process to produce prebiotic fructo-oligosaccharides from sucrose as a substrate.

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Fructo-oligosaccharides production by an *Aspergillus aculeatus* commercial enzyme preparation with fructosyltransferase activity covalently immobilized on Fe_3O_4–chitosan-magnetic nanoparticles

Rodrigo Lira de Oliveira[1], Marcos Fellipe da Silva[2], Suzana Pedroza da Silva[2], Ana Cláudia Vaz de Araújo[3], Jorge Vinícius Fernandes Lima Cavalcanti[4], Attilio Converti[5], Tatiana Souza Porto[2*].

[1]Northeast Biotechnology Network/RENORBIO, Federal Rural University of Pernambuco (UFRPE), Dom Manoel de Medeiros, Dois Irmãos, 52171-900 Recife, PE, Brazil.
[2]Academic Unit of Garanhuns/UAG, Federal Rural University of Pernambuco (UFRPE), Garanhuns, Av. Bom Pastor, Boa Vista, 55296-901 Garanhuns, PE, Brazil.
[3]Academic Unit of Cabo de Santo Agostinho/UACSA, Federal Rural University of Pernambuco (UFRPE), Rua Cento e Sessenta e Três, 300, Garapu, 54518-430, Cabo de Santo Agostinho, PE, Brazil.
[4]Department of Chemical Engineering, Federal University of Pernambuco (UFPE), Av. Prof. Moraes Rêgo, 1235, Cidade Universitária, 50670-901, Recife, PE, Brazil.
[5]Department of Civil, Chemical and Environmental Engineering, Pole of Chemical Engineering, Genoa University, via Opera Pia 15, 16145 Genoa, Italy.

*Corresponding author
Tel.: +55 81 33206345.
Email address: portots@yahoo.com.br

ABSTRACT

Pectinex Ultra SP-L, a commercial enzyme preparation with fructosyltransferase activity, was successfully immobilized by covalent binding to Fe_3O_4–chitosan- magnetic nanoparticles. Immobilization carried out according to a 2^3-full factorial design where glutaraldehyde concentration, activation time and time of contact between enzyme and support were selected as the independent variables and immobilization yield as the response. The highest immobilization yield (94.84 %) was obtained using 3.0 % (v/v) glutaraldehyde and activation and contact times of 180 and 30 min, respectively. The immobilized biocatalyst, which showed for both hydrolytic and transfructosylating activities optimum pH and temperature of 7.0 and 60°C, respectively, retained 70 and 86% of them after 6 cycles of reuse. A kinetic/thermodynamic study focused on thermal inactivation of the immobilized construct indicated high thermostability at temperatures commonly used for fructo-oligosaccharides (FOS) production. Maximum FOS concentration obtained in lab-scale experiments was 101.56 g/L, with predominant presence of 1-kestose in the reaction mixture. The results obtained in this study suggest that the immobilized-enzyme preparation may be effectively exploited for FOS production and easily recovered from the reaction mixture by action of a magnetic field.

Keywords: Fructo-oligosaccharides; fructosyltransferase; magnetic nanoparticles.

1. Introduction

Prebiotics are nondigestible food ingredients that selectively stimulate growth of beneficial bacteria in the colon conferring a series of health benefits to an individual [1]. Among the most explored prebiotics are fructo-oligosaccharides (FOS), which are extensively used in the production of food formulations with low or no added sugars such as ice creams, dairy desserts, yogurts and bakery products [2]. Their wide utilization is justified by the health benefits associated to their functional properties, among which are non-carcinogenicity, safety for diabetics, protection against colon cancer, stimulation of the absorption of Fe^{2+}, Ca^{2+}, Mg^{2+} and other ions and reduction of blood cholesterol [3,4].

FOS can be defined as a mixture of fructo-oligomers with two or three fructose units bound to the β-2,1 position of sucrose, mainly composed of 1-kestose (GF_2), nystose (GF_3), and 1-β-fructofuranosyl nystose (GF_4) [5]. These oligosaccharides can be produced from sucrose by the action of two enzymes possessing transfructosylating activity, namely fructosyltransferase (FTase, EC 2.4.1.9) and β-fructofuranosidase (FFase, EC 3.2.1.26), latter acting at higher sucrose concentrations [6]. However, FOS enzymatic synthesis has some drawbacks such as high costs and low availability of efficient FOS-forming biocatalysts on the market [7,8].

An alternative to minimize this problem is the use of low-cost commercial food-grade enzyme preparations with transfructosylating activity. Among them, the Pectinex Ultra SP-L preparation is a mixture of enzymes from *Aspergillus aculeatus* marketed for use in fruit juice processing, which was shown to display pectinase, cellulase, β-galactosidase and FTase activities [9]. In addition, it satisfies the purity specification recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which promotes its use for FOS synthesis [10].

A feasible alternative to develop FOS production is the utilization of immobilized enzymes. The immobilization techniques allow for reuse or application of enzyme in continuous processes, besides lowering their deactivation rate under different process conditions [11]. For successful enzyme immobilization, the choice of carrier is a crucial step, and recently nanomaterials, specially magnetic nanoparticles (MNPs), have attracted much attention due to their large specific surface area, easily modifiable surface by chemical methods, good stability in a broad range of temperature and pH values and easy separation from the reaction mixture by a magnetic field [12,13]. However, MNPs composed of Fe_3O_4 are highly

sensitive to acidic and oxidative conditions and tend to aggregate in liquid media due to the strong magnetic dipole–dipole attractions among particles, thereby requiring a modification or functionalization [12,14]. Among the polymers used to stabilize MNPs, chitosan (CS) stands out because of its low toxicity and good biocompatibility as well as the presence of amino groups that enable enzyme immobilization by crosslinking with bifunctional reagents such as glutaraldehyde [12].

Based on this background, the present study aimed to a) immobilize in Fe_3O_4-CS MPNs the commercial enzyme preparation Pectinex Ultra SP-L from *A. aculeatus*, having high transfructosylating activity, b) estimate the kinetic/thermodynamic parameters of sucrose hydrolysis and fructosyl transfer reactions and thermal inactivation of the immobilized enzyme, and c) use the immobilized construct to perform FOS synthesis.

2. Material and Methods

2.1 Materials and chemicals

Pectinex® Ultra SP-L (P2611), a commercial enzyme preparation from *Aspergillus aculeatus*, with hydrolytic (378.0 U mL^{-1}) and transfructosylating (301.7 U mL^{-1}) activities, sucrose and chitosan (CS) from shrimp shells (with deacetylation degree ≥ 75%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and all the other reagents used in this study were of analytical grade.

2.2 Analytical determinations

Hydrolytic and transfructosylating activities of the immobilized preparation were determined according to Sangeetha et al. [15] with some modifications. Briefly, 0.25 mL of immobilized enzyme dispersion and 0.75 mL of 60% (w/v) sucrose in 0.1 M acetate buffer, pH 5.0, were incubated at 55°C for 1 h. After reaction, the nanoparticles were separated by a permanent magnetic field, and samples were collected to determine the concentrations of glucose (*G*) by a commercial glucose oxidase colorimetric kit (Liquiform, Labtest, Lagoa Santa, MG, Brazil) and reducing sugars (*RS*) by the 3',5'-dinitrosalicylic acid method [16], from which the hydrolytic and transfructosylating activities were assessed. One unit of hydrolytic activity (U_H) was defined as the amount of enzyme required to hydrolyze 1 μmol sucrose per minute,

while one unit of transfructosylating activity (U_{TF}) as that required to transfer 1 μmol of fructose per minute. To calculate the concentration of transferred fructose needed to determine the transfructosylating activity, we used the equations initially proposed by Chen and Liu [17]:

$$F = RS - G \quad (1)$$

$$F' = G - F = 2G - RS \quad (2)$$

where *F* and *F'* are the concentrations of free and transferred fructose in the reaction medium.

Total protein content needed to calculate the k_{cat} value as described in section 2.7 was determined according to the Bradford method [18] using bovine serum albumin as the standard protein.

2.3 Immobilization of enzyme preparation

The immobilization process was performed in three steps: synthesis of Fe_3O_4-magnetic nanoparticles (MNPs), activation and coupling of enzyme and support. The synthesis of MNPs was performed by co-precipitation using ferric chloride and ferrous sulfate as described by Chen et al. [14] with adaptations. Briefly, Fe_3O_4-MNPs (2.0 g) were initially dispersed in 100 mL of 4.0 g L^{-1} chitosan solution in 0.35 M acetic acid, mixed in magnetic stirrer (500 rpm) with 40 mL of 2.0 g L^{-1} sodium tripolyphosphate, kept under vigorous shaking for 30 min and treated in an ultrasonic bath (Unique Group, São Paulo, SP, Brazil) at frequency of 40 kHz, for the same time. The Fe_3O_4-CS-MNPs were separated by a permanent magnetic field generated by a neodymium magnet (5.08 cm x 3.99 cm x 0.635 cm) with 4400 G intensity, washed thoroughly with distilled water and ethanol, and finally dried at 60°C for 1 h.

Fe_3O_4-CS-MNPs were activated by dispersing 1 g of them in 30 mL of glutaraldehyde solution at different concentrations for different times under shaking (130 rpm) at room temperature (25 ± 1°C). Then, the nanoparticles were submitted to permanent magnetic field and washed exhaustively with distilled water until no unbound glutaraldehyde absorbing at 245 nm was detected spectrophotometrically (Biochrom Libra S22, Biochrom Ltd., Cambridge, United Kingdom). Finally, activated Fe_3O_4-CS-MNPs were incubated together with the enzyme preparation (1:1 v/v) for different times. The immobilization conditions were investigated according a 2^3-full factorial design plus three additional repetitions of the central point, where glutaraldehyde concentration (1.0, 2.0 and 3.0 % v/v), activation time (60, 120 and 180 min)

and contact time (30, 60 and 90 min) were selected as the independent variables and the immobilization yield (*Y*) as the response. *Y* was defined as:

$$\text{Y (\%)} = \left(\frac{A}{A_f}\right) \text{x } 100 \quad (3)$$

where *A* is the hydrolytic activity of the immobilized enzyme and A_f the initial one of the free enzyme before immobilization. The statistical analysis of results was performed using the software package Statistica 7.0 (Statsoft Inc., Tulsa, OK, USA).

2.4 Support characterization

The crystalline phases and particle size of nanoparticles were characterized by X-ray diffraction (XRD), using a diffractometer, model DMAX 2400 (Rigaku, Tokyo, Japan), with the Cu K_α radiation ($\lambda = 0.154$ nm) at a scanning speed of $1.0^o\ s^{-1}$ in the range of $2\theta = 15\text{-}80°$ with a step of 0.02°. The average crystallite size was estimated from the integral intensity of the maximum X-ray diffraction peak using the Scherrer's equation. XRD profiles were classified according to patterns described in Crystallography Open Database. The FT-IR spectra were collected with a spectrometer, model IFS-66 (Bruker Optik, Ettingen, Germany), in the range of 4000-400 cm^{-1}. Samples were pressed using KBr pellets.

2.5 Effect of pH and temperature on immobilized enzyme activities

The optimal pH of hydrolytic and transfructosylating activities of immobilized enzyme preparation was investigated in different buffers, namely 0.1 M sodium citrate ($3.0 \leq pH \leq 4.0$), citrate-phosphate ($4.0 \leq pH \leq 6.0$), sodium phosphate ($6.0 \leq pH \leq 7.0$) and Tris-HCl ($7.0 \leq pH \leq 9.0$) at constant temperature (55°C). The effect of temperature on the same enzyme activities was assessed by activity tests carried out at temperatures ranging from 20 to 80°C. The thermal stability of immobilized enzyme preparation was investigated by incubating the enzyme at temperatures from 60 to75˚C for different time intervals (0-180 min). Aliquots were withdrawn every 60 min, except at temperature of 70 and 75°C (0, 15, 30, 45 and 60 min), rapidly cooled to room temperature (about 25°C) to refold the reversibly inactivated enzyme molecules and

analyzed for residual hydrolytic activity. These results were used for determination of kinetic and thermodynamic parameters of thermal denaturation (Section 2.8)

2.6 Reusability and storage stability

The immobilized enzyme reusability was checked by performing repeated cycles of enzyme hydrolytic and transfructosylating activities in accordance with section 2.2, assuming an initial relative activity of 100%. On the other hand, for storage stability tests enzyme preparations were immersed completely at 4°C in 0.1 M acetate buffer, pH 5.0, and their fructosyltransferase (FTase) activity profiles followed for 30 days.

2.7 Kinetic and thermodynamic parameters of reactions catalyzed by the immobilized enzyme

Kinetic parameters, namely Michaelis constant (K_m), maximum rate (V_{max}) and catalytic constant (k_{cat}), of both sucrose hydrolysis and fructosyl transfer catalyzed by the immobilized enzyme preparation were estimated from Lineweaver-Burk double reciprocal plots. Enzyme activities were determined at different sucrose concentrations ($25 \leq S_0 \leq 700$ g L^{-1}) and constant pH (5.0) and temperature (55°C).

The activation energies (E^*_a) of both catalyzed reactions were estimated from the slopes of typical Arrhenius type-plots of $\ln A_0$ versus $1/T$, being A_0 the starting activity and T the absolute temperature. The other thermodynamic parameters of reactions, namely activation enthalpy (ΔH^*), Gibbs free energy (ΔG^*) and entropy (ΔS^*), were estimated by the equations:

$$\Delta H^* = E_a^* - RT \tag{4}$$

$$\Delta G^* = -RT \ln\left(\frac{k_{cat} h}{k_b T}\right) \tag{5}$$

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T} \tag{6}$$

where h (6.626×10^{-34} J s^{-1}) is the Planck constant and k_b (1.381×10^{-23} J K^{-1}) the Boltzmann one.

The effect of temperature on the reaction rate was also investigated in terms of quotient of temperature (Q_{10}), which is the factor by which the enzyme activity increases due to a 10°C temperature increase and was calculated as Dixon and Webb [19]:

$$Q_{10} = \text{anti log}\left(\frac{E_a^* \text{x } 10}{RT^2}\right) \quad (7)$$

2.8 Kinetic and thermodynamic parameters of thermal inactivation of the immobilized enzyme

Enzyme thermal inactivation can be described as a temperature-dependent first-order irreversible reaction, with k_d as the rate constant, which can be kinetically depicted by the equation:

$$\ln \psi = - k_d t \quad (8)$$

where ψ is the residual activity coefficient (dimensionless), i.e., the ratio of residual enzyme activity after a given time (t) to that at the beginning of thermal treatment ($\psi = A/A_o$). Only the results of the hydrolytic activity were used to calculate the kinetic and thermodynamic parameters of thermal inactivation. Thus, the k_d values were estimated in the temperature range of 60–75°C from the slopes of the straight lines obtained by plotting the experimental data of lnψ vs. time.

Other two kinetic parameters such as the half-life ($t_{1/2}$) and *D*-value were estimated from k_d at a given temperature, the former being the time after which the enzyme activity is reduced to one-half the initial value and the latter that required for a 10-fold reduction:

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

$$D = \frac{\ln 10}{k_d} \quad (10)$$

Another useful kinetic parameter, the *Z*-value, which is defined as the temperature increase needed to achieve a 10-fold reduction in the *D*-value, was calculated from the slope of the Thermal-Death-Time plot of log*D* versus *T* (°C). As far as the thermodynamic parameters of the irreversible enzyme inactivation are concerned, the activation energy (E^*_d) was estimated from the slope of the straight line of the lnk_d vs. 1/*T* Arrhenius-type plot, while the others, namely activation enthalpy (ΔH^*_d), Gibbs free energy (ΔG^*_d) and entropy (ΔS^*_d), were calculated similarly to those of the enzyme catalyzed reactions using k_d instead of k_{cat} (Eqs. 4-6).

2.9. Enzymatic synthesis of fructo-oligosaccharides

FOS production was performed by addition of 0.1 mL of the immobilized enzyme dispersion in 0.9 mL of sucrose solution in 0.1 M phosphate buffer, pH 7.0. To select the best conditions, experiments were carried out according to a 2^2-full factorial design plus three central points, where sucrose concentration (500, 600 and 700 g/L) and reaction time (60, 120 and 180 min) were selected as the independent variables and total FOS concentration as the response. Samples of each run were analyzed in an HPLC system composed of a pump, model CG 480-E (Instrumentos Científicos C.G. Ltda, São Paulo, SP, Brazil), and equipped with refractive index detector (HP 1047A, Hewlett Packard) and a Phenomenex Luna C-18 (250 mm × 4.6 mm) column. 5.0 mM H_2SO_4 in ultrapure water was used as eluting solvent at a flow rate of 1.1 mL/min and 25 ± 1°C. FOS standards for the chromatographic analysis (1-kestose and nystose) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

3. Results and discussion

3.1 Enzyme immobilization by covalent binding to Fe_3O_4–chitosan-magnetic nanoparticles

The effects of three independent variables, namely glutaraldehyde concentration, activation time and time of contact between enzyme and chitosan (CS) beads, on the yield of Pectinex Ultra SP-L immobilization onto Fe_3O_4–CS-magnetic nanoparticles (MNPs) were investigated according to a 2^3-full factorial design. The results listed in Table 1 show that the highest immobilization yield (94.84 %) was obtained in run 4 using 3.0 % (v/v) glutaraldehyde and activation and contact times of 180 and 30 min, respectively. Likewise, Chen et al. [14] reported optimum conditions of immobilization of an alcalase/trypsin mixture onto the same support using the same glutaraldehyde concentration, but longer immobilization time (4 h).

Table 1. Experimental conditions and results of tests of Pectinex Ultra SP-L immobilization onto Fe_3O_4–CS-magnetic nanoparticles carried out according to a 2^3-full factorial design.

Run	Activation time (min)	Glutaraldehyde (%, v/v)	Contact time (min)	IY (%)
1	60	1.0	30	77.65
2	180	1.0	30	69.02
3	60	3.0	30	67.82
4	180	3.0	30	94.84
5	60	1.0	90	81.51
6	180	1.0	90	65.52
7	60	3.0	90	82.51
8	180	3.0	90	73.98
9 (C)	120	2.0	60	87.32
10 (C)	120	2.0	60	83.94
11 (C)	120	2.0	60	85.95

The analysis of variable effects and interactions showed that glutaraldehyde concentration was the only variable that exerted a statistically significant (positive) influence on the immobilization yield (Table 2), which means that an increase its concentration improved enzyme attachment to the support. Since glutaraldehyde is a bifunctional agent, whose two terminal aldehyde groups are able to react with the amino groups of D-glucosamine units of different chains, an increase in the cross-linking agent concentration was likely to cause more glutaraldehyde dimers to be formed, hence making the support more reactive and increasing the immobilization yield [12].

Table 2 Estimated effects and interactions of the independent variables used to immobilize Pectinex Ultra SP-L in Fe_3O_4–CS-magnetic nanoparticles according to the 2^3-full factorial design of Table 1.

Variables or interactions	Estimates
(1) Activation time	-1.27
(2) Glutaraldehyde concentration	5.30*
(3) Contact time	-1.21
1 x 2	8.98*
1 x 3	-8.94*
2 x 3	-1.35
1 x 2 x 3	-5.87*

*****Statistically significant estimates at 95% confidence level ($p < 0.05$).

Also, glutaraldehyde concentration synergistically interacted with the activation time and antagonistically with the contact time. While the former interaction is quite intuitive, the latter can be justified by excess crowding of enzyme molecules on the support surface with increasing the contact time, thus hindering the substrate entry to the active site [20]. The ternary interaction among all variables was statistically significant, which means that the effect of each of them was strongly influenced by the levels of the others, resulting in very complex interactions.

3.2 Characterization of immobilized enzyme constructs

The X-ray diffractogram of pure powdered Fe_3O_4 nanoparticles exhibited the characteristic magnetite peaks at $2\theta = 30.14°$, 35.55°, 43.17°, 53.60°, 57.10° and 62.72° (COD 96-900-5813) (Figure 1a). This Fe_3O_4 phase belongs to the cubic system, space group Fd-3m, with average crystallite size of 15.6 nm determined by the Scherrer's equation from the broadening (full width half maximum, FWHM) of the diffraction peaks. The XRD profiles of Fe_3O_4-CS-MNPs and Pectinex immobilized onto Fe_3O_4-CS-MNPs (Figures 1b and 1c, respectively) showed no detectable change from that of pure MNPs, which indicates that Fe_3O_4 underwent no phase change after interacting with CS and the commercial enzyme preparation. Furthermore, not even the average crystallite size changed in the systems under investigation [21,22].

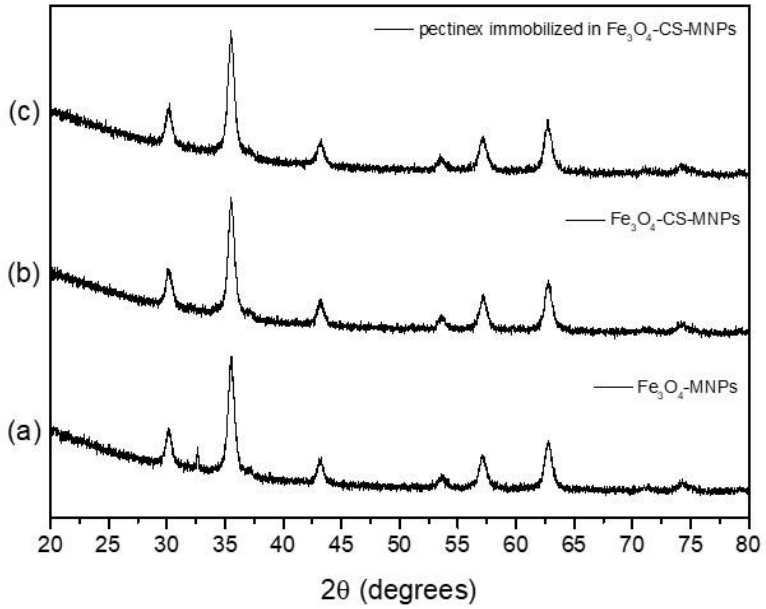

Figure 1. XRD patterns of (a) Fe_3O_4-magnetic nanoparticles (MNPs), (b) Fe_3O_4–CS-MNPs and (c) Pectinex immobilized in Fe_3O_4–CS-MNPs.

Figure 2 shows the FT-IR spectra of (a) pure Pectinex, (b) pure chitosan, (c) Fe_3O_4-MNPs, (d) Fe_3O_4-CS-MNPs, and (e) Pectinex immobilized in Fe_3O_4-CS-MNPs. One can identify in the Fe_3O_4-MNPs spectrum the characteristic absorption band of Fe-O stretching at around 572 cm^{-1} and in that of pure CS the absorption band of N-H bending vibration at around 1638 cm^{-1}. The interaction of Fe_3O_4-MNPs surface with CS is confirmed in the Fe_3O_4-CS-MNPs spectrum by the shift of the above absorption bands to lower wave numbers, namely 1617 and 558 cm^{-1}, respectively. The peak around 1638 cm^{-1}, assigned to C-O and N-H stretching in Pectinex spectrum, shifted to 1625 cm^{-1} when the enzyme preparation was immobilized in Fe_3O_4-CS-MNPs, suggesting interaction between enzyme functional groups and MNPs. These spectra taken together demonstrate that CS was bound successfully to MNPs and Pectinex to Fe_3O_4-CS-MNPs [12,13,21].

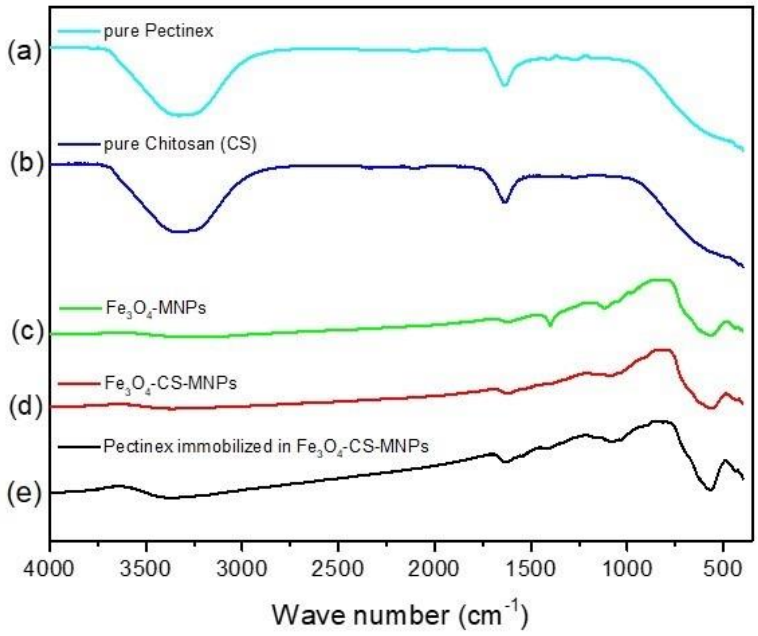

Figure 2. FT-IR spectra of (a) Pectinex, (b), chitosan (CS), (c) Fe_3O_4-magnetic nanoparticles (MNPs), (d) Fe_3O_4–CS-MNPs and (e) Pectinex immobilized in Fe_3O_4–CS-MNPs.

3.3 Effect of pH and temperature on immobilized enzyme activities

The individual effects of pH and temperature on relative hydrolytic and transfructosylating activities of the enzyme preparation immobilized in Fe_3O_4–CS- MNPs are illustrated in Figure 3A and B, respectively. The optimum pH of both activities (7.0) was much higher than that observed for the free enzyme preparation (4.5-6.0 and 5.0 for hydrolytic and transfructosylating activities, respectively - results not shown), which suggests a change in the enzyme conformation or interactions after binding to the nanoparticles surfaces [13]. Even though the optimum temperature of both activities was the same (60°C), an almost complete retention of relative hydrolytic activity was observed at 70°C (97.26 %), unlike the transfructosylating one that decayed abruptly. Chen et al. [23] reported the same optimum temperature for β-fructofuranosidase (FFase) from *Aspergillus japonicus* either free or immobilized in the same support.

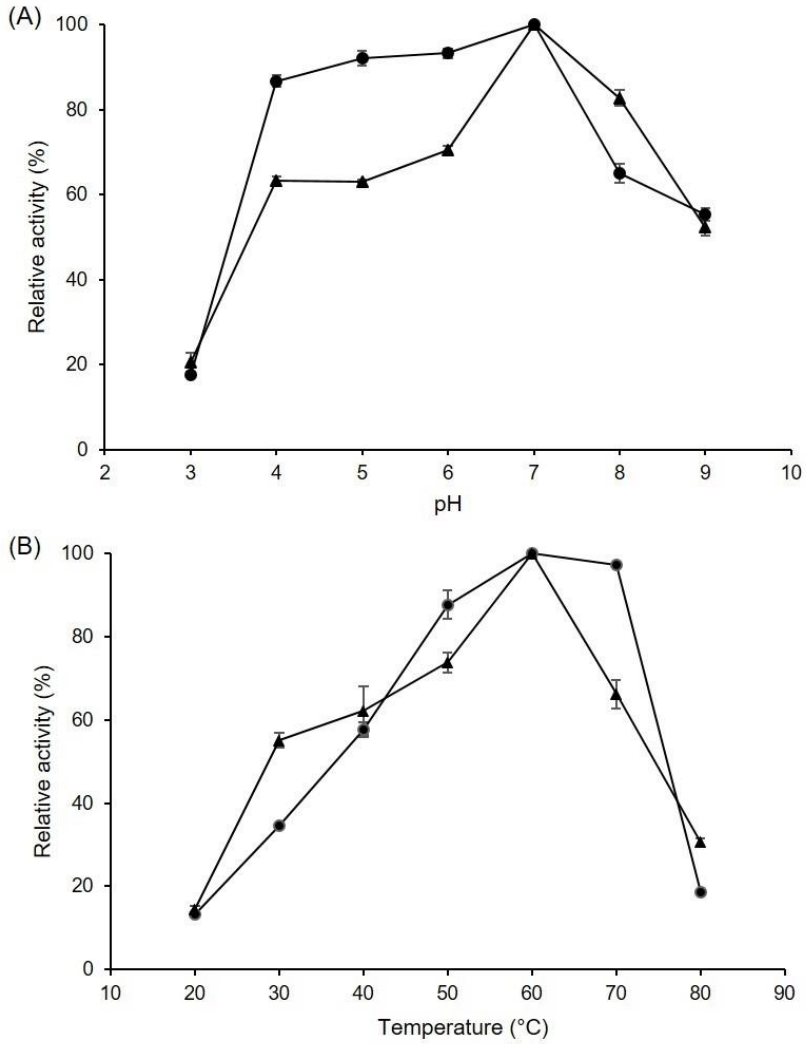

Figure 3. Effects of (A) pH and (B) temperature on relative (●) hydrolytic and (▲) transfructosylating activities of Pectinex immobilized in Fe_3O_4–chitosan-magnetic nanoparticles.

3.4 Reusability and storage stability of Pectinex–Fe_3O_4–chitosan-magnetic nanoparticles

Reusability is one of the most important advantages of enzyme immobilization that can determine the financial viability of an industrial bioprocess. After 6 cycles of reutilization, the immobilized enzyme construct showed no less than 70 and 86% of residual hydrolytic and transfructosylating activities, respectively. Such a promising feature may reduce the operational costs and improve the economic feasibility of fructo-oligosaccharides (FOS) synthesis. Another important parameter for satisfactory catalytic performance is the storage stability, which, in general, is much better for immobilized enzymes then for enzymes in solution [25]. The immobilized enzyme preparation showed 95.86 and 100% of hydrolytic and transfructosylating

residual activities after 30 days of storage at 4°C, respectively. Using the same support, Liu et al. [20] reported a storage stability (84.3 ± 1.2%) after 35 days of storage at the same temperature for *A. niger* α-glucosidase.

3.5 Kinetic and thermodynamic parameters of reactions catalyzed by immobilized enzyme preparation

The enzyme activities of the immobilized enzyme preparation tested at different sucrose concentrations exhibited the typical hyperbolic curve of Michaelis-Menten kinetics. The results were plotted according to Lineweaver-Burk with satisfactory correlation ($0.984 \leq R^2 \leq 0.986$), and the kinetic parameters were estimated (Table 3). The K_m value of the hydrolysis reaction (334.88 mM) was about one-third of that of fructosyl transfer (928.21 mM), which means that the immobilized enzyme had more affinity for sucrose than for fructose. Both K_m values were higher than those of the free enzyme (191.0 and 278.2 mM for hydrolysis and fructosyl transfer, respectively - results not shown), likely due to steric hindrance resulting from conformational changes in the enzyme tertiary structure induced by immobilization [25]. V_{max} and k_{cat} values were higher for the hydrolysis reaction than for transfructosylating, hence indicating that the former reaction occurs more easily. However, Ghazi et al. [26] reported higher k_{cat} values from transfructosylation in the free purified preparation. Probably, the presence of other enzymes in crude preparation as well as the immobilization process can be the cause of this different behavior.

Table 3. Kinetic and thermodynamic parameters of reactions catalyzed by Pectinex from *A. aculetus* immobilized in Fe_3O_4–chitosan-magnetic nanoparticles.

Parameters	Sucrose hydrolysis	Fructosyl transfer
K_m (mM)	334.88	928.21
V_{max} (mM.min^{-1})	400.00	238.09
k_{cat} (min^{-1})	8.15	4.85
E^*_a (kJ mol^{-1})	40.75	39.59
ΔH^* (kJ mol^{-1})	38.02	36.86
ΔG^* (kJ mol^{-1})	86.07	87.49
ΔS^* (J mol^{-1} K^{-1})	-146.43	-154.30
Q_{10}	1.0	1.0

Arrhenius plots of $\ln A_0$ vs. the reciprocal absolute temperature ($1/T$) allowed estimating with satisfactory correlation the activation energies (E_a) of both reactions catalyzed by the immobilized enzyme, namely sucrose hydrolysis (40.75 kJ mol^{-1}; $R^2 = 0.936$) and fructosyl transfer (39.59 kJ mol^{-1}; $R^2 = 0.944$) (Table 3). These values are lower than those reported for the free enzyme (42.21 and 47.41 kJ mol^{-1}, respectively), which suggests that enzyme immobilization by covalent bonding to a high-superficial area support like MNPs reduced the energy required to form the transition states of both reactions. The slightly lower activation enthalpy (ΔH^*) estimated for fructosyl transfer compared to sucrose hydrolysis can be explained by an easier transition state formation or suggests that an activated complex between the enzyme and the substrate occurred effectively in the transfructosylation reaction [27].

The ΔS^* is known to be correlated to the order (rigidity) degree of the enzyme-substrate activated complex. The ΔS^* values of both reactions catalyzed by either enzyme preparation were negative, which suggests that the structure of enzyme–substrate at transition state was more ordered than that of enzyme–substrate complex [28]. The Gibbs free energy (ΔG^*) is the thermodynamic parameter able to shed light on the actual feasibility and extent of a chemical reaction. The ΔG^* values of both sucrose hydrolysis and fructosyl transfer were positive and relatively high (>80 kJ mol^{-1}), which suggests that the favorably low enthalpic contribution could have been counterbalanced by the unfavorable entropic one [29]. Finally, the quotient of temperature (Q_{10}), which should always lie between 1 and 2 unless there is some interference of some other parameter [30], was 1.0 for both sucrose hydrolysis and fructosyl transfer reactions, which indicates that either reaction was kinetically controlled by temperature within the entire temperature range investigated.

3.6 Kinetic and thermodynamic parameters of thermal inactivation of immobilized enzyme preparation

The first-order rate constants (k_d) of irreversible thermal inactivation of the immobilized enzyme preparation were estimated with satisfactory correlation ($0.91 \leq R^2 \leq 0.98$) in the temperature range of 60-75°C from the slopes of straight lines of $\ln\psi$ vs. time semi-log plots (Figure 4). Whereas the half-life ($t_{1/2}$) and D-values decreased with rising temperature, k_d increased (Table 4), which means that enzyme inactivation became gradually more significant and that thermostability was reduced. In particular, the thermostability of the immobilized

enzyme at 60-65°C was very high ($t_{1/2}$: 594.13-322.57 min; *D*-value: 1973.64-1071.55 min) and higher than that of the free enzyme ($t_{1/2}$: 293.4-201.3 min; *D*-value: 974.6-668.7 min - results not shown), likely because the enzyme covalent attachment to the carrier prevented intermolecular aggregation, and the tertiary structure acquired higher rigidity [31]. The *Z*-value calculated from the Thermal-Death-Time curve (log*D* vs. *T*) was found to be 6.91°C, i.e., such a temperature increase was needed to obtain a 10-fold decrease of the *D*-value. Similar Z-value (6.81) was obtained for FFase with transfructosylating activity from *A. tamarii* [32].

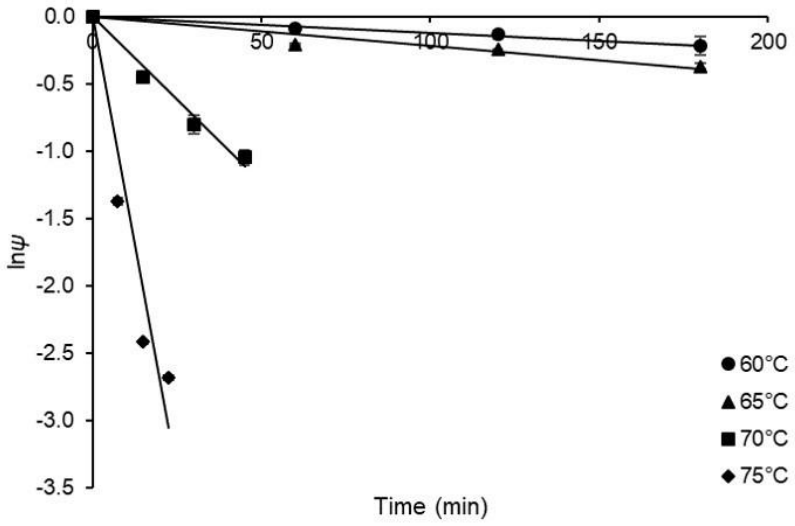

Figure 4. Semi-log plots of irreversible thermal inactivation of Pectinex immobilized in Fe_3O_4–chitosan-magnetic nanoparticles, using hydrolytic activity for determinations.

Table 4. Kinetic and thermodynamic parameters of thermal inactivation of Pectinex immobilized in Fe_3O_4–chitosan-magnetic nanoparticles.

Temperature (°C)	k_d (min^{-1})	R^2	$t_{1/2}$ (min)	*D*-value (min)	*Z*-value (°C)	E^*_d (kJ mol^{-1})	ΔG^*_d (kJ mol^{-1})	ΔH^*_d (kJ mol^{-1})	ΔS^*_d (J mol^{-1} K^{-1})
60	0.0012	0.98	594.13	1973.64	6.91	320.94	111.91	318.17	619.12
65	0.0022	0.90	322.57	1071.55			111.95	318.13	609.72
70	0.0249	0.98	27.95	92.86			106.67	318.08	616.11
75	0.1358	0.91	5.10	16.96			103.34	318.04	616.68

The thermodynamic parameters of irreversible thermal inactivation of the immobilized enzyme were also estimated. In particular, an activation energy (E^*_d) of 320.94 kJ mol^{-1} was estimated with satisfactory correlation ($R^2 = 0.952$) from the slope of the straight line obtained plotting lnk_d vs. 1/T (Figure 5). This parameter, which expresses how much energy is needed to thermally inactivate an enzyme under given conditions, was much higher than that of the free enzyme (237.0 kJ mol^{-1} - results not shown), which indicates that the immobilization process made the enzyme more thermostable, compact and resistant to heat [33]. Similar E^*_d values (293.74-319.44 kJ mol^{-1}) were reported by Aguiar-Oliveira and Maugeri [33] for immobilized fructosyltransferase (FTase) from *Rhodotorula* sp. immobilized in niobium ore at different pH values.

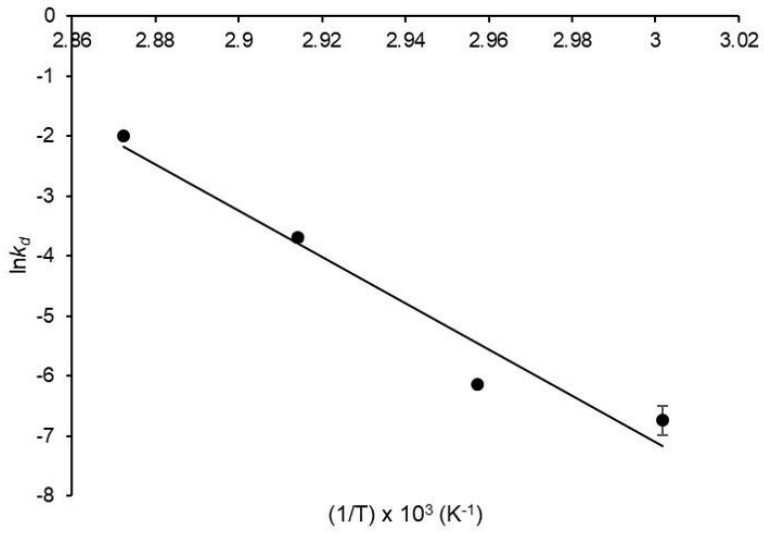

Figure 5. Arrhenius-type plots used to estimate the activation energy of irreversible thermal inactivation of Pectinex immobilized in Fe_3O_4–chitosan-magnetic nanoparticles, using hydrolytic activity for determinations.

The activation enthalpy of denaturation (ΔH^*_d) is another important thermodynamic parameter that expresses the total amount of energy required to denature an enzyme through disruption of non-covalent linkages, including hydrophobic interactions [33]. The immobilized enzyme preparation showed high and positive values of this parameter (318.04-318.17 kJ mol^{-1}) that indicates that high total energy was required to denature it. Oliveira et al. [32] reported slightly lower ΔH^*_d values for free crude β-fructofuranosidase from *Aspergillus tamarii* (290.26-290.44 kJ mol^{-1}).

The disruption of the enzyme structure is accompanied by an increase in disorder or randomness expressed by the activation entropy (ΔS^*_d). The quite high and positive values of this parameter estimated in the present study (616.68-619.12 J mol^{-1} K^{-1}) suggest an increased number of protein molecules in the transition active state, resulting in a) lower ability to order water molecules at higher temperatures, b) an increased disorder level through interference with the extension of the hydrogen-bonded network, and c) an easier water molecular rotation [27]. Another thermodynamic parameter, the Gibbs free energy (ΔG^*_d) is a very useful parameter to evaluate the enzyme thermal denaturation since it incorporates both enthalpic and entropic contributions. The estimated values of this parameter were especially high for the immobilized system but progressively decreased from 111.91 kJ mol^{-1} at 60 °C to 103.34 kJ mol^{-1} at 75 °C, thereby confirming that its resistance to denaturation tended to decrease with rising temperature.

3.7 FOS synthesis by Pectinex immobilized in Fe_3O_4–chitosan-magnetic nanoparticles

Table 5 lists the results of FOS production performed according a 2^2-full factorial design, where sucrose concentration and reaction time were selected as the independent variables and total FOS concentration as the response. Lower nystose production under all the tested conditions can be explained by an insufficient time for 1-kestose to act as fructosyl residue donor and then to complete the glycosidic chain elongation, hence causing almost only sucrose to be used as fructosyl donor and acceptor for FOS synthesis [35]. The highest total concentration of FOS (101.56 g L^{-1}), which was almost completely made up of 1-kestose (97.04%) plus other FOS including nystose, was obtained in run 4 carried out at sucrose concentration of 700 g L^{-1} for 180 min. The estimated effects showed that both independent variables exerted statistically significant positive effects (Table 6), which means that a separate increase of either promoted FOS production, and, accordingly, their interaction led to a synergism. The FOS concentration above described was higher than that reported by Csanádi and Sisak [37] for the same enzyme preparation immobilized onto the ion exchange resin Amberlite (Total FOS: 84 g L^{-1}; 1-kestose: 72.7 g L^{-1} nystose: 11.3 g L^{-1}) after 9 h of reaction using an initial sucrose concentration of 1.0 M. Tanriseven and Aslan [38], who studied FOS production by Pectinex immobilized onto Eupergit C, reported a different FOS profile after 24 h of reaction using 60 % (w/v) of initial sucrose concentration (1-kestose: 23.5%, nystose:

27.2% and 1-β-fructofunanosil nystose: 6.5% of total carbohydrates), confirming the strong influence of the reaction time on FOS production.

Table 5. Experimental conditions and results of FOS production from sucrose by Pectinex immobilized in Fe_3O_4–chitosan-magnetic nanoparticles performed according to a 2^2-full factorial design.

Run	Sucrose concentration ($g\ L^{-1}$)	Reaction time (min)	1-Kestose concentration ($g\ L^{-1}$)	Nystose concentration ($g\ L^{-1}$)	Total FOS concentration ($g\ L^{-1}$)
1	500	60	30.39	0.63	31.02
2	700	60	24.39	1.81	26.19
3	500	180	22.48	7.39	29.87
4	700	180	98.56	3.00	101.56
5	600	120	38.85	1.11	39.96
6	600	120	40.14	1.01	41.15
7	600	120	43.23	1.28	44.51

Table 6. Estimated effects and interaction of the independent variables used for FOS production by Pectinex immobilized in Fe_3O_4–chitosan-magnetic nanoparticles.

Variable or interaction	Estimates
(1) Sucrose concentration	14.15*
(2) Reaction time	15.71*
1 x 2	16.20*

*****Statistically significant estimates at 95% confidence level ($p < 0.05$).

4. Conclusions

Pectinex Ultra SP-L, a commercial enzyme preparation with high transfructosylating activity, was successfully immobilized onto Fe_3O_4-CS-magnetic nanoparticles with high immobilization yield (94.84 %). The enzyme preparation immobilized in this way proved to be very stable either at high temperatures (up to 75 °C) or under typical storage conditions (4 °C), besides displaying satisfactory reusability. These characteristics and its easy separation from the reaction medium by the application of a magnetic field demonstrate that the Fe_3O_4–CS-

magnetic nanoparticles may be used as an efficient support for Pectinex Ultra SP-L immobilization. In addition, the satisfactory FOS production in short reaction time is an additional desirable characteristic that points to possible application of this system for long-term FOS synthesis.

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CONCLUSÕES

- A nova FFase de *A. tamarii* com atividade de transfrutosilação apresentou características desejáveis para aplicação da produção de açúcar invertido e FOS. A utilização de um substrato de baixo custo como o farelo de soja aliado a estabilidade a enzima em temperaturas comumente empregada na produção de FOS é um atrativo para utilização deste biocatalisador.
- A utilização do planejamento de modelagem de mistura simplex centroide mostrou-se uma abordagem satisfatória para se estudar a produção da FFase utilizando diferentes substratos industriais e suas misturas binárias e ternária. Sendo capaz de obter modelos matemáticos satisfatórios para simular a produção enzimática.
- A imobilização da preparação comercial de grau alimentício Pectinex Ultra SP-L em quitosana (CS) e em nanopartículas magnéticas de Fe_3O_4-CS e posterior aplicação na produção de FOS por processos contínuos e descontínuos, respectivamente, mostrou-se satisfatória. A possibilidade de aproveitamento de uma enzima que já atende os requisitos de segurança em novos processos catalíticos para obtenção de FOS é animadora, uma vez que várias etapas do bioprocesso são evitadas.
- Foi observada uma elevada produção total de FOS por processo descontínuo utilizando a Pectinex imobilizada em nanopartículas magnéticas com quitosana (101.56 g L^{-1}). Apesar de ter apresentado resultados inferiores, a produção deste prebiótico pelo processo contínuo com a enzima imobilizada em quitosana (25.73 g L^{-1}) os resultados obtidos servirão de base para estudos de otimização posteriores.
- Os resultados envolvendo os parâmetros cinéticos e termodinâmicos envolvendo ambas as enzimas em suas diferentes formas vão contribuir para uma melhor compreensão do comportamento das enzimas formadoras de FOS, uma vez que os dados existentes ainda são escassos.

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Artigos completos publicados em periódicos

Título: Imobilização da frutosiltransferase de *Aspergillus aculeatus* por aprisionamento em alginato e ágar-ágar
Autores: Rodrigo Lira de Oliveira, Marcos Fellipe da Silva e Tatiana Souza Porto
Periódico: Revista Brasileira de Agrotecnologia
Ano: 2017
Qualis CAPES unificado: B4

Trabalhos completos publicados em anais de eventos

Título: Extraction and partitioning of β-fructofuranosidase from *Aspergillus tamarii* URM4634 with transfructosylating activity using PEG-Citrate Aqueous Two-Phase System
Autores: Rodrigo Lira de Oliveira, Karollayny Santos Couto, Yuri Matheus Silva Amaral e Tatiana Souza Porto
Evento: XXII Simpósio Nacional de Bioprocessos e XIII Simpósio de Hidrólise Enzimática de Biomassas
Cidade: Uberlândia
Ano: 2019

Resumos simples publicados em anais de eventos

Título: Immobilization of β-fructofuranosidase from *Aspergillus tamarii* URM4634 by covalent attachment on chitosan beads and application on sucrose hydrolysis in packed bed reactor
Autores: Rodrigo Lira de Oliveira, Vinícius Luiz Vilela dos Santos, Marcos Fellipe da Silva e Tatiana Souza Porto
Evento: 13º Simpósio Latino Americano de Ciência de Alimentos
Cidade: Campinas
Ano: 2019

Trabalhos de Conclusão de Curso de graduação

Título: Desenvolvimento de processo biotecnológico para obtenção de frutosiltransferase a partir de linhagens de *Aspergillus* spp. utilizando resíduos agroindustriais
Aluno: Marcos Fellipe da Silva
Curso: Engenharia de Alimentos (UFRPE/UAG)
Orientador: Tatiana Souza Porto
Coorientador: Rodrigo Lira de Oliveira
Ano: 2018

Título: Desenvolvimento de processo contínuo de produção de açúcar invertido utilizando um reator enzimático de leito empacotado
Aluno: Marcos Fellipe da Silva
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Coorientador: Rodrigo Lira de Oliveira
Ano: 2019

Apresentações em eventos
Título: Extração de β-frutofuranosidase de *Aspergillus tamarii* URM4634 utilizando sistemas de duas fases aquosas PEG-Sulfato
Autores: Maria Itais dos Santos Bernardino, Rodrigo Lira de Oliveira e Tatiana Souza Porto.
Evento: IV Encontro de Ciência e Tecnologia de Alimentos (ECTAL)
Cidade: Garanhuns
Ano: 2019

Premiações
Menção honrosa referente ao trabalho: **Extração de β-frutofuranosidase de *Aspergillus tamarii* URM4634 utilizando sistemas de duas fases aquosas PEG-Sulfato** no IV Encontro de Ciência e Tecnologia de Alimentos (ECTAL).