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Paula Chequer Gouveia Mól

Purificação e imobilização de β-glicosidase do fungo *Thermoascus aurantiacus* em criogéis supermacroporosos

São José do Rio Preto
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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Engenharia e Ciência de Alimentos, junto ao Programa de Pós-Graduação em Engenharia e Ciência de Alimentos, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

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Comissão Examinadora

Prof. Dr. Roberto da Silva
UNESP – Câmpus de São José do Rio Preto
Orientador

Prof. Dr. Vanildo Del Bianchi
UNESP – Câmpus de São José do Rio Preto

Prof. Dr. Gustavo Orlando Bonilla Rodriguez
UNESP – Câmpus de São José do Rio Preto

Prof. Dr. Silvio Silverio da Silva
USP – Câmpus de Lorena

Profª. Drª. Luana Pereira de Moraes
UENF – Campos dos Goytacazes

São José do Rio Preto
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RESUMO

As β-glicosidases são enzimas que hidrolisam ligações glicosídicas β-(1,4) terminais, função essencial a muitos processos biológicos, sendo, portanto, de grande interesse para a bioquímica e a biotecnologia. Este trabalho teve como objetivo a obtenção e o posterior estudo da purificação e imobilização da β-glicosidase do fungo termofilico *Thermoascus aurantiacus* em suportes supermacroporosos de poliacrilamida produzidos em condições de congelamento (conhecidos como criogel). A enzima foi produzida por fermentação em estado sólido utilizando sabugo de milho como substrato. A primeira coluna utilizada (Capítulo II) foi obtida por modificação química do criogel pela introdução dos grupos iônicos 2-(dimetilamino)etil metacrilamida (DMAEMA). Os experimentos de adsorção foram realizados em diferentes valores de pH e os resultados de rendimento (%) e fator de purificação foram calculados e submetidos a ANOVA a 95% de significância. O pH teve efeito significativo ($p<0,05$) no rendimento, sendo que o melhor resultado foi obtido em pH 5,0 (82%). O fator de purificação não variou e os resultados foram considerados baixos uma que vez que a eluição utilizada foi isocrática (1,25-1,33). Entretanto, por meio de SDS-PAGE verificou-se que o extrato bruto foi parcialmente purificado, independentemente do valor de pH. A segunda coluna sintetizada (Capítulo III) foi obtida pela incorporação do aminoácido L-fenilalanina na superfície do criogel, e utilizada em testes de imobilização de β-glicosidase via interação hidrofóbica. A adsorção foi inicialmente estudada em função do pH e verificou-se que o maior fator de purificação foi obtido em pH 3,0. Em seguida, o efeito da temperatura e da forca iônica de diferentes soluções salinas na adsorção e consequentemente na atividade da enzima imobilizada foi avaliado. Além disso, a reutilização do biocatalisador foi investigada por sete ciclos consecutivos e não foi observado decréscimo na sua atividade específica. Nos dois casos, as colunas de criogéis produzidos foram caracterizadas em termos de suas propriedades morfológicas e hidrodinâmicas. Os resultados obtidos no trabalho mostram que os criogéis produzidos podem ser um potencial meio de separação e imobilização de proteínas.

Palavras-chave: β-glicosidase. Adsorção. Criogel. Interação hidrofóbica. Troca iônica

ABSTRACT

β-glucosidases are enzymes that hydrolyze terminal β-(1,4) glycosidic bonds, an essential function to many biological processes, that is of great interest in biochemistry and biotechnology. The purpose of this study encompasses the obtaining and posterior purification and immobilization studies of β-glucosidase from thermophilic fungus *Thermoascus aurantiacus* in supermacroporous supports of polyacrylamide produced in freezing conditions (knows as cryogel). The enzyme was produced by solid state fermentation using corn cob as substrate. The first cryogel column used (Chapter II) was obtained by chemical modification of the cryogel by the introduction of the ionic groups 2-(dimethylamino)ethyl methacrylate. The adsorption experiments were carried out in different pH conditions and the results of yield (%) and purification factors were calculated and submitted to ANOVA at 95% of significance level. The efficiency was considerably affected ($p<0.05$) by the pH and the best result was achieved at pH 5.0 (82%). Purification factors did not vary and the results were low since isocratic elution was performed (1.25-1.33). However, SDS-PAGE was also realized to investigate purity and it was verified that the crude extract was partially purified, regardless of the pH. The second column synthetized (Chapter III) was obtained by the incorporation of the aminoacid L-phenylalanine on the cryogel surface and was used in β-glucosidase immobilization tests via hydrophobic interaction. The adsorption was initially studied as a function of the pH and the highest purification factor was found at pH 3.0. Then, the effect of the temperature and ionic strength of different saline solutions on adsorption and consequently on the activity of the immobilized enzyme was evaluated. In addition, the reuse of the biocatalyst was investigated for seven consecutive cycles and no decrease on its the specific activity was observed. In both cases, the cryogel columns produced were characterized in terms of their morphological and hydrodynamic properties. The results obtained in this work show that the cryogels produced can be potential supports of protein separation and immobilization.

Keywords: β-glucosidase. Adsorption. Cryogel. Ion Exchange. Hydrophobic interaction

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LISTA DE ABREVIATURAS

***p*NPG**	*p*-nitrophenyl-β—D-glucopyranoside
DMAEMA	2-(dimethylamino)ethyl methacrylate
Aam	Acrylamide
AGE	Allyl glycidyl ether
APS	Ammonium persulfate
ANOVA	Analysis of variance
ATR	Attenuated total reflectance
CV	Column volume
DSC	Differential scanning calorimeter
FTIR	Fourier transform infrared spectrophotometry
HETP	Height equivalente to theoretical plate
TEMED	N,N,N´,N´-tetramethyl-ethylenediamine
MAAm	N,N´-methylene-bis-acrylamide
UV	Ultraviolet
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide Gel Electrophoresis

LISTA DE SÍMBOLOS

U	Activity unit
U/mg	Activity unit per milligram
C	Celsius degree
cm	Centimeter
g	gram
X g	Gravitational force unit
h	Hour
kg	Kilogram
L	Liter
m	Meter
min	Minute
µL	Microliter
µm	Micrometer
µm	Micromol
mg	Milligram
mM	Millimeter
m	Minute
nm	Nanometer
Pa	Pascal
%	Percentage
S	Second
V	Volt
v/v	Volume – volume ratio
w/v	Weight – volume ratio

SUMÁRIO

1. INTRODUÇAO GERAL

As β-glicosidases são enzimas amplamente distribuídas na natureza e que são responsáveis pela hidrólise de ligações β-glicosídicas em dissacarídeos e oligossacarídeos, liberando moléculas de glicose. As β-glicosidases desempenham papéis em diversos estágios biológicos e por isso são consideradas enzimas biologicamente e industrialmente importantes. Dentre suas aplicações biotecnológicas, a que mais se destaca é a degradação da biomassa para produção de etanol de segunda geração. Entretanto, aplicações nas áreas farmacêuticas, de alimentos e de bebidas vêm cada vez mais recebendo atenção, tanto em pesquisas quanto em aplicações tecnológicas.

Em processos industriais, a utilização de enzimas que apresentem termoestabilidade é desejada uma vez que grande parte destes processos ocorrem em altas temperaturas devido às suas vantagens, tais como altas taxas reacionais, diminuição da viscosidade dos fluidos, aumento da solubilidade de substratos e minimização dos riscos de contaminação. Dessa forma, o estudo e a obtenção de enzimas termoestáveis são importantes na enzimologia aplicada. Enzimas estáveis em altas temperaturas são geralmente secretadas por microrganismos termofilicos, como o fungo filamentoso *Thermoascus aurantiacus*, o qual cresce bem em biomassa lignocelulósica proveniente de resíduos como bagaço de cana, farelo de trigo e sabugo de milho. A fermentação em estado sólido é um processo fermentativo particularmente adequado para substratos sólidos, uma vez que apresenta vantagens como alta produtividade, estabilidade dos produtos finais, baixos consumos de água e energia, entre outros.

Os processos catalisados por enzimas constituem uma alternativa aos processos químicos convencionais, entretanto, as enzimas ainda são mais onerosas, principalmente em função do grau de pureza desejado e das várias etapas de purificação requeridas. Assim, o desenvolvimento de novos materiais e técnicas é necessário visando a diminuição dos custos finais de obtenção. Dentre as etapas de purificação, as separações cromatográficas são bastante utilizadas devido à eficiência e à pureza obtida.

A imobilização de enzimas é uma estratégia onde, teoricamente, a enzima fica retida em um suporte, seja por interações químicas de baixa energia ou por ligação covalente, para ser utilizada por tempo indefinido. A imobilização apresenta muitas vantagens como reutilização do biocatalisador, diminuindo os custos de aquisição de novos, facilidade de separação dos produtos e aumento da estabilidade química e térmica das enzimas. Além disso, o processo de imobilização possibilita a aplicação do sistema em processos contínuos ou semi-contínuos.

Porém, o método de imobilização e o tipo de interação entre o suporte e a enzima afetam diretamente a estabilidade e a atividade catalítica recuperada.

A escolha do material a ser utilizado como suporte de separação e ou imobilização é um dos fatores determinantes na eficiência do processo. Os criogéis são géis poliméricos produzidos em condições de congelamento e têm sido considerados promissores na separação de biomoléculas. Suas propriedades como elevada porosidade e baixa resistência ao escoamento, permitem o processamento de extratos brutos e meios viscosos e ou pouco clarificados, o que pode contribuir para a diminuição do número de etapas de *downstream*. Além disso, são materiais quimicamente estáveis, de fácil preparo e baixo custo e, dessa forma, têm sido testados na purificação e imobilização de uma grande variedade de enzimas com resultados satisfatórios. Contudo, estudos referentes ao preparo dos suportes e da sua reutilização ainda são necessários para ampliar o conhecimento acerca destes materiais. Além disso, enzimas diferentes apresentam estruturas moleculares diferentes, resultado também, em interações diferentes com os suportes.

Dessa forma, o objetivo deste trabalho foi estudar a utilização de criogéis supermacroporosos, baseando-se tanto nas técnicas de adsorção por troca iônica quanto na de interação hidrofóbica, como um possível protocolo de purificação e imobilização da enzima β-glicosidase.

2. OBJETIVOS

2.1. Objetivo geral

Purificar a enzima β-glicosidase do fungo termofílico *Thermoascus aurantiacus* por meio de cromatografia de troca iônica e/ou por interação hidrofóbica, empregando criogéis supermacroporosos e, posteriormente, estudar a viabilidade da imobilização desta enzima no criogel sintetizado.

2.2. Objetivos específicos

• Produzir um suporte supermacroporoso pela técnica de crio-polimerização, utilizando monômeros de acrilamida, bis-acrilamida e alil glicidil éter.

• Modificar quimicamente o criogel por meio da incorporação do grupo trocador iônico N,N-dimetilaminoetil metacrilamida (DMAEMA);

• Modificar quimicamente o criogel por meio da introdução do aminoácido L-fenilalanina, que apresenta cadeia lateral hidrofóbica.

• Caracterizar os suportes sintetizados em termos de suas propriedades morfológicas e hidrodinâmicas.

• Verificar o efeito do tempo de agitação no processo de extração da enzima;

• Purificar a enzima por cromatografia de troca iônica utilizando o criogel sintetizado, avaliando o efeito do pH;

• Avaliar o efeito do pH na adsorção da β-glicosidase na coluna de criogel funcionalizado com L-fenilalanina;

•Determinar o conteúdo de proteína total presente e atividade especifica em todas as frações obtidas (extrato bruto e amostras eluídas pelas duas colunas);

• Imobilizar a β-glicosidase proveniente do extrato bruto no criogel, por meio de interação hidrofóbica, avaliando o efeito da temperatura e da força iônica de diferentes soluções salinas no rendimento e na atividade específica da enzima imobilizada;

• Determinar os parâmetros cinéticos da enzima imobilizada e da enzima livre;

• Verificar a possibilidade de reutilização da enzima imobilizada.

3. ESTRUTURA E ORGANIZAÇÃO DOS CAPÍTULOS

Esta tese está organizada em três capítulos para melhor distribuição e entendimento do assunto abordado. **Capítulo 1: β-glucosidase: An overview of structure, action, immobilization and its application (Revisão de Literatura):** A revisão procurou demonstrar a relevância científica e o estado da arte do tema abordado. Foram apresentadas as características de estrutura e ação da β-glicosidase, a importância e principais aplicações biotecnológicas da enzima livre e imobilizada e as vantagens e desvantagens da imobilização, além da descrição dos diferentes materiais de suporte e métodos utilizados sua imobilização, com ênfase em suas vantagens e desvantagens. Finaliza-se com uma conclusão e menção de perspectivas e direções futuras. **Capítulo 2: Production and capture of β-glucosidase from *Thermoascus aurantiacus* using a tailor made anionic cryogel**. Neste capitulo, descreveu-se a preparação de um criogel de troca aniônica, o qual foi caracterizado usando técnicas morfológicas, hidrodinâmicas e térmicas. O mesmo foi usado para capturar β-glicosidase de *Thermoascus aurantiacus* produzida por fermentação em estado sólido. **Capítulo 3: Immobilization of β-glucosidase from crude extract of *Thermoascus aurantiacus* on macroporous cryogel by hydrophobic interaction.** Neste capítulo, descreveu-se a produção, purificação e imobilização por interação hidrofóbica da β-glicosidase extracelular do fungo *Thermoascus aurantiacus* em criogel macroporoso contendo L-fenilalanina. A enzima imobilizada no criogel foi avaliada quanto à atividade e reutilização. Também, foram avaliados os efeitos da temperatura e das diferentes concentrações salinas sobre a adsorção da enzima no suporte. Todos os Capítulos foram redigidos na forma de artigos científicos. O segundo capítulo já foi publicado na revista **Process Biochemistry** (vol. 82, p. 75–83, 2019) conforme anexo no final da tese. Os outros dois capítulos serão submetidos, após devidas correções e traduções ao inglês, à publicações em periódicos internacionais. Finalmente, após as seções de capítulos, uma seção de conclusões gerais apresenta uma análise dos principais resultados a as conclusões obtidas no presente trabalho.

CAPÍTULO I

β-glucosidase: An overview of structure, action, immobilization and its application

Abstract

β-glucosidases constitute a heterogeneous group of well-characterized and biologically important hydrolytic enzymes. β-glucosidase acts on different substrates, and therefore, it has been widely utilized in various biotechnological processes such as bioethanol production, isoflavone hydrolysis, flavor enhancement and the synthesis of oligosaccharides. Nowadays, immobilization of β-glucosidase has become one of the most important biocatalytic systems used for many industrial purposes, especially in the food, pharmaceutical, and beverage industries. Immobilized β-glucosidase has advantages such as it usually being chemically and thermally more stable, it is easily separated from the product, its possible reuse and, additionally it can replace chemical catalysts in some industrial applications. So, currently, both from a scientific and applied point of view, interest in developing new support materials and new protocols for β-glucosidase immobilization has grown. The main drawback of using immobilized β-glucosidase directly in the hydrolysis of insoluble (biomass) cellulose is the enzyme recovery since both enzyme and product are insoluble. Using an immobilized enzyme on a magnetic nanomaterials support, which allows for the recovery of the supported biocatalyst by an external magnet, has become an attractive way for enzyme immobilization. In this review, we present the principal methods used for the immobilization of β-glucosidase and the description of the different support materials already employed, with emphasis on their advantages and disadvantages. The main physical-chemical characteristics of immobilized enzymes, as well as an extensive discussion on applications of the pure or mixed immobilized β-glucosidase are presented. Then, we end with an overall conclusion and mention of perspectives and future directions.

1. Introduction

Enzymes have the attractive properties that can make the chemical and biochemical reactions of efficient catalytic specificity, selectivity, biodegradability and activity. The current requirements for sustainable industrial processes that include the principles of green chemistry, as well as the limitations existing in obtaining specific products or intermediaries of industrial importance, have made enzyme technology an increasingly popular alternative for application in various industrial sectors (Souza et al., 2017). The global enzymes market size was valued

at USD 9.9 billion in 2019 and is expected to reach USD 10.6 billion in 2020. Increasing demand from end-use industries such as food and beverage, biofuel, animal feed, and home cleaning, is projected to drive the market growth over the forecast period. Also, increasing health awareness among consumers has resulted in the growing consumption of functional food products, which is expected to propel the product demand in the near future (Grand View Research, 2020).

The main problems with the use of soluble enzymes are high cost of production and purification, instability of the three-dimensional structure and loss of activity under some environmental conditions or inhibition by substrate or product. This results in the use of high amounts of catalysts and consequently an increase in the final cost. So the use of expensive catalysts, such as enzymes, requires recovery and reutilization in order to make the process economically viable. The biocatalyst reuse is only possible when the enzyme preparation is stable enough. The required recovery and stability can be achieved by enzymatic immobilization procedures, making it a promising technique in the application of enzymes in large scale. In fact, the development of strong, stable and ideally insoluble biocatalysts is a major demand in industrial biocatalysts. Moreover, immobilization to solid support helps the maintenance of the enzymes activities by maintaining their structure. As a consequence, immobilized enzymes are more powerful and resistant to fluctuations in the reaction medium, which is essential for many industrial processes that occur under extreme pH and temperature conditions (Leite et al., 2008; Souza et al, 2017; Irfan et al., 2019).

β-glucosidase or β-D-glucoside glycohydrolase (EC 3.2. 1.21) is a ubiquitous enzyme found in a wide diversity of sources such as animals, plants and microorganisms, where it plays several functions, including biochemical, physiological, and nutritional roles depending of the organism (Cairns and Esen, 2010). It has the ability to hydrolyze β-D-glucosidic bonds of various compounds, including the hydrolysis of cellobiose into fermentable sugar for renewable bioethanol production, and due to this, β-glucosidase is mainly reported in the area of biofuels. However, besides biofuel production, β-glucosidase has the potential to be utilized in various biotechnological processes such as isoflavone hydrolysis, flavor enhancement and oligosaccharides synthesis (Ahmed et al., 2017a). So, due to this range of applications the demand of β-glucosidase immobilized is increasing day by day for different uses (Verma et al. 2016; Deng 2020; Pinotti et al, 2020).

Many methodologies involving different techniques and supports have been developed over the years aiming the efficient immobilization of β-glucosidase.

Vaz et al. (2016) prepared a review covering holocellulase immobilization studies and focusing on the production of second generation ethanol. However, as mentioned, these enzymes can be utilized in various relevant applications. Therefore, this work includes a general summary about β-glucosidase, as well as the main aspects related to enzyme immobilization and its main characteristics, ending with a comprehensive review of the immobilization protocols used for β-glucosidase and the principal applications.

2. β-glucosidase structure and function

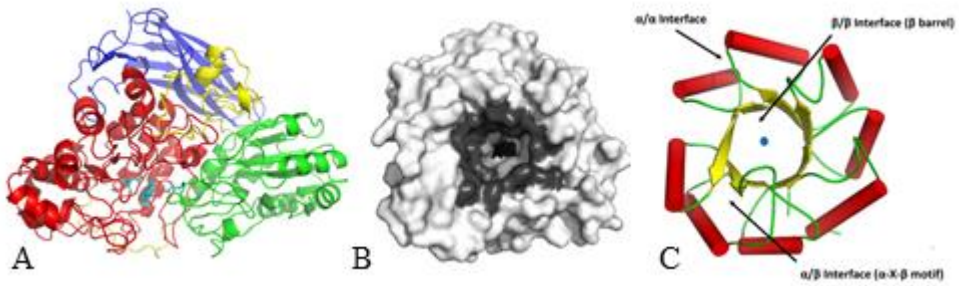
β-glucosidases are common among plants, fungi and bacteria, and showed an identical similarity with respect to their sequences and structures. According to Singh et al. (2016), they can be classified on the basis of their substrate activity or their nucleotide sequence identity. Based on substrate specificity, β-glucosidases are grouped into three classes: (i) aryl-β-D-glucosidases (display strong affinity for aryl-β-D-glucosides), (ii) cellobiases (hydrolyze only disaccharides and are considered true cellobiases) and (iii) broad specificity glucosidases (hydrolyze a wide variety of substrates and are one of the most common found β-glucosidases) (Rajan et al. 2004, Singh et al. 2016). On the basis of sequence homology, β-glucosidases have been divided into two subfamilies (i) BGA (β-glucosidases and phospho-β-glucosidases from bacteria to mammals) and (ii) BGB (β-glucosidases from yeasts, molds and rumen bacteria) (Cantarel et al. 2009; Krisch et al. 2010). These earlier methods have been largely replaced by the classification on the basis of nucleotide sequence identity (NSI), proposed by Henrissat and Bairoch (1996). In this system, those enzymes with overall amino acid sequence similarity and well conserved sequence motifs are considered in a single family. At this writing, 168 glycoside hydrolase families are listed in the frequently updated **C**arbohydrate **A**ctive en**ZY**me (CAZY, 2021). The β-glucosidases that have been described in the literature fall in glycoside hydrolase families GH1, GH3, GH5, GH9, GH30 and GH116 (Karkehabadi et al., 2018). These families are further classified into clans. A "clan" is a group of families that have a common ancestry and are recognized by significant similarities in catalytic domain structures, conserved catalytic amino acids residues and catalytic mechanism (Henrissat and Bairoch, 1996).

The clan GH-A contains largest number of families including β-glucosidase containing families GH1, GH5 and GH30. Largest number of characterized β-glucosidases belongs to GH1 family, which includes β-glucosidases from archaea, plants and mammals. Family GH3 comprises β-glucosidases of some bacterial, mold and yeast origin (Cairns and Esen 2010; Krisch et al. 2010), based on the sequence and folding similarities (hydrophobic cluster

analysis, HCA) of these enzymes. HCA of a variety of such enzymes suggested that the α-helices and the β-strands were localized in similar positions in the folded conformation. Moreover, a number of highly conserved amino acids were also clustered near the active site. A classical $(\alpha/\beta)_8$ TIM barrel folds has been reported as a key feature of GH1 family β-glucosidases. The TIM barrel received this name from the enzyme **T**riose phosphate **i**so**m**erase (TIM), which was the first protein possessing the fold to be crystallized (Vijayabaskar and Vishveshwara, (2012). TIM barrels has typically 200-250 amino acid residues folded into 8 α-helices and 8 β-strands (Wierenga, 2001).

The β-strands are arranged into a parallel β-barrel, and are surrounded by the 8 α-helices. The defining property of TIM β-barrels is that they always possess a sheer number of 8. The TIM barrel has been reported in X-ray crystallographic structure of β-glucosidase BGL1A from basidiomycete (*Phanerochaete chrysosporium*) (Nijikken et al., 2007), human cytosolic β-glucosidase (Tribolo et al., 2007), GH1 β-glucosidases from *T. reesei* (TrBgl2), *Clostridium cellulovorans* (CcBglA), and *Neotermes koshunensis* (NkBgl) (Jeng et al., 2011), recombinant β-glucosidase (rThBgl) from *Trichoderma harzianum* (Santos et al. 2016), and GH3 β-glucosidases from *A. aculeatus* (Segato et al., 2014), *Neurospora crassa* (Karkehabadi et al., 2018) and *Talaromyces emersonii* (Murray et al., 2004). A schematic representation of the overall structure of β-glucosidase from *T. reesei* is presented in Figure 1.

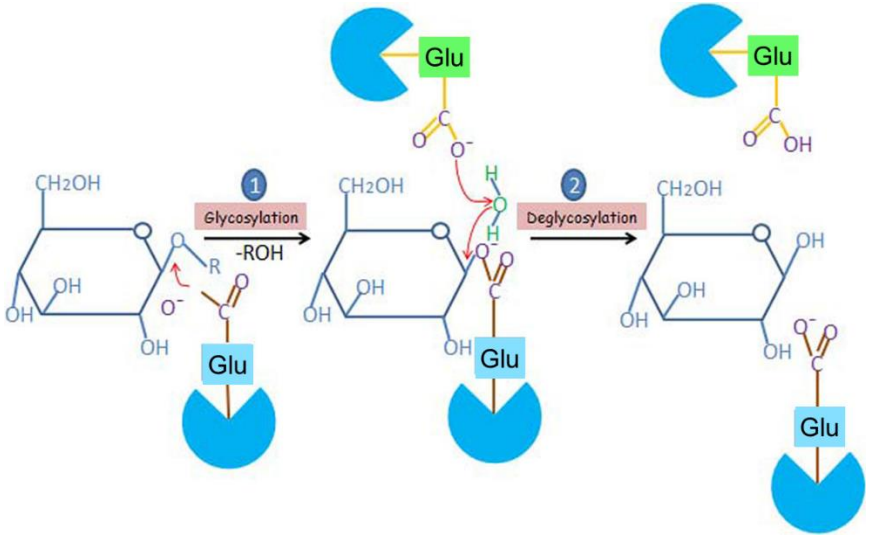
Figure 1. Schematic representation of the overall structure of β-glucosidase (*T. reesei*). **A**). Cartoon representation highlighting the three domains colored, red (domain one), green (domain two), and blue (domain three). The two domain linker regions are shown in yellow. The glucose bound in the active site (depicted using a ball-and-stick representation) and the two catalytic residues (depicted in sticks only) are colored cyan. **B**) Surface representation. The amino acids located in the catalytic center shown in black, substrate-binding region shown in light gray and substrate entrance region shown in dark gray. **C**) The three-dimensional structure of a typical (α/β)8-TIM barrel fold of GH1 enzymes.



Source: A) KARKEHABADI et al. (2014); **B**) LEE et al. (2012); **C**) VIJAYABASKAR and VISHVESHWARA (2012).

β-glucosidases cleave β-glycosidic bonds between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. The enzymatic hydrolysis of a glycosidic bond requires two critical residues: a proton donor and a proton acceptor which can also be called a nucleophile/base and the catalytic residues are aspartate and glutamate. (CAZY, 2021). β-glucosidases in all of these GH families, except for GH9, perform hydrolysis by a double-displacement reaction mechanism with net retention of the configuration at the anomeric carbon (Gebler et al., 1992). Most reported β-glucosidases are GH1 and GH3 families. Although, both act on similar substrates, the GH1 β-glucosidases use a glutamate residue as the catalytic nucleophile, whereas the GH3 β-glucosidases use an aspartate residue as the nucleophile (Jeng et 2011, Santos et al, 2016). Most reported β-glucosidases accomplish the catalysis in two steps, glycosylation and deglycosylation and are retaining enzymes. The catalytic mechanism of action of β-glucosidases is described in Figure 2. For this catalysis two glutamate residues carry out the overall reaction. The first one acts as a nucleophile and the second residue acts as a general acid/base catalyst (Davies and Henrissat, 1995). In the initial (glycosylation) step, glutamate undergoes a nucleophilic attack on the anomeric carbon and results in a glucose–enzyme intermediate product. In the second (deglycosylation) step, a water molecule, which is activated by acid/base catalyst glutamate residue, acts as a nucleophile and breaks the glycosidic bond to release glucose (Geronimo et al. 2018; Litzinger et al. 2010).

Figure 2. Proposed ‘‘retaining’’ mechanism for hydrolysis of b-glycosidic bond by β-glucosidase: (1) during first glycosylation step, a conserved glutamate residue acts as nucleophile and attacks on the glycosidic bonds or cellobiose and other oligosaccharides formed by the hydrolytic action of other enzyme of cellulase system. This results into the formation of an enzyme-substrate intermediate complex, (2) during second step called deglycosylation, an another conserved glutamate residue activates a water molecule present in the proximity by general acid/base catalyst reaction and now this activated water molecule acts on the intermediate complex to release the free glucose residue.



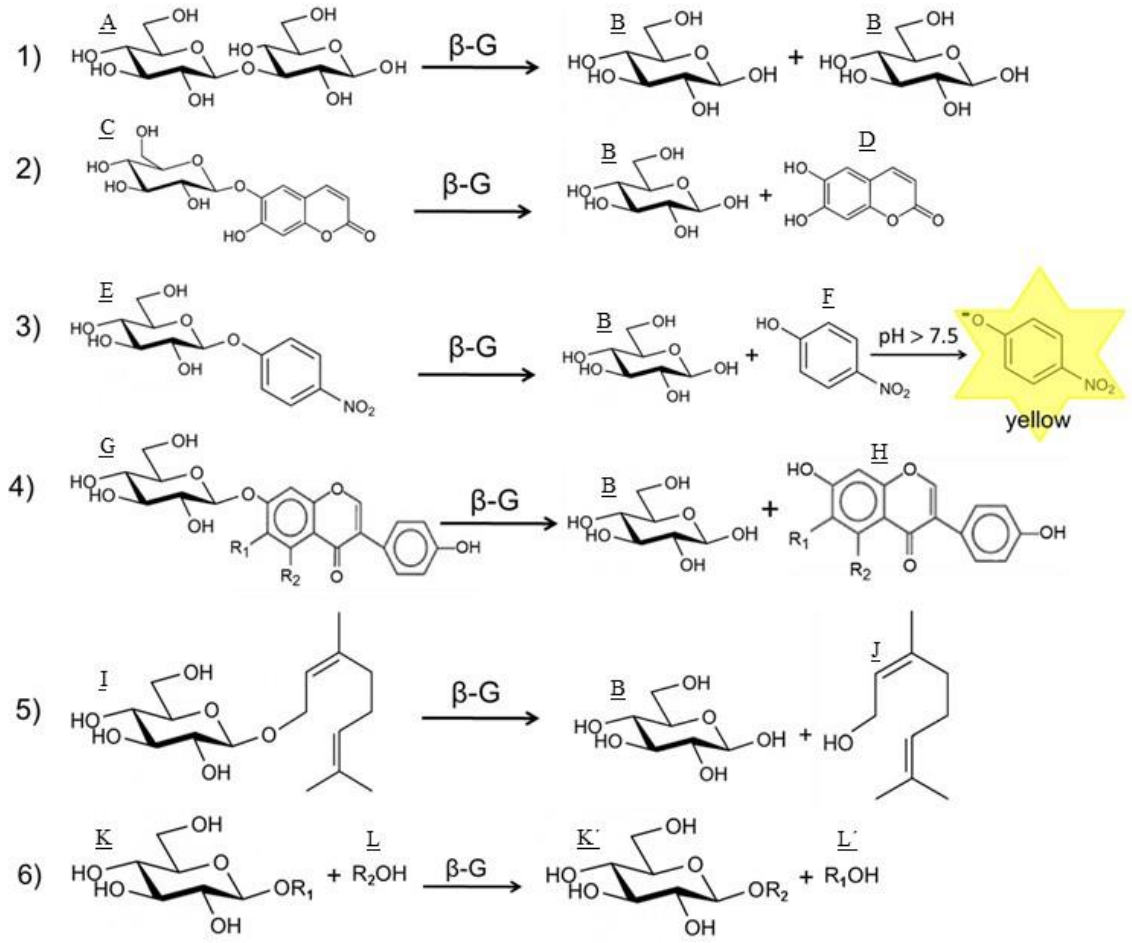
Source: SINGH et al. (2016) and GERONIMO et al. (2018).

3. β-glucosidase activity and screening

β-glucosidases or β-D-glucoside glycohydrolases (EC 3.2. 1.21) catalyzes the hydrolysis of β-(1,4) glycosidic bonds between glucose terminals of disaccharides and oligosaccharides or β-glycosidic bonds between glucose and an alkyl or aryl aglycones.

Since there are many compounds containing nonreducing terminal glucosides in organic compound, β-glucosidases have been suggested to have many functions in nature These include biomass conversion in microorganisms, breakdown of glycolipids and exogenous glucosides in animals, and lignification, catabolism of cell wall oligosaccharides, defense, phytohormone conjugate activation, and scent release in plants, as well as both sides of plant–microbe and plant–insect interactions (Cairns and Esen, 2010). Some examples of the wide range of activities β-glucosidases are shown in Figure 3.

Figure 3. Different actions of β-glucosidase (β-G). 1) Hydrolysis of cellobiose (A) liberating glucoses (B); 2) Hydrolysis of esculin (C) into glucose and esculetin (D); 3) Hydrolysis of p-NPG (E) liberating glucose (B) and nitrophenol (F); 4) Hydrolysis of soy isoflavones (G) into glucose (B) e aglycones (H); 5) Hydrolysis of terpene glycoside (I) into glucose (B) and the flavoring compounds such as nerol (J); 6) Transglycosylation reaction of glycosidic conjugates (K and K´) involving a nucleophile such as alcohols (L and L´).



Source: Prepared by the author.

Different methods can be used in order to estimate the β-glucosidase activity. Cellobiose is a disaccharide derived from cellulose. β-glucosidase hydrolyzes cellobiose into two glucose units, which is measured using glucose specific test such as glucose oxidase/peroxidase or by High Performance Liquid Chromatography (HPLC). Since cellobiose is the natural substrate for β-glucosidase, it is preferred by some researches Other prefers the use of chromogenic substrate-based assays producing high sensitivity, low cost and easy operation. Among the tests based on chromogenic substrates, β-glucosidase activity has been routinely determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG), as previously described (Wood and Bhat 1988; Leite et al., 2008; Mól et al., 2019). In this assay, β-glucosidase hydrolyzes *p*-nitrophenyl-β-D-glucopyranoside liberating nitrophenol (pK 7.15)

that in pH > 7.5 results in the formation of a colorimetric (405 nm) product, proportional to the β-glucosidase activity present. Activity is determined by monitoring release of *p*-nitrophenyl at 405 nm with reference to a standard curve prepared using *p*-nitrophenol. Activity is generally expressed in International Units (IU), where 1 IU releases 1 μmol *p*-nitrophenol per min.

Despite being a ubiquitous enzyme found in all living organisms, microbial β-glucosidases have been preferred for industrial applications because they generally exhibit more diverse properties and robust activity. Screening β-glucosidases has been described by various biochemical methods (Ahmed et al., 2017b). Cellobiose as sole carbon source can be used for screening and selection for microorganism producing β-glucosidase, as only microorganism with β-glucosidase production capability can grow on this media (Liu et al., 2009). Other of the most used methods is the use of esculin (8-hydroxyquinoline-β-D-glucoside) in medium containing agar. Microorganism producing β-glucosidase hydrolyzes esculin releasing glucose and the aglycone moiety, esculetin, which, then chelates ferric ions supplemented to the growth medium and forms dark brown complex against clear background (Ahmed et al., 2017b).

4. β-glucosidase general applications

The dual activity of β-glucosidase leading to cleavage and synthesis of glycosidic bonds is pivotal in many biological pathways such as cellular signaling, biosynthesis and degradation of structural and storage polysaccharides, host-pathogen interactions, as well as in a high number of biotechnological applications, which can be divided based on the hydrolytic activity or based on the synthetic activity (Bhatia et al., 2002).

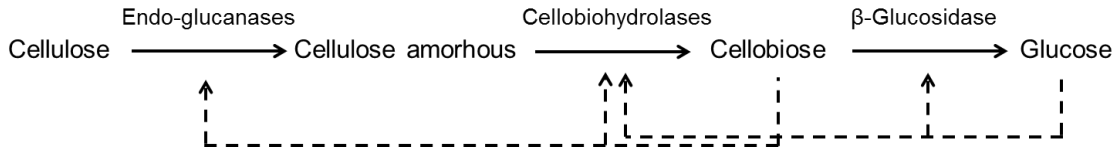
β-glucosidases are widely distributed in nature, being found in bacteria, fungi, yeasts, plants and animals (Bhatia et al., 2002). However, for industrial applications, microorganisms are considered the best source for the production of the enzyme. Microorganisms grow quickly, which speeds up the production of the enzyme, and are easier to manipulate than animals and plants, requiring less space and thus lowering costs. In addition, microorganisms can be easily modified by genetic engineering, producing enzymes with desirable characteristics in the face of drastic conditions of temperature and alkalinity (Singhania et al., 2010; Ahmed et al., 2017a). β-glucosidases from various sources have been widely used in industrial applications. Filamentous fungi such *Trichoderma reesei, Penicillium oxalicum, M. purpureus, M. sanguineus,* and *Aspergilus* spp. are the most prominent producers of β-glucosidase (Ahmed et al., 2017b). The demand of β-glucosidase for different applications is described below:

Bioenergy: β-glucosidase is mainly used for agriculture and biotechnology, particularly in the employment of lignocellulosic waste to produce biofuels and other fermentative products of sugar. Cellulose hydrolysis for bioethanol production is undoubtedly one of the most attractive applications of β-glucosidase, since cellulose is the most abundant organic compound in nature and is a source of renewable energy (Beguin and Aubert, 1994).

The production of bioethanol from biomass involves a first step of pretreatment to destroy biomass recalcitrance and improve substrate accessibility, followed by enzymatic hydrolysis of holocellulose (cellulose and hemicelluloses that is obtained by removing the extractives and the lignin) by the use of several enzymes acting synergistically to degrade the substrate into fermentable sugars, which will be transformed into ethanol in the fermentation step. Cellulases and xylanases are considered the major enzymes for hydrolysis of holocellulose.

Cellulase is a system of enzymes that can convert the cellulose, the most abundant biopolymer in biosphere and the major constituent of plants, into glucose and are found in insects, plants, fungi and bacteria. This enzymatic system, presented by many filamentous fungi, consists of different glycoside hydrolases (GHs) and accessory proteins (e.g, lytic polysaccharide monooxygenase (LPMOs), which act synergistically. *Trichoderma reesei* (the most studied filamentous fungi for cellulose saccharification) produces several GHs that synergistically act in cellulose hydrolysis. The most accept concept today is endo/exo synergism, which include four endo-β-1,4-glucanase acting in amorphous regions of cellulose and two exo-cellobiohydrolases, CBHI (acting in R-terminal) and CBHII (acting in NR-terminal). Endoglucanase hydrolyzes internals amorphous regions liberating extremities for attack by exo-cellobiohydrolases (Payne 2015). The endo and exo enzymes hydrolyze cellulose chains typically into oligos, and mainly, disaccharide units of cellobiose (which are not fermentable). Finally, cellobiose and cellooligos are hydrolysed by β-glucosidase into glucose monomer unit, ending the cellulose hydrolysis (Figure 4). As cellobiose is a strong inhibitor of cellobiohydrolases and endoglucanases, β-glucosidase plays an important role for efficient process of cellulose hydrolysis (Bhatia et al., 2002; Singhania et al., 2013; Tu et. al. 2013; Ahmed et al., 2017; Irfan et al., 2019).

Figure 4. Products inhibition of cellulases by sugars in the hydrolysates.



Source: XIN et al. (2016).

Some microbial cellulases system are deficient in β-glucosidase, thus for commercial application preparations require supplementation in order to relieve product inhibition caused by accumulation of cellobiose and for efficient saccharification. Immobilization of β-glucosidase could facilitate enzyme recycling in sequential batchwise or semi-batchwise saccharification processes, thereby reducing cost (Tu et al., 2006; Borges et al., 2014).

Soil quality: As participant of the carbon cycle, β-glucosidase plays an important role in soil fertility and has been proposed as a soil quality indicator. β-glucosidase activity is sensitive to a variety of managements and various soil types, textures and moisture. This enzyme is viewed as an indicator of turnover of soil organic carbon compounds from crop residues, biotechnological byproducts, animal manure and sewage sludge. It is the driving force in the decomposition of carbohydrates in soil and the resulting hydrolysis sugars are important energy sources for microorganisms in soils. The changes in management and soil use, such as deforestation or conventional agriculture, can lead to large reduction in the soil organic matter content. Thus, in soils with low amount of organic matter there are less simples sugar for the microbial population (De Almeida et al., 2015).

Isoflavone production: β-glucosidases have also been used in the hydrolysis of isoflavone present in soy and its derivatives. Isoflavones are phenolic compounds, secondary metabolites of plants that differ in chemical structure and biological function and that have gained the attention of several researchers in the field of health and food technology due to their antioxidant, anticancer, antiallergic, anti-inflammatory and anti-hypertensive properties, among others (Chang et al., 2013).

Isoflavones are naturally found in glycosidic forms, which increases their solubility in water and their stability, but decreases their absorption and biological activity when compared to aglycone. The release of the aglycone molecule requires the action of specific enzymes, such as arabinosidase and β-glucosidase, and the released aglycone can be easily absorbed, increasing its biological potential (Ahmed et al., 2017a).

Food and beverage: In the food industry, β-glucosidases can be used as flavoring substances in wines, teas and fruit juices, improving the quality of the taste, aroma and coloring of food.

Most aroma compounds found in plants and fruits such as grapes, plums, peaches, strawberries and papaya are present in the form of glycosidic conjugates, making them non-volatile and tasteless. β-glucosidases act in the hydrolysis of glycosidic bonds, releasing volatile terpenes and contributing to the increase in aroma production during processing (Spagna et al., 2002; González-Pombo, 2011; Singhania et al., 2013; Ahmed et al., 2017a).

Oligosaccharides: Oligosaccharides are widely distributed in nature and are used in the food and medical industries as therapeutic or probiotic agents. Besides, to their catalysis of action, β-glucosidases also catalyze reverse hydrolysis and transglycosylation reactions, which can be used to synthesize oligosaccharides and glycosides of interest (Cairns and Esen, 2010). Cellobiose, gentiobiose, sophorose and laminaribiose have been synthesized by β-glucosidase via reverse hydrolysis reaction (Bhatia et al., 2002; Ahmed et al., 2017a, Niu et al., 2020). Various proteins engineering of β-glucosidases have been developed to eliminate hydrolytic activity and improve synthetic activity to maximize the products of these transglycosylation reactions (Niu et al., 2020).

5. β-glucosidase immobilization

The term "immobilized enzymes" is a generic term that refers to enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously (Brena et al., 2013).

To efficiently develop immobilization processes, especially those based on bioconjunction, that is, the process of chemically joining two or more biomolecules by chemical bonding or crosslinking, it is very important to know, not only the chemical characteristics of the support, but also the chemical nature of the functional groups in the protein. Therefore, the enzyme bioconjugation process depends on the reactivity of the protein component and the specificities of the selected crosslinker, which contain reactive ends for specific functional groups (for example, primary amines, sulfhydryl, etc) in proteins. The chemical reactivity of proteins depends not only of side chains of their amino acid composition, but also of the free amino and carboxyl groups of the N and C-terminal residues, respectively. Eight types of side chains of amino acids contribute to chemical reactivity in proteins, all of which have hydrophilic side chain that comprise imidazolyl group of histidine, guanidinyl group of arginine, phenolic group of tyrosine, sulfhydryls (side chain of cysteine), ε-amino group of lysine, indolyl group of tryptophan, thioether moiety of methionineas, as well as γ-carboxyl

groups of glutamic and β-carboxyl groups of aspartic acid (Souza et al., 2017; Wahab et al., 2020).

The immobilization of enzymes on solid supports increases the prospect of savings in the final cost of the process due to use in a continuous process, improves operational stability and the reuse of the biological material (Da Silva et al., 2014).

The ability to be used repeatedly without significant drop in activity is one of the most important characteristics of immobilized enzymes (Tan and Lee, 2015). This is because most of the enzyme used in the industry are very expensive. Storage stability for the immobilized enzyme is also one of the significant indexes to evaluate the properties of an enzyme, which can make the immobilized enzyme more advantageous than that of the free one (Su et al., 2010). Immobilization also increases thermal stability and provides better stability under pH and temperature ranges. In general, the immobilization support has a protecting effect at high temperatures when enzyme denaturation usually occurs. The immobilization increases the rigidity of the enzyme, preserving its tertiary structure and avoiding conformational changes due to environment (Chang et al., 2007; Vaz et al., 2016). Alternatively, immobilization in many cases may be a step for purifying enzymes (Barbosa et al., 2015).

In addition to the advantages mentioned above, the immobilization approaches allow continuous operations, when successfully implemented (Fernandes, 2010; Wei et al., 2018; Sui et al., 2019). This was verified by Wei et al. (2018), who compared the biochemical characterization of a β-glucosidase immobilized microreactor operating in continuous mode with free β-glucosidase in batch mode. The microreactor exhibited greater stability than the free enzyme over the time and its activity decreased more slowly. The long-term operational stability of the microreactor was assessed for 12 days and the residual activity was 92% after the experiment. Also, after storage at 4 °C for 2 months, the residual activity of the free enzyme declined to 1% while that of the microreactor decreased to only 60%.

Despite the great benefits, some limitations hamper the applications of enzyme immobilized in commercial scale, such as high cost, low productivity and stability and narrow range of substrates (Fernandes, 2010).

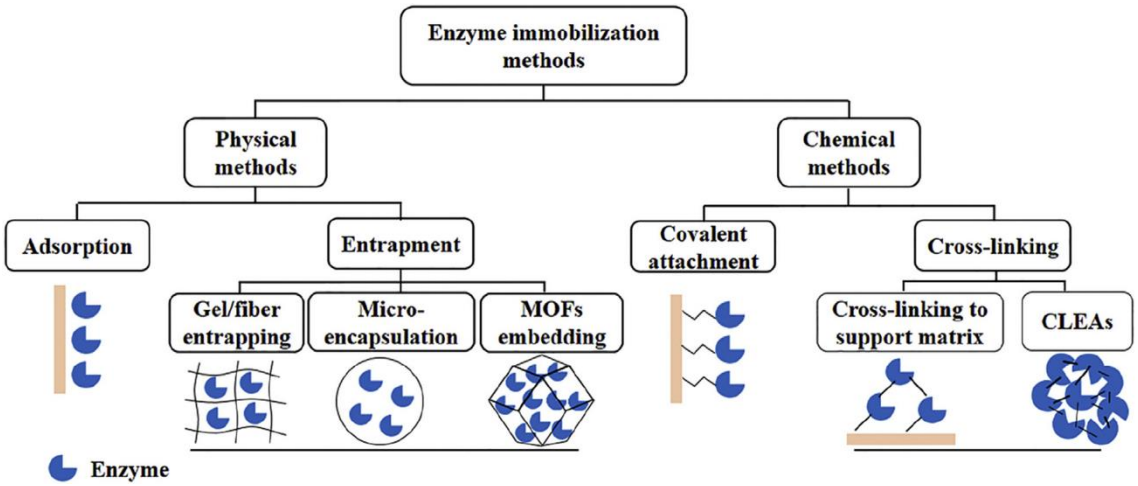
As a consequence of immobilization, some properties of enzymes such as catalytic activity may change. Such modifications can be caused both by changes in the intrinsic activity of the enzyme and by the fact that the interaction of immobilized enzyme with the solid carrier occurs in a different microenvironment than the original. Changes observed in catalytic activity after immobilization can be attributed to conformational changes in the protein caused due to

the connection with the matrix (Brena et al., 2013; Liese and Hilterhaus, 2013). Thus, the selection of the support and correct choice of the immobilization protocol are essential to obtain the desired advantages aiming at the implementation of the industrial biocatalyst (Mendes et al., 2011).

5.1. Techniques of immobilization

Briefly, there are four different methods of enzyme immobilization: adsorption, physical entrapment, covalent attachment, and cross-linking of enzyme aggregates (Figure 5). There are also some other methods which may be combinations of the ones mentioned (Irfan et al., 2019), but only the conventional ones will be addressed.

Figure 5. Schematic representation of the methods of enzyme immobilization.



Source: LIU et al. (2018).

These methods can be divided into physical, which includes adsorption and entrapment, and chemicals, which involve covalent bonding and cross-linking. Adsorption is so far the most common way to immobilize enzymes and covers both affinity adsorption, ion adsorption and physical adsorption. The enzymes are taken or trapped in the support pores by van der Walls forces, hydrogen bonding, hydrophobic and or electrostatic interactions. These forces of binding are usually sensitive to environmental variations, resulting in biocatalysts with relative low stability (Nguyen and Kim, 2017; Souza et al., 2017; Guo et al., 2019; Irfan et al., 2019). In entrapment, enzyme leaching is not a drawback since the polymeric network keeps the enzymes retained, improving the stability, and allows the traverse of substrate and products. However, the main disadvantage is the diffusional problems that hinder the substrate of flow into the network (Nguyen and Kim., 2017).

Among chemical methods, covalent binding is a traditional method where the enzyme is attached due to chemical reactions with the support. The strength of this connection is high and usually involves several residues of the protein, providing a great rigidity that can maintain the structure unchanged from denaturing agents (Souza et al., 2017). Meantime, partial inactivation and consequently activity reduction can occur due to changes in the enzyme´s native conformation imposed by the connections between the enzyme and the support, which can also block the active sites of the enzyme (Souza et al., 2017; Irfan et al., 2019). The binding of the enzymes to solid support is generally carried out by initial activation of the surface using multifunctional reagents followed by enzyme coupling to the activated support, then the removal of excess and unbound biomolecules. Normally, glutaraldehyde is one of the most commonly used (Sassolas et al., 2012; De Andrades et al., 2019).

Immobilization of enzymes by crosslinking is another well-known approach. The enzyme is cross-linked with each other in the presence of bifunctional agents or in the presence of a functionally inert protein such as bovine serum albumin (Sassolas et al., 2012, Nguyen and Kim, 2017). The crosslinking agent, such as glutaraldehyde, glyoxal or hexamethylenediamine, is a molecule that contains at least two reactive ends that bind to specific groups of the enzyme surface. This method is attractive due to the simplicity and the strong chemical binding between the molecules and, just like in covalent binding, the main hindrance is the occurrence of activity losses due to alterations of the native conformational of the enzyme.

A generalized characterization of the immobilization methods is shown in Table 1. Crosslinking and covalent attachment are long-lasting and successful but costly and effortlessly aggravating the implementation of the enzyme. On the other hand, adsorption is inexpensive, easy and productive but commonly reversible due to the weakness of interaction between the support carrier and the enzyme (Irfan et al., 2019).

The selection of immobilization method should be based on parameters such as the total activity of the biocatalyst, regeneration and inactivation, cost of the procedure, toxicity of the reagents, operational stability, hydrodynamics properties and the desired final characteristics of the immobilized enzyme, each method having a unique nature (Brena et al., 2013; Souza et al., 2017).

Table 1. A generalized characterization of the immobilization procedures

Parameter	Immobilization procedure			
	Covalent	**Adsorption**	**Crosslinking**	**Entrapment**
Activity	High	Low	Intermediate	High
Range of application	Low	Intermediate	Low	Intermediate/ High
Efficiency	Low	High	Intermediate	Intermediate
Cost	Low	High	Intermediate	Low
Preparation	Easy	Difficult	Intermediate	Intermediate
Substrate specificity	Cannot be changed	Can be changed	Cannot be changed	Can be changed
Regeneration	Possible	Impossible	Impossible	Impossible

Source: Fernandes (2010)

5.1.1. Glutaraldehyde as activating agent

Many of the works presented used glutaraldehyde as an activating agent at some stage of the immobilization process. Glutaraldehyde is a very versatile agent that reacts with primary amino groups of the enzyme and the support and helps preventing enzyme leaching, obtaining active and stable enzyme preparations (De Andrades et al., 2019).

In general, protocols for covalent immobilization begin with the modification of the support surface through activation reactions with glutaraldehyde, in which the functional groups of the support are modified to produce reactive intermediates. The enzymes as covalently immobilized to the support through its amino groups (α-NH_2 of the end chain, ε-NH_2 of lysine and or NH_2 from chemical amination), which binds to the aldehyde groups of the support, forming the Schiff bases. Other functional groups of the enzyme such as carboxylic groups (β-COOH of aspartic acid, γ-COOH from glutamic acid and α-carboxylic), tyrosine phenolic groups, cysteine sulfhydric group, hydroxyl group from serine, threonine and tyrosine, imidazole group from histidine and tryptophan indole group can also covalently bind to reactive support groups (Souza et al., 2017).

Enzymes can also be immobilized by crosslinking via reaction of glutaraldehyde with reactive amine groups on the protein surface. In some of these approaches, the enzymes are immobilized without the necessity of supports (Souza et al., 2017). However, the enzymes can still be crosslinked even after its attachment to the support.

Naseer et al. (2019) conducted a study comparing the immobilization of β-glucosidase extracted from the fruiting bodies of *Agrocybe aegirit* through the crosslinking method, using genipin or using glutaraldehyde. Genipin is an aglycone derivative also capable of promoting crosslinking in materials with primary amine groups such as proteins and is produced by the hydrolysis of geniposide by β-glucosidase. The enzymes treated with genipose were previously adsorbed on SiO_2 nanoparticles and then submitted to crosslinking, although the enzymes treated with glutaraldehyde were directly immobilized on the previously activated support. The concentration of added geniposide was monitored and decreased 3% and 82% after 2 and after 48 hours of reaction, indicating the hydrolysis catalyzed by the enzyme. As expected, after immobilization, pH and thermal stability were greatly improved and the residual activity stayed around 80% and 50% after 5 and 10 operation cycles, respectively. However, the immobilization method using geniposide showed higher yield (96%) and had better substrate affinity, observed by the kinetic parameters determination, than the other method.

De Andrades et al. (2019) studied the immobilization of three β-glucosidases (Pectinex Ultra SP-L, Pectinex Ultra Clear and a homemade obtained from *Aspergillys niger*) on MANAE-agarose using different strategies of glutaraldehyde activation. In some cases, the enzyme was previously adsorbed on the support and then treated with glutaraldehyde and in others the support was pre-activated. The pH value of the immobilization and ionic strength was studied. Generally, the activities recoveries were higher when the pre activated supports were used. The stability of the immobilized enzyme was higher than that of free enzyme. The stability was enhanced by immobilization on the pre activated support, but the stabilization of Pectinex Ultra Clear and β-glucosidase from *Aspergillus* was even higher when the enzymes were adsorbed and then cross-linked. For Pectinex Ultra SP-L, the opposite was verified.

They concluded that optimal protocol for immobilizations using glutaraldehyde chemistry depends a lot on the enzyme type and thus must be individually tested. Also, the identification of an ideal immobilization technique for β-glucosidase is crucial for achieving the desired characteristics (Borges et al., 2014), whatever the application.

Nonetheless, not a single approach or support is perfect for all types of enzymes and their utilizations due to the wide range of chemical composition and characteristics of enzymes, difference in the properties of products and substrates, and difference in the use of the product (Irfan et al., 2019). Prior knowledge of the support characteristics and the effects of the methods used are also essential when selecting an immobilization technique for a given purpose (Souza et al., 2017).

5.2. Supports

As the number of applications employing enzyme immobilized has increased, the understanding of the factors that impact activity and the strategies to manipulate enzyme-support interaction had also been well-understood (Talbert and Goddard, 2012). According to Talbert and Goddard (2012), the main factors are related to the nature of the enzyme (amphiphilicity, ionizable groups, rigidity and multimeric structures), the nature of the material of the support (surface hydrophobicity/hydrophilicity, surface charge, material size and topography) and the nature of the interface formed (microenvironment effects, attachments chemistry and protein loading).

The characteristics of the carriers are also fundamental in the biocatalyst performance (Brena et al., 2013, Zdarta et al., 2018). Physical and chemical resistance, hydrophilicity, biological resistance, low cost and availability are examples of desired properties. The classic supports are usually classified into organic or inorganic. The organic may be natural, such as polysaccharides (cellulose, dextran, agar, agarose, chitin, chitosan, alginate, dextran, starch and pullulan) and proteins (collagen, albumin and gelatin) or synthetic such as polyacrylamide, polyurethane, polypropylene, polystyrene and polyvinyl alcohol (PVA). The most common example of natural inorganic biopolymers as carriers are supports based on silica materials, ceramics, diatomaceous earth (celite), titania, zirconia and alumina (Brena et al., 2013; Irfan et al., 2019, Aggarwal et al. 2020).

Various of these materials, such as alginate (Su et al., 2010; Tsai and Meyer, 2014), chitosan (Spagna et al., 2002; Chang et al., 2007; Zheng et al., 2013), silica (Agrawal et al., 2015), and synthetic polymers (González-Pombo et al., 2011; Khan et al., 2012; Sui et al., 2019) have been used as supports for β-glucosidase immobilization.

Besides these classic materials, the discovery and use of new materials with desired properties has recently become extremely important (Zdarta et al., 2018, Srivastava et al., 2020). Examples of excellent candidates for biomacromolecules encapsulation are the mesoporous nanomaterials which can be of the type non-magnetic and magnetic mesoporous nanocarriers (Aggarwal et al. 2020, Venezia et al., 2020).

According to Zdarta et al. (2018), with the use of these so called new materials as enzyme supports, the control of the technological process is improved, the immobilized enzyme exhibit enhanced catalytic efficiency and the purity and quality of the reaction products increased compared to the process catalyzed by the enzyme immobilized in classic materials. A complete review conducted by Singh et al. (2020) describes the use of nanomaterials as

supports for immobilization of enzymes. According to these authors, β-glucosidase coimmobilized on superparamagnetic nanoparticles achieved 100% immobilization efficiency and better stability of enzymes for the conversion of cellulosic biomass into sugar for bioethanol production.

Others examples of new supports used for β-glucosidase immobilization include magnetic particles (Zheng et al., 2013, Ferner et al., 2016, Ferner et al., 2018), nanoparticles (Cherian et al., 2015), mesoporous materials (Venezia et al., 2020), macroporous materials (Khan et al., 2012), carbon nanotubes (Gómez et al., 2005), inorganic-organic hybrids (Chang et al., 2007), and others. Numerous studies have been reported to date on platforms and a review of methodologies for enzyme immobilization was recently reported by Aggarwal et al. (2020).

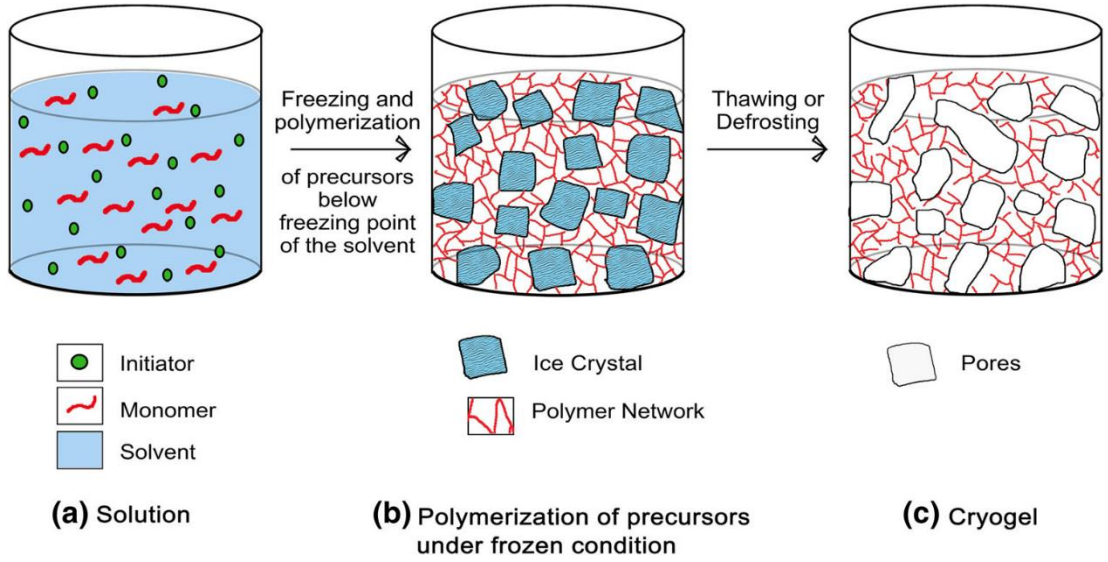
5.2.1 Cryogels as versatile support for enzyme immobilization

The so called cryogels (from the greek *kryos*, frozen or ice) are polymeric gels formed in cryogenic conditions and were reported about 50 years ago (Lozinsky et al., 2001; Lozinsky et al., 2003). These polymeric gels have several applications in many areas of biotechnology, such as chromatographic materials, supports for immobilization of biomolecules and cells, matrices for electrophoresis, among others.

These supports are obtained due to the polymerization, under freezing, of a monomer solution containing the appropriate catalysts. With freezing, there is an increase in the concentration of monomers in a microphase that has not frozen yet, allowing the formation of a more resistant polymeric network. When the ice thaws, the cavities of the cryogels are formed, resulting in a supermacroporous and spongy structure (Figure 6). Ice crystals are then porogenic agents, and the shape and size of these crystals determine the shape and the size of the pores that will develop after thawing the present water (Plieva et al., 2008).

The formation of cryogels is a complex process and involves several variables, such as concentration of monomers, temperature control, freezing speed of the mixture, amount of crosslinking agent and copolymerization of other monomers, for example (Yao et al., 2006a; Plieva et al., 2008).

Figure 6. Scheme of cryogel formation.



Source: JAIN et al. (2017).

Cryogels can be produced essentially from any precursor of gel formation and can have different morphologies and porosities. Among the possible polymers used in the synthesis of these compound, polyacrylamide monoliths stand out, which are obtained from the polymerization of acrylamide molecules and the crosslinking agent N,N´-methylene-bis-acrylamide. The catalysts for this reaction are ammonium persulfate and TEMED and other monomers can also be added, such as allyl glycidyl ether, responsible for the rigidity of the structure and for providing epoxy groups where some binders can be immobilized. The pore size in acrylamide gel is determined by the content of monomers present in the solution and the degree of crosslinking (Lozinsky et al., 2003; Yao et al., 2006b; Plieva et al.; 2008; Erturk and Mattiasson, 2014).

Cryogels have several advantages when compared to conventional matrices. Its structure is composed of interconnected pores ranging from 10 to 100 µm, allowing the passage of even cells and the use of uncleared media. Due to the high porosity, cryogels allow flow through the material to occur at a reasonable pressure and, as a result, have a low pressure drop. Because they are cheap and easily prepared material on laboratory benches, cryogels can still be disposable, avoiding cross-contamination between batches (Arvidsson et al., 2002; Turu et al., 2016).

In relation to immobilization procedures, cryogels have been efficiently used as support for the immobilization of biomolecules such as proteins (Khan et al., 2012; Uygun et al., 2014; Veríssimo et al., 2018; Erol et al., 2019).

Khan et al. (2012) immobilized two variants of a thermostable β-glucosidase from *Thermotoga neapolita* in polyacrylamide cryogel by covalent bonding. The authors observed that more than 91% of the initial activity was maintained after ten cycles of operation and that after 3 months of storage the immobilized enzymes showed no significant loss of activity. In addition, the immobilization allowed the recycling of the biocatalyst and increased thermostability. Such results corroborate the use of the enzyme immobilization technique using cryogels as support.

Uygun et al. (2014) demonstrated that a cryogel functionalized with dye Cibracon Blue F3GA was efficiently used for reversible immobilization of alcohol dehydrogenase. The cryogel was reused and after 25 cycles of adsorption/desorption there was no decrease in the adsorptive capacity. Tüzmen et al. (2012) also used a polyacrylamide cryogel containing the covalently bound Cibracon Blue F3GA dye for catalase immobilization. The incorporation of ions slightly increased the adsorptive capacity of the cryogel (1.2). The researchers found that the immobilized enzymes had a higher maximum reaction velocity and were stable over a wider temperature and pH range.

Veríssimo et al. (2018) developed a bioreactor composed of lipases encapsulated in a polyacrylamide cryogel to be used in the synthesis of structured lipids. The bioreactor proved to be efficient in relation to esterification and interesterification reactions, demonstrating its potential for biotechnological applications.

Although there is still a lot of work about cryogel manufacture to be carried out, the studies already conducted suggest that such material may be appropriate support for the construction of bioreactors (Hixon et al., 2017).

6. Main changes in the characteristics of the immobilized enzymes

Although the characteristics of the biocatalysts produced may vary widely, most of studies highlight the increase in thermal stability, one of the major advantages and flagship of enzyme immobilization. One can observe that improvement in the thermal stability following immobilization has been reported in practically all studies listed. For β-glucosidase, this is extremely relevant because when renewable sources such as agricultural crops or wood are utilized for extraction of high values products, thermostable enzymes have an obvious advantages as higher temperatures often promote better enzyme penetration and cell-wall disorganization of the raw material (Irfan et al., 2019). According to Borges et al. (2014), the

degree of stabilization of an enzyme depends on various factor, among them the microorganism from which it originates and the type of binding to the support, as well as temperature and pH.

Regarding the changes obtained in the parameters K_M and V_{max} with the immobilization protocols, the results obtained by all the studies described in the work show that there is still no well-defined pattern about what could happen, each immobilization technology has a particular effect. These changes upon immobilization is a critical point for investigating the success of a protocol (Çelik et al., 2016). Normally, the immobilization procedures lead to structural changes in the enzyme and, consequently, decrease the accessibility of the substrate to its the active site. In other words, the affinity of the enzyme for the substrate decreases and the K_M value increases (Liang et al., 2012. Da Silva et al., 2014; Çelik et al., 2016). In addition to conformational changes, factors such as ionic strength, steric effects and diffusional limitations increases the K_M value. Some authors, however, obtained lower K_m values for the immobilized enzymes or did not observe significant differences (Su et al., 2010; Agrawal et al., 2015, Sui et al., 2019). Sannino et al. (2020) found that the apparent K_M was about half of that of the free enzyme and attributed it to a creation of a very favorable micro-environment in the interior of nanoparticles. The decrease in the maximum reaction rate may be observed and attributed to reduction of active sites and limitation of the enzyme flexibility (Sui et al., 2019). On the other hand, Liang et al. (2012) and Çelik et al. (2016) reported an increase in V_{max} upon immobilization, which might also be due to enhanced rigidity of the covalently bound enzyme. In these cases, the immobilization prevents conformational changes increasing the stability of the enzyme. Summarizing, different behaviors can be obtained because many factors are involved.

7. Applications of immobilized β-glucosidase

As discussed in section 3, free-β-glucosidases have general applications in different process, however, enzyme immobilization on support materials not only increases the catalytic efficacy of the enzyme but also offers other advantages. In this section, we review and discuss the diversity of immobilized β-glucosidase used for biotechnological applications.

Enzymes can be immobilized according to many principles and on many supports. β-glucosidase has already been immobilized by all the principles mentioned, but the choice of the support as well as the origin of the enzyme may vary a lot, generating infinite possibilities. In most cases, the resulting biocatalyst presents at least one advantage compared to free enzyme,

such as temperature and pH optimization, thermostability, reusability, higher shelf-life and gain of activity.

According to Vaz et al. (2016), one of the first report of β-glucosidase immobilization was conducted by Chakrabarti and Storey in 1989. From there to here, several studies describing β-glucosidase immobilization are found in the literature. In this section, some examples of the work performed over the more recent years and the most important resulting applications from them will be presented.

In the first work covered here, β-glucosidase was immobilized by adsorption on beads prepared from chitosan mixed with activated clay and subjected to crosslinking with glutaraldehyde (Chang et al., 2007). The addition of activated clay was intended to make the support more rigid and insoluble, facilitating its separation from the product solutions. The authors investigated the effect of enzyme loading, immobilization time, glutaraldehyde amount and crosslinking time to optimize the process. As expected, immobilized enzyme had a broader tolerance range to pH and heat than free enzyme, which is explained by the decrease in conformational flexibility of the enzyme when immobilized. The storage stability was also superior for immobilized enzyme, which maintained an activity at least 5 times higher than that of free enzyme after 60 days of storage.

An example of β-glucosidase immobilized by physical adsorption was conducted by Da Silva et al. (2014), where the purified enzyme obtained from *Aspergillus japonicus* were adsorbed by two different anionic exchanger support (MANAE-agarose and DEAE-cellulose). Both procedures presented good operational stability: 50 and 60% of residual activity, respectively, after 5 cycles of enzymatic activity determination with synthetic substrate. Furthermore, MANAE-agarose and DEAE-cellulose preparations were 75 and 120 times more stable then free enzyme by measuring the activity in repeated hydrolysis experiments. The enzyme was also immobilized by entrapment with sodium alginate beads and covalent binding using CNBr-agarose. According to the authors, 100% of the enzyme was immobilized in all cases.

Another method that involves adsorption is the immobilized metal affinity (IMA), where enzyme functional groups such as amino acids and imidazole groups attach to the support by affinity to a specific metal ion. In the study conducted by Chen et al. (2014), magnetic (Fe_3O_4) nanoparticles were synthesized, coupled with agarose and further chelated with metal ions ($Cu^{+2,}$ Mn^{+2}, Mg^{+2}, Co^{+2} or Ni^{+2}). β-glucosidase was then immobilized and the carrier containing Co^{+2} presented the best results of activity recovery (117%), which means that this

ion may still be involved in the enzyme activation. Compared to free enzyme, the immobilized form showed activity in a broader pH range and enhanced thermostability. The immobilized enzyme also exhibited high operational stability, once 90% of the initial activity were retained after 15 hydrolysis runs utilizing synthetic substrate.

Sannino et al. (2020) studied the development of an immobilization protocol through covalent binding of β-glucosidase into silicate porous nanoparticles. The novelty of the work is that the immobilization process was performed in anhydrous acetone, an organic solvent in which the enzyme is insoluble so to restrict its conformational freedom and thus preserve its native structure (Sannino et al., 2020). Thermal stability of the immobilized system was higher than free enzyme. The biocatalyst also showed operational stability as it could be used in six consecutive cycles of cellobiose hydrolysis.

The examples above tested the biocatalyst activity only on synthetic substrates. Although the results were interesting and relevant, the direct application of biocatalysts in more real and complex systems is essential to demonstrate the potential within an industry. Table 2, located at the end of this section, shows the studies already developed and divided by application.

7.1. Application in degradation of cellulosic biomass

The main application of immobilized β-glucosidase, extensively reported in the literature, is in the degradation of cellulosic biomass.

Zheng et al. (2013) prepared magnetic (Fe_3O_4) chitosan microspheres for β-glucosidase immobilization and study its potential use in biomass hydrolysis. First, the concentrations of the reagents of the microspheres was optimized, as well as the ratio of the magnetic material Fe_3O_4, and then the parameters of immobilization such as activity yield (83.2%) and efficiency (90.2%) were determined. The application of free and immobilized β-glucosidases supplementing cellulases in the hydrolysis of corn straw were studied and the results showed no difference in the rates of hydrolysis during the initial 5 h. However, the addition of β-glucosidase (both forms) increased the final rate and consequently the level of reducing sugar produced. The authors observed that the granular form of immobilized enzyme could cause less homogeneous distribution than that of free enzyme, leading to a slightly lower yield. Reuse of the immobilized β-glucosidase in batch hydrolysis were studied and the conversion rate remained greater than 76.5% after eight rounds.

Khan et al. (2012) went further e resorted, in addition to immobilization, to gene expression of β-glucosidase from *Thermotoga neapolitana* in *Eschericia coli* to obtain thermostable enzymes. The enzymes (wild and cloned) were covalently binding to three acrylic supports (Eupergit® C, Eupergit® C250L, and polyacrylamide cryogel, the first two was obtained commercially and the third was synthesized). Part of the supports were activated with the glutaraldehyde space arm. Cryogels showed low values of immobilization efficiency, although the addition of glutaraldehyde had a significant effect using this support, and the best results were obtained using Eupergit® C250L. The β-glucosidase immobilized without glutaraldehyde was recycled 3 times while the enzyme immobilized with glutaraldehyde could be used 10 times. Regarding storage, the biocatalysts were stored for three or six months and then reused. The authors also verified by thermogravimetric analysis that the support materials are stable for systems operating at very high temperatures. The application was in the hydrolysis of yellow onion extract to produce quercetin, an antioxidant compound.

In the study accomplished by Tsai and Meyer (2014), β-glucosidase was cross-linked using glutaraldehyde and then entrapped in calcium alginate particles. An interesting result obtained is that BSA was added to glutaraldehyde in order to provide groups for crosslinking and thus prevent β-glucosidase from having its active site blocked, however this did not affect the activity of the immobilized enzyme. After determining the optimal conditions, the residual activity was more than 60%. Operational stability was evaluated using a cellulose-derived substrate with the addition of commercial cellulases and the immobilized β-glucosidase was stable up to 20 rounds of 48 h each. Then, the performance of the biocatalyst was tested in the hydrolysis of pre-treated barley straw and both forms of the enzyme efficiently hydrolyzed the substrate.

Borges et al. (2014) immobilized β-glucosidase by ionic adsorption on polyacrylic resin activated by carboxyl groups and by covalently attached to glyoxyl-agarose. It is worth mentioning that the enzymes were modified by inserting amino groups to increase the availability of groups involved in the attachment and thus improve the values of immobilization yields, which had been low. Another interesting alternative was the use of glucose, an inhibitor, during the immobilization process to avoid the formation of covalent bonds that can distort the active sites of the enzyme and consequently decrease the activity. Although the recovery activities of the enzyme immobilized by covalent bonding was low (from 5.1 to 18.2), the recovery when the adsorption technique was used reached 97%. The authors observed a considerable increase in thermal stability of the enzymes covalently immobilized but, on the

other hand, thermostability of the enzyme immobilized on polyacrylic resins was lower compared to free enzyme, since significant changes in the enzyme during adsorption process is not common due to the weakness interactions. The efficiency of both biocatalysts were evaluated firstly in cellobiose hydrolysis and then in the hydrolysis of pretreated sugar cane bagasse containing soluble cellulase. In general, the results were satisfactory and the enzyme immobilized on the resin was even more efficient, reaching almost 100% conversion of the bagasse in 96 of reaction and maintaining stability in up to three operational cycles.

Agrawal et al. (2015) studied the covalent immobilization of β-glucosidase from a mutant strain of *Bacillus subtitlis* on SiO_2 nanoparticles aiming to improve enzyme thermostability. The authors observed higher storage stability for the biocatalyst, which was reusable for up to 10 cycles with 70% residual activity. In addition, the application of the immobilized β-glucosidase in sugarcane treatment allowed an increase in the phenolic compounds content, which shows an eco-friendly way to increase the extractability of phenolic compounds.

Tan et al. (2015) prepared κ-carrageenan beads to immobilize β-glucosidase via covalent bonds using polyelectrolytes followed by glutaraldehyde to bind the enzyme. The immobilization parameters involving activation time, polyelectrolyte concentration and pH of the solution were first optimized and the immobilization yield achieved 98.4%. The authors observed that immobilized β-glucosidase can tolerate a broader range of pH and higher reaction temperature in relation to free enzyme. Recycling was determined and no sharp drop in activity was observed, the enzyme maintained 84.9% of the initial activity after 10 operation cycles and 75.6% after the 12^{th} cycle. The final test was the application of either free or immobilized β-glucosidase along with commercial cellulase in the enzymatic hydrolysis of pretreated microalgae cellulosic residue. Without supplementation, the yield of hydrolysis catalyzed by cellulase was 62.8%. The addition of β-glucosidase elevated the yield value to 79.4% and 71% using free and immobilized, respectively. The fact that the yield was slightly lower using immobilized enzyme may be irrelevant when considering the advantages of its use, some of them already mentioned in the study.

The purification of the enzymes prior to immobilization is a complicated process that increases cost and limits industrial applications of the enzymes once these process can also decrease stability. Liang et al. (2012) synthesized a novel polyacrylate copolymer to immobilize cellulase by covalent bindings. The maximum activity yields of the biocatalyst reached 63% after the optimization of the conditions such as the copolymer concentration and pH, time of

reaction, amount of crosslinking reagent and amount of protein. The immobilized cellulase showed higher storage and thermal stabilities. After five cycles of hydrolysis of corn stover, the immobilized cellulase retained 83% of the initial activity.

Cherian et al. (2015) studied the immobilization of cellulases from *Aspergillus fumigatus* in magnetic nanoparticles. The enzymes were produced by submerged fermentation using jackfruit waste as substrate and then covalently immobilized onto the MnO_2 nanoparticles using glutaraldehyde as binding agent, with maximum immobilized yield obtained equal 75%. The authors noticed an increase in optimum temperature range for immobilized cellulases, which were even more stable as well. To complete, the study used agricultural waste such as sugar cane leaves to produce reducing sugar and bioethanol. The contents were higher using immobilized enzyme (24 g/L and 21.96 g/L, respectively) against free enzyme (20 g/L and 18 g/L, respectively), showing again that cellulase immobilization improved enzymatic activity.

Nishida et al. (2018) also conducted a study immobilizing β-glucosidase without previous clarification of the extract. The enzyme was produced by *Aspergillus awamori* in solid-state fermentation using pineapple crown leaves (80%) and wheat bran (20%) as substrates. The crude enzyme was immobilized via glutaraldehyde cross-linked using commercial gelatin with 99.8% yield and presenting 83% of residual activity after ten cycles, making the process very attractive in terms of efficiency and economy, since gelatin is abundant and cheap and a few upstream steps were conducted. Also, the immobilized form of enzyme was more thermostable. The authors applied free and immobilized β-glucosidase in the hydrolysis of cellobiose for 12 h and obtained 70% and 100% of hydrolysis, respectively. After that, both enzymes were added to *Tricoderma reesei* cellulases and applied in cellulose hydrolysis and similar result were obtained, the immobilized β-glucosidase were more efficiency in releasing glucose. According to many authors, higher efficiency of immobilized β-glucosidase is probably due to lower inhibition by free glucose and higher thermostability (Verma et al., 2013; Cherian et al., 2015; Tan et a., 2015; Nishida et al., 2018).

More recently, Sui et al. (2019) reported a simple method for covalent immobilization of commercial cellulase on polyurea microspheres via the glutaraldehyde method. The optimization of the immobilizations conditions was conducted through the study of the glutaraldehyde concentration, activation time, enzyme concentration and immobilizing time. Compared to free enzyme, immobilized enzymes presented greater pH tolerance and higher temperature stability over 50 °C and showed residual activity 3.5 higher during stability test. The biocatalyst maintained 75% of its initial activity after eight cycles of CMC hydrolysis

operation and, during the continuous process, presented constant release of reducing sugar for 36 h, which may be attributed to multipoint covalent immobilization that improves the stability and increases the possibility of using immobilized enzymes in industrial applications (Sui et al., 2019)

Zhou et al. (2019) developed an enhanced ionic liquid-tolerant immobilized cellulase in hydrogel microspheres and then applied it in biomass saccharification. The commercial enzymes were immobilized in poly(N-isopropylacrylamide), PNIPAM), with maximum immobilization efficiency of 95.6%, a significantly high value. Ionic liquids act in the disorganization of the chemical structure of cellulose, reconstructing it in a more hydrolysable form (Samayam et al., 2011), thus being very useful in the treatment of biomass. In general, the lower the ionic liquid concentration, the higher the enzyme activity. The authors showed that immobilized cellulase presented superior performance than free enzyme, retaining more activity during the hydrolysis of both cellulose microcrystalline and bagasse, and so presenting a better tolerance to ionic liquid.

In addition to reducing the number of steps by not performing purification, the synergistic action of cellulase must also be considered. Carli et al. (2019) studied the synergistic effect of β-glucosidase from *Humicola insolens* and endoglucanase from *Scytalidium thermophilum* immobilized in ferromagnetic nanoparticles. Both proteins were recombinant. When the enzymes were immobilized individually, the immobilization yields, in terms of protein, were approximately 20%. However, the co-immobilization of the enzymes had 49% yield. When applied in the hydrolysis of CMC, β-glucan and pretreated fractions of sugarcane, the enzymes exhibit a significant synergistic effect in both arrangements, when combined individually and when applied in a co-immobilized form.

7.2. Application in beverages

Most of terpenes in wines are conjugated to various sugar, representing a significant reservoir of aromatic precursor, and the release of these terpenes can be catalyzed by enzymes such as β-glucosidase (Ferner et al., 2018). Spagna et al. (2002) studied the application of a mixture of immobilized glycosidases from *Aspergillus niger* in oenological applications. Three purified enzymes (β-glucosidase, α-arabinofuranosidase and α-rhamnopyranosidase) were immobilized by inclusion using chitosan gels and subsequent cross-linking with glutaraldehyde. First, the effect of the presence of additives (lysine, agar, arabic gum, polyvinyl alcohol, gelatine and silica gel) on the immobilization protocol were investigated. Silica gel and gelatine

had the best results in terms of low release of the enzymes, immobilization yields and stability, wherein the first was preferred due to better gel consistency. Free and immobilized enzymes were added to a model solution wine containing aromatic precursors extracted from *Moscato* skin grapes and the results showed that both enzymes considerably increased the content of the terpene alcohols linalool, nerol, geraniol and citronellol, the more fragrant volatile compounds found in wine. When testing immobilized enzymes by covalent bonds on a previous work, the same group found unsatisfactory results as a reduction in the enzymatic activity, which they attributed to diffusion problems and reduced accessibility of the substrate to the active site of the enzyme, a recurring limitation in such method. This problem was not observed when immobilizing by inclusion because the enzymes were capable of maintain their freedom (Spagna et al., 2002).

Almost ten years later, González-Pombo et al. (2011) used Eupergit C 250L for covalent immobilization of β-glucosidase isolated from *Issatchenkia terricola* by multi-point attachment. The biocatalyst was incubated with Muscat wine. Comparing the treated wine with the control wine, the analysis showed significant effect on the hydrolysis of glycosides. As the content of monoterpenes and norisoprenoids has increased after incubation, it is expected that the varietal aroma of the wine will also increase. Beyond that, the immobilization has notorious improved the pH stability and increased the time of storage in model wine solution.

Ferner et al. (2016) and (2018) also used the immobilized enzymes aiming potential application in wine aromatization. In the first work, they studied a simple method for immobilizing several glycosidases from *Aspergillus niger* (β-glucosidase, α-arabinofuranosidase, α-rhamnopyranosidase and β-xylopyranosidase) on commercial magnetic microparticles using carbodiimine, which can form amine bonds between amine groups of the enzymes and carboxyl groups of the particles surface. Based on the results, the enzymes were successfully immobilized and β-glucosidase had the highest affinity for the magnetic microparticles. Then, free and immobilized enzymes were characterized in terms of β-glucosidase activity and no significant differences were detected. The stability of the biocatalyst was investigated in model wine environment for 70 days and β-glucosidase presented the highest loss in activity (64.1%), which the author attributed to the considerable amount of tartaric acid content. On the other hand, the activity of α-rhamnopyranosidase slightly increased (8.6%).

In a subsequent work, Ferner et al. (2018) used the immobilized biocatalyst in the treatment of model wine with different glycosides and in white wine and analyzed the volatile

compounds. Contrary to what happened in the model wine, the stability of immobilized β-glucosidase in wine decreased only 10% in 70 days, and the authors verified that the reduction was due to inactivation of enzyme and not due to loss of enzyme since no release of bound enzyme was observed. The magnetic separation efficiency was almost 100% and the biocatalyst was used six time without a loss of activity. The addition of both free and immobilized enzymes in model wine resulted in increased amount of total terpenes, although the release promoted by immobilized enzymes was 45% lower. In the white wine treatment, the increase of total terpenes was also relevant, but the difference between the enzyme forms were insignificant, evidencing the efficiency of the biocatalyst. The enzyme-treated wine and the untreated wine were compared by sensory analysis and the results showed higher odour intensity due to the aroma release.

Su et al. (2010) investigated the immobilization of β-glucosidase on alginate aiming to increase the aroma of tea beverages. The authors chose two types of carrier and tested four immobilization methods in each one, as follows: using sodium alginate, it was tested direct entrapment, entrapment-crosslinking, crosslinking-entrapment and crosslinking-entrapment-crosslinking, and using chitosan they studied direct adsorption, adsorption-crosslinking, crosslinking-adsorption, crosslinking-adsorption-crosslinking. Among the options, the results with alginate were better and the crosslinking-entrapment-crosslinking was the best immobilizing methods in terms of activity recovery (31.4%). Then, the immobilization methodology was optimized and recovery achieved 46%. Infusion of three types of tea (green, black and oolong) was treated with immobilized β-glucosidase and the total amount of the aroma components present increased in all cases, suggesting that the glycosidic aroma precursors were hydrolyzed by the enzyme, each one with a different rate according to the tea processing technique. The reusability of the immobilized enzyme was evaluated using green tea infusion in a 2 h cycle. The biocatalyst could be used for 50 times and the residual activity was still 93.6%. Besides that, thermal and pH stabilities as well as storage stability increased in relation to free enzyme.

Also wanting the aroma-increasing in beverages, Çelik et al. (2016) characterized the biocatalyst formed by β-glucosidase immobilized on chitosan-multiwalled carbon nanotubes and applied it on tea extracts. The enzyme was covalently bonded to the support by the glutaraldehyde method with 95% of immobilization yield. As expected, the immobilized enzyme showed better storage stability when compared with free enzyme, with 68.4% of residual activity after 50 days at 4 °C, as well as better pH and thermal stabilities. The

biocatalyst showed 72.83% of residual activity after 10 cycles of reuse. In relation to the application on tea beverages, the results indicated that the amount of aromatic components in all tea samples (black tea, green tea, yarbe mate, sage and balm tea) increased, although different hydrolysis degree occurred due to process technique of each tea, same result observed by Su et al. (2010).

Besides aromatization, β-glucosidases can be used in food industries in the clarification process, especially of grape juice and wine. Monteiro et al. (2019) conducted the first work using β-glucosidase from thermophilic fungi aiming at this application. The enzymes were produced from *Malbranchea pulchella* and were immobilized on MANAE-agarose via ionic adsorption and on ConA-Sepharose via affinity adsorption. Both biocatalyst showed optimum temperature and pH similar to free enzyme and less inhibition in the presence of glucose and ethanol. The strong interaction of the enzyme with both supports were proved by the desorption assays, where approximately 50% of the enzyme remained adsorbed on MANAE-agarose even with 3 M of NaCl and, on the other hand, β-glucosidase was not able to desorb from ConA-Sepharose even with 3 M of α-methyl-D-mannopyranoside, In relation to clarification of the beverages, it was observed decrease in the absorbance of all samples, indicating the loss of the reddish purple color and hydrolysis of anthocyanins. The β-glucosidase immobilized on MANAE-agarose showed betters results, clarifying 52% red wine, 71% diluted wine, 77% grape juice and 56% diluted grape juice, against 41%, 46%, 63% and 23% obtained by β-glucosidase on ConA-Sepharose. As a conclusion, the results demonstrated the effectiveness of the use of β-glucosidase immobilized for this purpose (Monteiro et al., 2019).

7.3. Application in isoflavone glycosides

The conversion of soybean isoflavone glycosides into their aglycon forms is another relevant biotechnological application, since the originated compounds are bioavailable because of their unimpeded intestinal adsorption. Although various physical and chemical strategies are available to convert isoflavone glycosides into aglycones, the hydrolysis of isoflavones with β-glucosidase is the most attractive because of the high efficiency and operational safe (Chang et al., 2018). Normally, high concentration of ethanol has to be added into the isoflavone hydrolysis due to the low solubility of isoflavone, which requires that the enzyme to show a certain tolerance.

Chang et al. (2013) studied β-glucosidase immobilization considering this application. The enzyme was immobilized on chitosan-carbon beads cross-linked with glutaraldehyde, and

the optimal conditions in terms of cross-linked time, glutaraldehyde concentration, and then pH and immobilization time were determined. Both crude and purified enzyme (recombinant β-glucosidase from *Exiguobacterium* sp. DAU5 expressed in *Escherichia coli*) were immobilized and although the yield was higher using purified enzyme, β-glucosidase from crude extract was chosen for further studies considering the cost of the process. Immobilized β-glucosidase presented better thermal and pH stability than free enzyme and could maintain 80% residual activity after seven cycles of activity, besides to retain 95% of initial activity after 60 days stored. Next, genistin and daidzin, isoflavone glycosides, were dispersed in an aqueous-organic two phase system and the immobilized β-glucosidase was incubated for different times in the aqueous phase. The presence of genistein and daidzein, hydrolysis products of genistin and daidzin, respectively, were found in the organic phase. The conversion yields for genistein and daidzein after 60 min were 72.4% and 74.6%. The use of a biocatalyst combined with the technology of a two phase system was to ensure that neither the isoflavone dissolved in solution nor the hydrolysis products were not adsorbed by chitosan.

Chen et al. (2013) reported covalent immobilization of β-glucosidase from *Aspergillus niger* on spent coffee grounds and application in black soymilk. After the determination of optimum pH and temperature, the reusability was confirmed for more than 30 operation batches using synthetic substrate. In relation to the hydrolysis of isoflavone, the biocatalyst was capable of increasing the aglycones concentration even 70% after 60 min of incubation with the soymilk.

Grade et al. (2014) also used β-glucosidase immobilized in soy drinks. The enzyme was extracted from soy cotyledon flour and immobilized on chitosan beads. The activation of the support with glutaraldehyde was optimized in terms of the reagent concentration, pH and incubation time, and the highest efficiency was equal to 41.5%. Then, the immobilization process was studied as a function of the protein content, pH and linkage time and it was obtained 73.8% of efficiency. The chitosan beads containing the β-glucosidase immobilized were incubated with commercial soy drink and, after 60 min, the aglycones content increased 24%.

Chang et al. (2018) investigated the purification and immobilization of β-glucosidase immobilized onto cellulose and its application in soybean isoflavone hydrolysis. In order to improve the biochemical properties of the enzyme and also reduce the number of purification steps, β-glucosidases was firstly fused with an affinity tag, such as carbohydrate-binding molecule module (CBM). Six CMB sequences were chosen and fused to the C-terminal of β-glucosidase and the fusion proteins were expressed in *Eschericia coli*. The immobilized

enzymes showed higher stability towards ethanol than free enzyme. The better fusion protein reached 93% of immobilization efficiency and was then used in the hydrolysis of soybean. The hydrolysis rate of daidzin and genistin was 85% and 82%, respectively, with concentrations of daidzein and genistein increased by 6 mM and 4 mM. After four cycles of operation, the concentrations of daidzein and genistein still increased.

In the work conducted by Hu et al. (2018), β-glucosidase gene from *Thermoascus aurantiacus* was recombinantly expressed in *Pichia Pastoris* and immobilized via adsorption on regenerated amorphous cellulose. The immobilized enzyme was less sensitive to environmental variations and after 30 rounds of isoflavone hydrolysis, 96.9% of daidzin and 98.9% of genistin could be hydrolyzed.

Phadungcharoen et al. (2019) described a simple method for β-glucosidase immobilization on chitosan beads aiming at the cleavage of sugar moieties from isoflavone genistin into genistein. The enzymes were crosslinking using both the common reagent glutaraldehyde and genipin. The concentrations of cross linkers, the mixing procedure (containing one or two steps) and the immobilization time were studied. The researches verified that the biocatalyst produced with genipin showed greater stability and broader tolerance to pH and temperature compared to the one that was produced using glutaraldehyde. The reusability was studied using the biocatalyst for repeated batches hydrolysis of the pNPG substrate and the biocatalyst prepared with genipin showed more than 90% of the initial activity after ten rounds of reaction. It also hydrolyzed genistin to genistein and could be reused by exhibiting 85% of the initial substrate conversion after five cycles.

7.4. Application in resveratrol production

Some studies have investigated the production of resveratrol via biotechnological synthesis using β-glucosidase, but the use of immobilized form of the enzyme is still quite limited (Zhang et al., 2014). Resveratrol is a natural phenol, common in red grapes skin, and that showed popularity in traditional herbal medicine. In the study conducted by Zhang and coworkers (2014), the authors investigated the production of resveratrol using immobilized β-glucosidase. The enzyme was immobilized on cross-linked chitosan microspheres modified by L-lysine. After the optimization of the immobilization procedure, the authors applied the biocatalyst on the hydrolysis of polydatin to produce resveratrol and obtained 85.8% of hydrolysis and 92.9% of enzymatic activity after 8 h of continuous reaction.

8. Conclusion and future prospects

β-glucosidase catalyzes a myriad of hydrolysis of β-(1,4) glycosidic bonds between glucose terminals, sugars and various aglycones molecules. Due to this range of action, β-glucosidase has been widely used in various biotechnological processes and its immobilization has become one of the most important biocatalytic systems used for many industrial purposes. In comparison with soluble enzymes, immobilized enzyme shows important advantages such as the possibility of reusing and facility of separation and interruption of the process. Besides that, the immobilized form tends to be robust and more tolerant to changes in the reaction environment including temperature, pH and organic solvents influences. However, depending on the biocatalyst stability, the immobilization process can be even more costly. Furthermore, despite the promising results of application of β-glucosidase immobilized, most of them have been tested only at laboratory scale. That is why the number of cited applications for immobilized enzymes is substantial, but only a limited number are deployed in marketplace, which requires the screening of new supports and the use of methods and enzymes sources that are most suitable for an industrial application. Thereby, the development of an efficient protocol for β-glicosidase immobilization to be applied mainly in the hydrolysis of complex substrates, such as lignocellulosic materials from agroindustrial wastes has become a necessary goal (Vaz et al., 2016).

Table 2. Summary of the approaches of β-glucosidase immobilization with the corresponding application.

Enzyme	Support	Technique	Application	Reference
Mixture of purified glycosidases from *Aspergillus niger*	Chitosan	Inclusion and crosslinking	Oenological applications	Spagna et al. (2002)
Commercial β-glucosidase *from Aspergillus niger* (Novozymes)	Eupergit C	Covalent	Lignocellulosic hydrolysis	Tu et al. (2006)
Commercial β-glucosidase (Novozymes)	Silica gel	Covalent	Enzymatic conversion of biomass	Karagulyan et al. (2008)
Commercial β-glucosidase (Novozymes)	Kaolin carriers	Adsorption		Karagulyan et al. (2008)
Commercial β-glucosidase (Fluka)	Alginate	Crosslinking- entrapment-crosslinking	Tea aromatization	Su et al. (2010)
Mixture of commercial cellulases	Agarose matrix functionalized with distinct reactive	Ionic adsorption	Bioconversion of lignocellulosic biomass	Vieira et al. (2011)
Mixture of cellulases	Magnetic silicia nanoparticles	Covalent	Cellulose hydrolysis	Cho et al. (2012)
β-glucosidases from *Thermotoga neapolitana* expressed in *Escherichia coli*	Eupergit Polyacrylamide cryogel	Covalent	Production of quecertin	Khan et al. (2012)
Commercial β-glucosidase from *Aspergillus niger* (Novozymes)	Silica gel	Covalent	Cellulose hydrolysis	Jung et al. (2012)

Table 2 Continuation

Enzyme	Support	Technique	Application	Reference
Commercial Cellulase (Novozymes)	Polyacrylate copolymer	Covalent	Cellulose hydrolysis	Liang et al. (2012)
Recombinant β-glucosidase from *Exiguobacterium* sp.	Chitosan carbon	Crosslinking	Hydrolysis of isoflavone	Chang et al. (2013)
β-glucosidase from *Aspergillus niger*	Coffee grounds	Covalent	Application in soymilk	Chen et al. (2013)
Comercial β-glucosidase from *Aspergillus niger* (Sigma)	Magnetic nanoparticles	Covalent	Biofuel production	Verma et al. (2013)
Commercial β-glucosidase (Novozymes)	Fe_3O_4 chitosan microspheres	Entrapment	Cellulose hydrolysis	Zheng et al. (2013)
Commercial β-glucosidase from *Trichoderma reesei* (Genencor)	Polyacrylic resin	Adsorption	Cellulose hydrolysis	Borges et al. (2014)
Commercial β-glucosidase from *Trichoderma reesei* (Genencor)	Glyoxyl agarose	Covalent	Cellulose hydrolysis	Borges et al. (2014)
Commercial β-glucosidase from almonds (Nuohui)	Metal ios chelated Fe_3O_4 nanoparticles coupled with agarose	Adsorption	Hydrolysis of isoflavone	Chen et al. (2014)
β-glucosidase from *Aspergillus fumigatus*	Alginate beads	Entrapment	Bioethanol production	Das et al. (2014)

Table 2 Continuation

Enzyme	Support	Technique	Application	Reference
β-glucosidase extracted from soy cotyledon flour	Chitosan beads	Covalent	Hydrolysis of isoflavone	Grade et al. (2014)
Commercial β-glucosidase *from Aspergillus niger* (Novozymes)	Calcium alginate	Crosslinking and entrapment	Cellulose hydrolysis	Tsai and Meyer et al. (2014)
Commercial β-glucosidase from almonds (Sigma)	Chitosan microspheres	Crosslinking	Production of resveratrol	Zhang et al. (2014)
β-glucosidase from *Bacillus subtilis* mutant	SiO_2 nanoparticles	Covalent	Cellulose hydrolysis	Agrawal et al. (2015)
Commercial β-glucosidase *from Aspergillus niger* (Novozymes)	κ-carrageenan hybrid matrix	Covalent	Production of reducing sugar from macroalgae cellulosic residue	Tan and Lee (2015)
Mixture of commercial glycosidases	Magnetic beads	Covalent	Oenological applications	Ferner et al. (2016) and (2018)
Commercial β-glucosidase from almonds (Aldrich)	Chitosan multiwalled carbon nanotubes	Covalent	Tea aromatization	Çelik et at. (2016)
β-glucosidases expressed in *Escherichia coli*	Cellulose	Affinity adsorption	Hydrolysis of isoflavone	Chang et al. (2018)
Commercial β-glucosidase (Novozymes)	Hydroxyapatite	Adsorption	Hydrolysis of cellobiose	Coutinho et al. (2018)
β-glucosidases expressed in *Pichia pastoris*	Amorphous cellulose	Adsorption	Hydrolysis of isoflavone	Hu et al. (2018)
β-glucosidase from *Aspergillus awamori*	Gelatina	Crosslinking	Celulose hydrolysis	Nishida et al. (2018)

Table 2 Continuation

Enzyme	Support	Technique	Application	Reference
β-glucosidase from *Malbranchea pulchella*	MANAE-agarose	Ionic adsorption	Clarification of beverages	Monteiro et al. (2019)
β-glucosidase from *Malbranchea pulchella*	ConA-Sepharose	Affinity adsorption	Clarification of beverages	Monteiro et al. (2019)
Commercial β-glucosidase from almonds (Sigma)	Chitosan beads	Crosslinking	Hydrolysis of isoflavone	Phadungcharoen et al. (2019)
Commercial cellulase from *Trichodema viride* (Sinopharm)	Hydrogel microspheres	Encapsulation	Saccharification of biomass	Zhou et al. (2019)
Commercial β-glucosidase from almonds (Sigma)	Synthesized tannic acid template mesoporous silicia nanoparticles	Adsorption	Bioconversion of lignocellulosic biomass	Venezia et al. (2020)

CAPÍTULO II

Production and capture of β-glucosidase from *Thermoascus aurantiacus* using a tailor made anionic cryogel

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CAPÍTULO II

Production and capture of β-glucosidase from *Thermoascus aurantiacus* using a tailor made anionic cryogel

Abstract:

In this study, an anion exchange cryogel was prepared and characterized using morphological, hydrodynamic and thermal techniques and it was used to capture β-glucosidase from *Thermoascus aurantiacus*. The enzyme was produced by solid state fermentation. The effect of the stirring time (15, 30 and 60 min) on the β-glucosidase extraction was assessed and was not significant ($p>0.05$). The adsorption behavior of the β-glucosidase produced was studied as a function of pH (3.0, 5.0 and 7.0), and the results of yield (%) and purification factor were calculated and submitted to ANOVA at a significance level of 95%. The efficiency was considerably affected ($p<0.05$) by the pH and the best result was achieved at pH 5.0 (82%). Purification factors did not vary and the results were low since isocratic elution was performed (1.25 to 1.33). SDS-PAGE was also realized to investigate purity. The point of zero charge of the adsorbent was found at pH 8.0. The evaluation of functional groups confirmed the incorporation of the ion exchanger onto the cryogel surface. Thermal degradation of the cryogel started at 220 °C. These results indicated that the cryogel exhibited good properties to be used as a chromatographic media to purify biomolecules such as β-glucosidase.

Keywords: β-glucosidase, adsorption, cryogel, DMAEMA, ion-exchange

1. Introduction

β-glucosidase is an important glycoside hydrolase, which selectively catalyzes the hydrolysis of β-glycosidic linkages in disaccharides and oligosaccharides. It is commonly found in bacteria, fungi, plants and animals. β-glucosidases are members of the cellulase system and together with cellobiohydrolase and endo-β-glucanase play important roles in lignocellulosic biomass degradation to produce ethanol (Borges et al., 2014; Watanabe et al., 2016, Xia et al., 2018). β-glucosidase can hydrolase soy isoflavones, producing aglycones, compounds that are bioactive (Chang et al., 2013; Singhania et al., 2013). These enzymes have been used in many biotechnological applications, including the production of aromatic compounds in wine and other beverages (González-Pombo et al., 2011; Zhou et al., 2017). In this industry, they are capable of degrading anthocyanins, producing free sugar and aglycone anthocyanidins, which

are less soluble than anthocyanins, being easily removed during filtration (Palma-Fernandez et al., 2002) Due to their various important roles, they are considered to be biologically and industrially important enzymes (Watanabe et al., 2016).

Many studies have looked for more efficient and thermostable β-glucosidases for industrial applications (Leite et al., 2007; Borges et al., 2014; Watanabe et al., 2016). Most of the industrial processes occur at high temperatures, especially due to high reaction rates, decreased viscosity in fluid processes, increased solubility of the substrate and reduced contamination risk by undesired organisms. Hence, the use of thermostable enzymes is convenient as they maintain their catalytic activity (Bruins et al., 2001; Leite et al., 2007; Pereira et al., 2015).

Various thermophilic fungi have been reported to produce hydrolases characterized by valuable properties such as superior thermal stability and optimum activity at elevated temperatures (Dave et al., 2013). *Thermoascus aurantiacus* is a thermophilic fungus, which grows efficiently on lignocellulosic biomass from agricultural wastes (Kalogeris et al., 2003; Da Silva et al., 2005; Leite et al., 2008). These substrates are attractive since they are readily available at low cost.

Purification of enzymes presents one of the major challenges in order to characterize its structure and physical-chemical properties with a view to its biotechnological application. Chromatographic processes are far and away the most common technique when products with high purity and high yield are desired. Ion exchange chromatography (IEC) is the most widely used technique in industrial bioprocessing. In IEC, the pH of the samples is one of the most important parameters in determining protein binding, as it determines the change in both the protein and the ion exchanger (Janson e Rydén, 2011; Lenhoff, 2016).

The choice of the material to be used as carrier is also an important factor in separation efficiency. Among the materials available, a supermacroporous monolithic cryogel is an interesting alternative (Lozinsky et al., 2003; Bakhshpour et al., 2019)

Cryogels are polymeric gels produced under freezing conditions and were first documented about 50 years ago (Lozinsky et al., 2003). During defrosting, ice or solvent crystals leave empty spaces of pores between the polymeric chains, forming a network of interconnected macropores, which allow the unobstructed passage of solutes. Pore sizes up to 200 μm may be found (Petrenko et al., 2011; Jain et al., 2017). The properties such as high porosity and low flow resistance allow direct processing of crude extracts without prior clarification, optimizing time, saving water and reducing others expenses. In addition, reducing

the number of steps in the process helps maintain the integrity of some compounds. Due to these features, the development of cryogels for use in purifying biomolecules is growing. They are also used in various fields such as drug delivery systems, dentistry, pharmaceuticals, agriculture (Jain et al., 2017), chromatography (Yun et al., 2007; Carvalho et al., 2014; Mól et al., 2017, immobilization (Arvidsson et al., 2002; Veríssimo et al., 2018) and tissue engineering (Bakhshpour et al., 2019).

The monomer composition and the variation in the cryogelation process can result in cryogels with slightly different properties. The adequate characterization of these matrices is advisable in the studies aiming a better understanding of flow inside its structure and also the interactions between the functional groups of the matrix and the target molecules (Fontan et al., 2018). The characterization includes the determination of physical, hydrodynamical, chemical and thermal properties, which allows predicting the behavior of the chromatographic material during its storage and under operational conditions (Mól et al., 2017; Fontan et al., 2018).

These adsorbents are usually chemically modified by the incorporation of functional groups on its surface, in order to increase their adsorptive capacity and/or their selectivity towards a compound of interest. Thus, cryogels can be used in capture or purification of molecules by exploiting various types of interactions, such as affinity (Mól et al., 2017; Gonçalves et al., 2017) ion exchange (Arvidsson et al., 2002; Fontan et al., 2018; Nascimento et al., 2019) and hydrophobic interaction (Türkmen and Denizli, 2014), among others, just by the chemical binding of a suitable ligand.

Thus, this work reports the preparation of a polyacrylamide cryogel grafted with 2-(dimethylamino)ethyl methacrylate (DMAEMA), aiming to investigate the capture of β-glucosidase produced by solid fermentation of the fungus *Thermoascus aurantiacus*. The effect of the stirring time on the β-glucosidase extraction was assessed. The adsorbent was also characterized in terms of its morphological and hydrodynamic properties and then the adsorption study was conducted.

2. Material and methods

2.1. Materials

Acrylamide (AAm, 99%), N,N'-methylene-bis-acrylamide (MAAm, 99%), ammonium persulfate (APS), N,N,N',N'-tetramethyl-ethylene-diamine (TEMED, 99%), allyl glycidyl ether (AGE, 99%) and 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98%) were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade

and Milli-Q System deionized water was used throughout the experiments (MilliporeSigma, Burlington, USA).

2.2. Cryogel preparation

The cryogel was prepared as described by Mól et al. (2017), except for the addition of 0.25 mL of AGE instead of 1.0 mL. The monomers of AAm and MBAAm (6% m/v) and AGE (0.01% v/v) were dissolved in deionized water and the mixture was degassed. The polymerization reaction was started with TEMED and APS. The mixture was immediately poured into a Tricorn glass column 10/50 (GE Healthcare, Uppsala, Sweden). The column was sealed and immersed in an ethanol bath at -12 °C for 24h. Afterwards, the frozen cryogel was thawed at refrigeration temperature, washed with 200 mL of deionized water and dried for 24 h at 60 °C.

The dried cryogel was reinserted in the glass column for grafting polymerization of DMAEMA, which was performed as described by Savina et al. (2005) with modifications. First, solutions of potassium diperiodate 0.0562 mol/L (Zhang et al., 1999) and NaOH 1.0 mol/L were prepared, mixed in the ratio 2:1 and filtered. Then, the solution was recirculated through the cryogel using a peristaltic pump at 0.25 mL/min for 1.5 h at 46 °C. Next, a solution of DMAEMA 1.5 mol/L was pumped until the removal of the previous solution. The ends of the column were closed and the cryogel matrix was immersed in the DMAEMA solution for 3 h at 46 °C. The column was then washed with 200 mL of HCl 0.1 mol/L at a flow rate of 1.0 mL/min and then with 200 mL of distilled water to remove any residual monomers.

2.3. Characterization of the ion-exchange cryogel

2.3.1. Swelling capacity and expansion degree: The swelling capacity of the cryogel (g water/g dried cryogel) was determined by immersing dried cryogels samples (m_d) in 50 mL of distilled water. After 24 h, the excess of water was gently removed from the cryogel and the wet weight (m_w) was measured. The swelling capacity was determined using Eq. (1) (Savina et al., 2005).

$$S = \frac{m_w - m_d}{m_d} \quad (1)$$

The degree of expansion (mL water/g dried cryogel) was calculated according to Eq. (2). The same samples used above, after being saturated with water, were transferred to a

graduate cylinder containing water (V_1) and the final volume was determined (V_2) (Fontan et al., 2017).

$$ED = \frac{V_2 - V_1}{m_d} \quad (2)$$

2.3.2. Scanning electron microscopy: Macroporous structure and pore sizes of the cryogel were evaluated by scanning electron microscopy (SEM). A small sample cut from the center of the dried cryogel was coated with a gold layer and the gel structure was examined using an LEO EVO 40 XVP scanning electron microscope (Zeiss, Oberkochen, Germany).

2.3.3. Ionic capacity (Λ): Ionic capacity expresses the maximum capacity of the adsorbent in exchanging monovalent ions. It was determined by a titrimetric method (Fontan et al., 2017). 50 mg of cryogel were weighed and put into assay tubes containing 45 mL of HCl 1.0 mol/L. These systems were kept under orbital agitation (25 rpm for 12 h) at room temperature. Then, the solution was filtered and the samples were submitted to a triple washing with 45 mL of ultrapure water followed by 3 h of agitation. The samples were dried at 60 °C for 24 h, transferred to new tubes containing 45 mL of NaCl 2.0 mol/L and left under agitation (25 rpm for 12 h). Then, an aliquot of 40 mL of the solution was collect from each tube and titrated with a standardized HCl 0.01 mol/L (for anionic capacity) or NaOH 0.01 mol/L (for cationic capacity). The NaCl solution was also titled for control.

The anionic capacity was determined in terms of mass of dried cryogel (Λ_m), expressed in mol/kg, utilizing Eq. 3:

$$\Lambda_m = \frac{V_T}{m}\left[\left(\frac{M_{HCl} f V_{HCl}}{V}\right)_{sample} - \left(\frac{M_{HCl} f V_{HCl}}{V}\right)_{NaCl}\right] \quad (3)$$

where: V_T is the volume of HCl added to the tubes (L), m is the mass of dried cryogel added to the tubes (kg), M_{HCl} is the concentration of the HCl solution used for titration (mol/L), f is the correction factor of the HCl solution, V_{HCl} is the volume of the HCl solution used for titration (L) e V is the volume of the solution titrated with HCl. The parentheses of the notation "sample" indicates the saline solution after the contact with the activated carbon and the parentheses with the notation "NaCl" indicate the saline solution before the contact with the adsorbent.

2.3.4. Fourier transform infrared spectrophotometry: The functional groups of the cryogel surface were analyzed using Fourier transform infrared spectrophotometry (FTIR) using a

Vertex 70 FTIR spectrophotometer (Bruker, Billerica, USA). Direct readings were performed by attenuated total reflectance (ATR) in the infrared region of 4000 to 500 cm^{-1}

2.3.5. Thermal analysis: Thermal properties of the cryogel were investigated using a TA 60 differential scanning calorimeter (DSC) (Shimadzu, Kyoto, Japan). The samples (5 mg) were heated from 25 to 215 °C in a nitrogen (N_2) atmosphere at a heating rate of 10 °C/min. Thermogravimetric analysis (TGA) was performed on a DTG 60/60 thermal analyzer (Shimadzu, Kyoto, Japan). Samples of cryogel (10.0 mg) were heated from 35 ºC to 750 ºC, under a dynamic nitrogen atmosphere, with a heating rate of 10 °C/min.

2.3.6. Point of zero charge: The point of zero charge (pH_{pzc}) was determined based on the "experiment of 11 points" (Vieira et al., 2010). 0.03 g of dried adsorbent were added to centrifuge tubes containing 20 mL of aqueous solution at pH varying from 1.0 to 12.0 and were kept under mild agitation for 24 h. Then, the final pH of each solution was measured and the point of zero charge was identified in the experimental condition without pH variation.

2.3.7. Porosity and axial dispersion: The residence time distribution (RTD) was determined according to the method described by Yao et al. (2006a, 2006b) with modifications. The RTD was measured on a Äkta Purifier preparative chromatographic system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using a Tricorn 10/50 glass column (GE Healthcare, Uppsala, Sweden) at various fluid velocities (0.6 cm/min to 10.2 cm/min) at 25 °C, using acetone pulse (100 µL, 10% v/v) as a tracer. A UV detector monitored the corresponding peak (280 nm) at the column exit. Assays were conducted in duplicate.

The mean residence time (t_R) and variance (σ_t^2) were obtained from the RTD curves by the momentum method. Then, porosity (ε_T) was determined by linear regression of Eq. (3) (Furusawa et al., 1976):

$$t_R = \varepsilon_T . \frac{L}{U} \qquad (3)$$

where L (m) is the column length and U (m/s) is the superficial fluid velocity.

Axial dispersion coefficients at various flow velocities were determined by non-linear regression of Eq. (4) using the residence time and the variance of the RTD curves under closed-vessel boundary conditions (Levenspiel 1999).

$$\frac{\sigma_t^2}{t_R^2} = 2\left(\frac{D_{ax}}{uL}\right) - 2\left(\frac{D_{ax}}{uL}\right)^2\left[1 - exp\left(-\frac{uL}{D_{ax}}\right)\right] \quad (4)$$

where D_{ax} is the axial dispersion coefficient and u is the interstitial fluid velocity through the column ($u = U/\varepsilon_T$). Height equivalent to theoretical plate (HETP) (Eq. 5) was also calculated from the RTD curves:

$$HETP = L\frac{\sigma_t^2}{t_R^2} \quad (5)$$

2.3.8. Flow resistance: The flow resistance provided by the cryogel in the laminar region was determined, in duplicate, by the Darcy-Weisbach equation (Eq. 6). For that, the pressure drop of the column was estimated using degassed deionized water as mobile phase at different flow rates (0.6 to 12.7 cm/min) and the hydraulic permeability was obtained by linear regression of the equation (Yao et al., 2006a; Yao et al., 2006b).

$$\frac{\Delta P_w}{L} = -\frac{\mu_w}{K_w}.U \quad (6)$$

where ΔP_w is the pressure drop through the column (Pa), μ_w is water viscosity (Pa·s) and K_w is hydraulic permeability (m^2).

2.4. β-glucosidase production

β-glucosidase was produced based on the methodology described by Leite et al. (2008). The fungus *Thermoascus aurantiacus* was cultivated in 250 mL erlenmeyers flasks containing 40 mL of Sabouraud dextrose agar slope for 48 h at 50°C. The suspensions of microorganisms were obtained by scraping the medium surface using 25 mL of mineral solution (0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4.7H_2O$ and 0.1% NH_4NO_3, w/v).

The inoculation was carried out by transferring 3 mL of the culture suspension to polypropylene packages containing 5 g of corn cob mixed with the previously described mineral solution to achieve an initial humidity of 60%. Solid-state fermentation was carried out for 48 h at 50°C. All the materials were sterilized at 120 °C for 20 min.

2.4.1. Enzymatic extract preparation

The extraction of β-glucosidase was performed by adding 50 mL of deionized water to each of the polypropylene packages, followed by stirring on a rotatory shaker (80 rpm) for varied times and at room temperature. The effect of the stirring time required to extract the β-glucosidase (15, 30 and 60 min) was tested. After shaking, the samples were filtered in a

Musseline fabric filter. Then, the samples were centrifuged at 4 °C at 10000 × *g* for 20 min and the supernatant was recovered and stored at 7 °C for further adsorption studies.

2.5. Adsorption studies

2.5.2. Effect of pH

The capture of β-glucosidase by the anion-exchange cryogel was studied as a function of the pH of the adsorption (3.0, 5.0 and 7.0). The enzyme was extracted according to the results of the previous section but a sodium phosphate buffer (0.02 mol/L, variable pH) was used instead of water. For the preparation of the phosphate buffer at pH 3.0, the pH of a monobasic sodium phosphate solution (0.02 mol / L) was adjusted to 3.0 using pure phosphoric acid. A phosphate buffer at pH 7.0 was obtained by pH adjustment of a dibasic sodium phosphate solution (0.02 mol/L) to 7.0, using a monobasic sodium phosphate solution (0.02 mol/L).

The cryogel column was placed into a Tricorn 10/50 glass column (GE Healthcare, Chicago, USA) and was equilibrated with 4 column volumes (CV) of sodium phosphate buffer solution (0.02 mol/L; variable pH). Then, 1 mL of crude enzymatic extract was injected into the column at a flow rate of 1.0 mL/min. The column was washed with 4 CV of the equilibrium buffer and isocratic elutions were performed with 3 CV of same buffer containing NaCl at 1.5 mol/L. The column was re-equilibrated by applying 4 CV of the equilibrium buffer for complete removal of salt and so was prepared for the next assay. The adsorption and elution profiles were monitored at 280 nm. The peak corresponding to the elution was collected and stored for subsequent analysis. The assays were done in triplicate using an Äkta Pure chromatographic system (GE Healthcare Life-Sciences, Uppsala, Sweden) at room temperature. The same procedure was used for the all pH values tested.

2.6. Quantification of total protein

Total protein content of the samples was determined following the Bradford method (Bradford, 1976), using bovine serum albumin as standard. For the crude extract, the total protein is total protein loaded and, for the eluted fractions, it is the total protein recovered.

2.7. β-glucosidase activity

The activity (U) of the β-glucosidase was determined by incubating 50 µL of the enzymatic extract in 250 µL of acetate buffer (0.1 mol/L; pH 5.0) and 250 µL of *p*-nitrophenyl-

β-D-glucopyranoside 4 mmol/L. The reaction mixture was kept at 60 °C for 10 min. The enzymatic reaction was stopped by the addition of 2 mL of sodium carbonate 2 mol/L, and the released nitrophenol was quantified using a spectrophotometer at 410 nm [13]. One unit of enzymatic activity was defined as the quantity of enzyme needed to release 1 µmol of nitrophenol per minute of reaction, using the standard curve for nitrophenol.

2.8. SDS-PAGE

The purity of the fractions collected from the capture of β-glucosidase was evaluated by SDS-PAGE. Electrophoresis was conducted using a vertical gel electrophoresis system (BioRad, Hercules USA) for 1 h at 150 V and with a 10% polyacrylamide gel. The gel was stained using the silver method according to See and Jackowski et al. (1989). All samples were treated with 2X Laemmli Sample Buffer (BioRad, Hercules, USA) and β-mercaptoethanol and then heated at 100 °C for 5 min. Aliquots of 20 µL of the samples or 2 µL of the molecular weight standards (Broad Range, BioRad, Hercules, USA) were applied to the gel.

2.9. Data analysis

The adsorption yield (*Y, %*) and purification factor (P_F) were calculated by Eqs. (8) and (9):

$$Y = \frac{TA_e}{TA_i} \tag{8}$$

where TA_e is the total activity recovered in the eluted fraction (U) and TA_i is the total activity of the crude extract injected (U).

$$P_F = \frac{SA_e}{SA_i} \tag{9}$$

where SA_e is the specific activity of the eluted fraction (U/mg) and SA_i is the specific activity of the crude extract injected (U/mg)

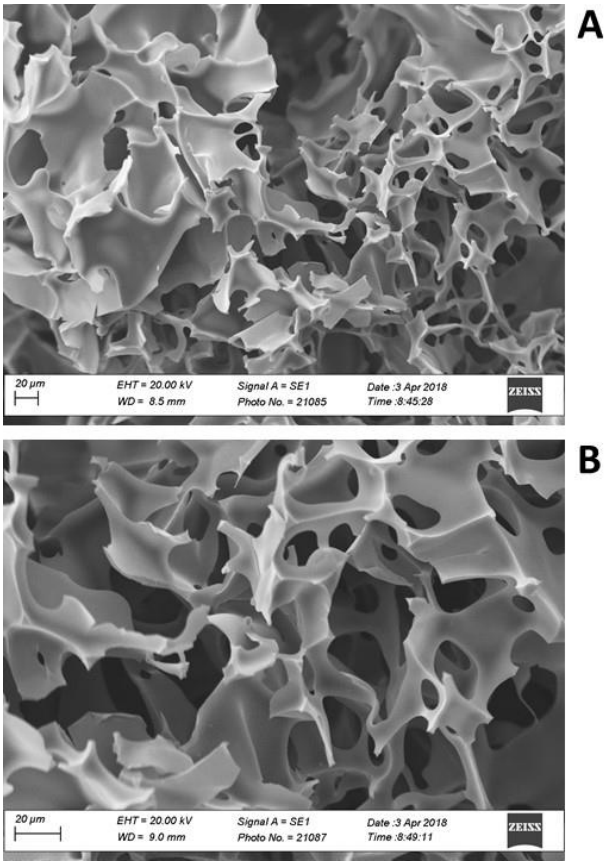
The effect of pH on these parameters was evaluated using analysis of variance (ANOVA) at a 5% level of significance. Statistical analyses were performed using the Minitab 16 software package (Minitab, State College, US).

3. Results and discussion

3.1. Characterization of the cryogel

The internal structure of the cryogel is shown in Figure 1. It shows a porous structure of uniform and interconnected pores with diameters ranging from 10 to 100 µm. The swelling capacity was determined as 8.9 kg/kg expressed as the amount of water absorbed by dry cryogel. The degree of expansion is useful for converting the dehydrated mass of the cryogel and its hydrated volume (Fontan et al., 2018) and the value obtained was 18.6 L/kg.

Figure 1. Scanning electron micrographs of the anionic cryogels produced. (A) 500x and (B) 2000x.

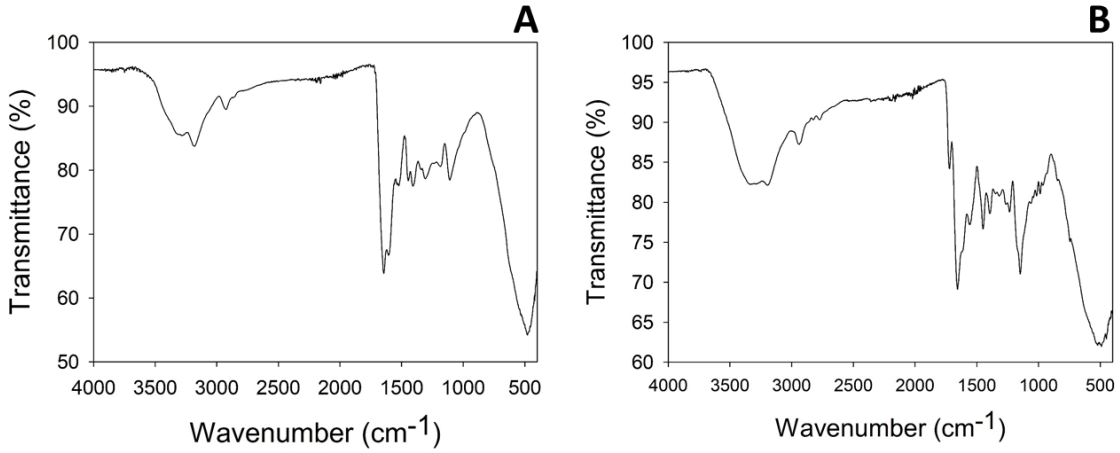


Source: Prepared by the author.

Figures 2 shows the IR spectra of the ungrafted cryogel (control) and the cryogel grafted with DMAEMA. The pure cryogel presented a strong infrared absorption band at 1645 cm^{-1} (Figure 2 A), which are characteristic of carbonyl groups (C=O) of acrylamide. The absorption found at 1103 cm^{-1} may be related to the epoxy group of the allyl glycidyl ether. After the grafting process with a DMAEMA anionic ligand, absorption bands at 1149 and 1235 cm^{-1} are observed and may be attributed to C-O bonding stretching, an ester characteristic, confirming

the presence of this group in the cryogel structure and, consequently, the incorporation of DMAEMA (Figure 2 B) (Barbosa, 2007). The absorption bands obtained at 1656 and 1722 cm^{-1} (Figure 2 B) are typical of carbonyl groups of amide and ester function, which are functional groups of acrylamide and DMAEMA, respectively [40]. Vibration frequencies at the region of 2926 cm^{-1} are characteristic of sp^3 and sp^2 carbon stretching (Figure 2 A and 2 B). The peak observed at 1435 cm^{-1} is associated to bending vibration of (C-H) and the weak absorption at 1560 cm^{-1} may be assigned to alkenes (C=C) (Barbosa, 2007; Jain et al., 2017). A broad peak due to absorption in the region from 3100 to 3500 cm^{-1} is evident (Figure 2 B), thus suggesting the presence of water (O-H band) and this can also be associated to N-H stretching of amides (Jain et al., 2017). The IR spectra also shows an absorption close to 500 cm^{-1} (Figure 2 B) which can be attributed to angular deformation outside the plane of the N-H bond (Barbosa, 2007).

Figure 2. Infrared absorption spectra of the pure cryogel (A) and anionic cryogels produced (B).

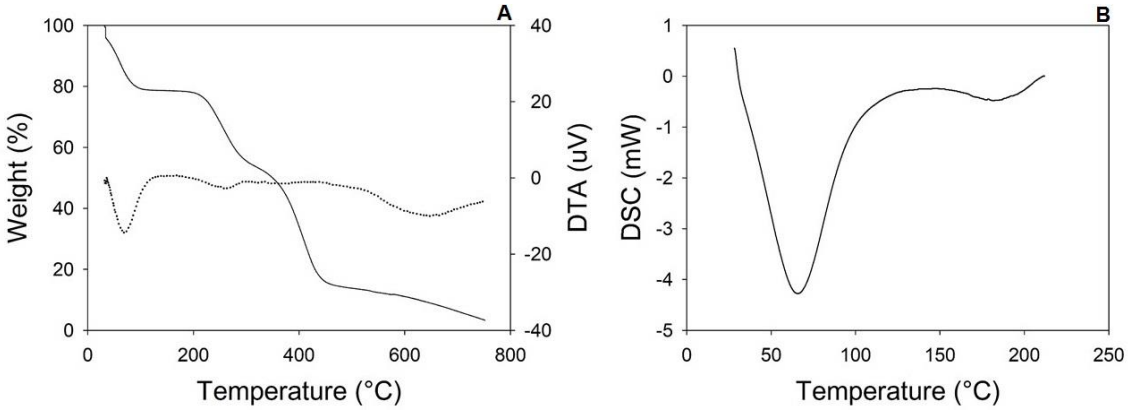


Source: Prepared by the author.

The thermal stability of the adsorbent was evaluated by TGA. Samples of cryogel were weighed at different temperatures and the results are shown in Figure 3 A. The first noteworthy step (up to 100 °C) may be attributed to the loss of hydration water present in the porous of sample. In the second thermal event (from 220 to 550 °C), the cryogel undergoes severe thermal degradation, with a pronounced mass loss (approximately 87.0%). Then, the residual mass of the samples is small enough to indicate nearly complete thermal decomposition. A weight loss of 50% starting at 365 °C is similar to the result found by Silva et al. (2014) who study a polymer network based on polyDMAEMA. The results obtained show the thermal stability usually presented by cryogels and imply the possibility of its use on an industrial scale up to 220 °C. Similar behavior for mass loss of nanosheets functionalized with polyDMAEMA was

observed by Bak et al. (2012) in the same range of 250-400 °C resulting from the decomposition of the polyDMAEMA.

Figure 3. Thermal analysis of the anionic cryogel samples. (A) Thermogravimetric curve and (B) Differential scanning calorimetry curve of the anionic cryogel.



Source: Prepared by the author.

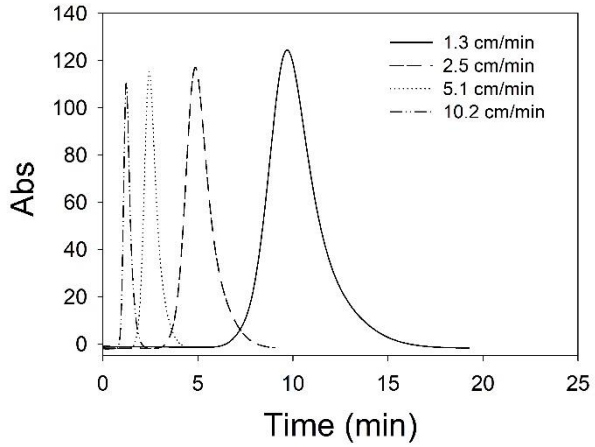
Differential scanning calorimetry (DSC) is a very versatile tool in the analysis and characterization of polymeric materials. So a DSC thermogram was generated to study the thermal behavior of the cryogel. During heating, an acute endotherm peak with an onset at 37 °C and ending at 120 °C (Figure 3 B) is observed and can be assigned in the melting of crystallites in the cryogel due to hydrogen bonding between the cryogel and water (Bajpai and Saini, 2006; Jain et al., 2017). A second peak observed at 170 °C may be related to the loss of ammonia. According to Convely (1970), polyacrylamide degrades in the temperature range of 175-300 °C by the formation of imide groups via cyclization.

The residence time distribution at different flow velocities of the mobile phase was determined by a tracer pulse method. The mean residence time and variance were determined for each flow velocity using the momentum method. The porosity was determined using these results (Eq. 3) and was 0.92. Figure 4 shows the RTD curves with symmetrical peaks independent of the water velocity.

From the RTD curves, axial dispersion coefficients were calculated and varied from 10^{-7} to 10^{-6} m^2/s. The HETP and dispersion number were also calculated and did not vary with flow velocity. HETP varied from 2.8 to 3.0 mm (Figure 5). These values are similar to those found by other authors (Mól et al., 2017; Fontan et al., 2018) but slightly higher than those reported by Yun et al. (2007) for a similar anion exchange cryogel. The results obtained suggest that most of mass transfer occurs predominantly by convection. In terms of process, these

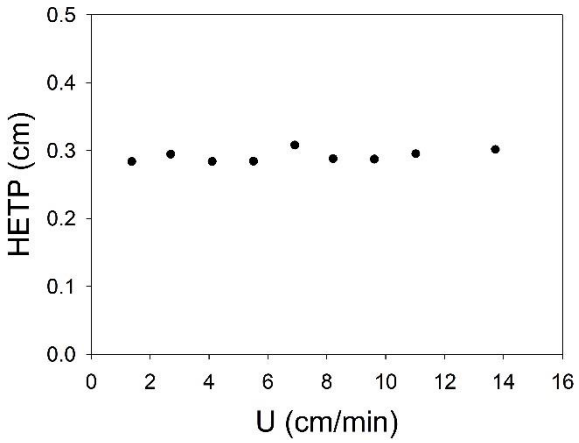
results indicate small dispersion, contributing to high column efficiency and consequently high yields.

Figure 4. Residence time distribution curves for acetone pulses at different flow velocities, in the anionic cryogel column (1.3 cm/min to 10.2 cm/min).



Source: Prepared by the author.

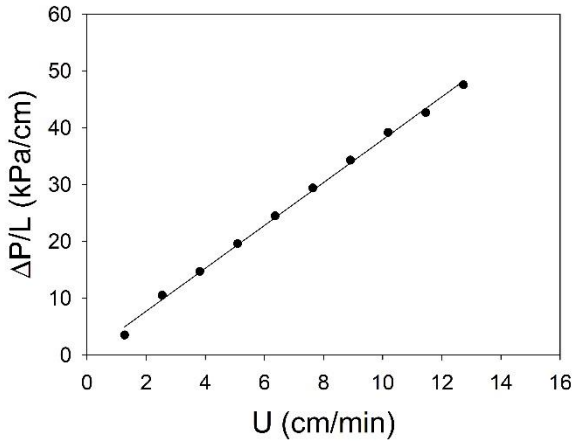
Figure 5. Height equivalent to a theoretical plate at different mobile phase flow rate (0.6 cm/min to 10.2 cm/min), in the anionic cryogel column.



Source: Prepared by the author.

Hydraulic permeability is related to the resistance to flow through the cryogel column. Experimental data for flow resistance is shown in Figure 6. The value determined by the Darcy-Weisbach equation was 5.0 x 10^{-13} m^2, indicating that flow resistance within the cryogel is low. The value is close to others found for polyacrylamide cryogels (Yun et al., 2007; Carvalho et al., 2014; Mól et al., 2017; Fontan et al., 2018).

Figure 6. Experimental data of pressure drop at different water flow velocities (●) and fitted equation to determine permeability (-). (μ_w=1.003 Pa·s; K_w=5.9 x 10^{-13} m^2).

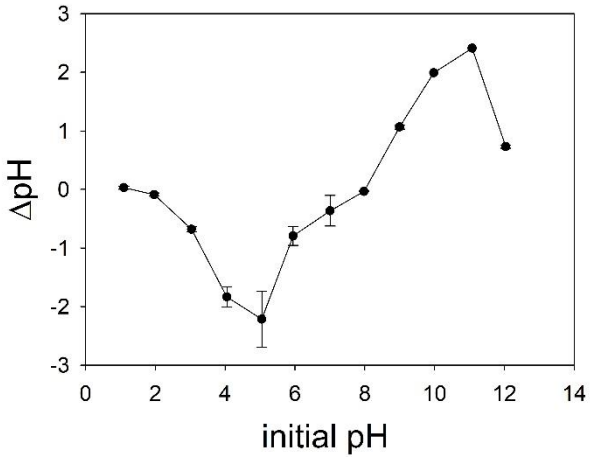


Source: Prepared by the author.

The highly porous structure of the cryogels and the presence of large pores allow the unobstructed passage of solutes and, consequently, the low flow resistance normally observed.

The determination of the point of zero charge is an important measure since the pH affects the dissociation of the active sites present on the adsorbent surface. The surface is protonated in solutions at pH below the point of zero charge, favoring the adsorption of negatively charged molecules. However, at pH values above the point of zero charge, the surface is deprotonated, which favors the adsorption of positively charged compounds. At pH equal to the point of zero charge, the adsorbent acts as a buffer (Vieira et al., 2010). The point of zero charge found for the cryogel grafted with DMAEMA in this study was close to pH 8.0 (Figure 7). This result is very close to the pKa of DMAEMA (8.44). At pH values below the pKa, most of the ion exchanger binders are positively charged and thus adsorb negatively charged molecules of the solution. Most of the proteins are negatively charged at pH 8.0, so the DMAEMA is commonly used as an anion exchanger ligand (Arvidsson et al., 2002; Savina et al., 2005; Nascimento et al., 2019), although we found higher cationic than anionic capacity.

Figure 7. Determination of the point of zero charge of the anionic cryogel. ΔpH is the difference between the final pH of aqueous solution after 24 h and the initial pH of aqueous solution.



Source: Prepared by the author.

Ionic capacity represents the number of sites available for adsorption. Although adsorbents produced with DMAEMA have commonly been used as an anion exchanger (Arvidsson et al., 2002; Savina et al., 2005) it was found that the adsorbent produced had a higher cationic (0.329 mmol/g) than anionic (0.06 mmol/g) capacity. In general, the values obtained were lower than those reported in the literature.

3.2. Enzyme extraction

After the solid fermentation of *Thermoascus aurantiacus* had been developed, water was added to the packages containing the substrate and the grown fungi and the systems were left under stirring to extract the enzymes. The stirring time required to extract the β-glucosidase was tested and the results of enzymatic activity are shown in Table 1.

Table 1. Results of total protein content (P_T, mg), total activity (A_T, U), specific activity (A_S, U/mg) as a function of the stirring time used in the β-glucosidase extraction from the fermented broth.

Stirring time	P_T (mg)	A_T (U)	A_S (U/mg)
15 min	3.65±0.00	58.05±5.31	15.91±1.18
30 min	3.29±0.24	61.51±3.36	18.71±1.02
60 min	3.93±0.45	62.61±5.99	15.92±1.52

Source: Prepared by the author.

The data were submitted to regression analysis and it was verified that stirring time did not affect the extraction process of β-glucosidase (p=0.5460). Thus, a stirring time of 15 min was fixed as suitable to extract the β-glucosidase present in the fermented medium. Leite et al. (2007, 2008) also produced β-glucosidase and left the solid fermentation media under stirring for 2 h. This work suggests the use of one eighth of that time. According to the results, the stirring time used can be significantly reduced without compromising the amount of enzyme extracted. Multiple steps are generally necessary in the purification processes of biomolecules, and a decrease in the number of steps or a simple reduction in the time of each one can save total time and the use of materials, often allowing new experiments to be done due to savings of both time and other resources (Straathof, 2011).

3.3. Effect of pH

Adsorption assays were conducted at different pH values and the experimental results of yield and purification factor shown in Table 2 were subjected to analysis of variance at 95% of significance level.

Table 2. Results of total protein content (P_T, mg), total activity (A_T, U), specific activity (A_S, U/mg), purification factor (P_F) and yield (Y, %) of samples of β-glucosidase in the unpurified extract and in the eluted samples from anionic cryoel column as a function of pH.

Sample	pH	P_T (mg)	A_T (U)	A_S (U/mg)	P_F	Y (%)
Extract	3.0	0.10	0.65	6.37	1	100
Eluted	3.0	0.05±0.00	0.43±0.03	8.46±1.11	1.33±0.17	67.19±4.21
Extract	5.0	0.14	1.24	8.84	1	100
Eluted	5.0	0.08±0.00	0.97±0.06	11.01±0.61	1.25±0.07	82.03±1.89
Extract	7.0	0.18	2.08	11.71	1	100
Eluted	7.0	0.10±0.01	1.49±0.02	15.21±2.11	1.30±0.17	71.76±1.00

Source: Prepared by the author.

According to the ANOVA, the pH affected the adsorption yield of β-glucosidase (p=0.0015). The highest value was obtained at pH 5.0 (82%), suggesting that this is the ideal pH for subsequent analysis. pH variation affects the adsorption process by the dissociation of the adsorbent counterions, as well as providing the change in the surface charge of the proteins according to their isoelectric point (pI). The optimum pH found (5.0) is higher than the isoelectric point of β-glucosidase (3.9-4.5) (Bedino et al., 1985; Kalogeris et al., 2003) and, at

this pH, this protein has a negative charge. Consequently, the adsorption occurs due to anion exchange between the adsorbent surface and the protein. This result is in agreement with the point of zero charge found, which is around 8.0. At pH values below the point of zero charge, the surface of the adsorbent is positively charged and adsorbs anions from the solution.

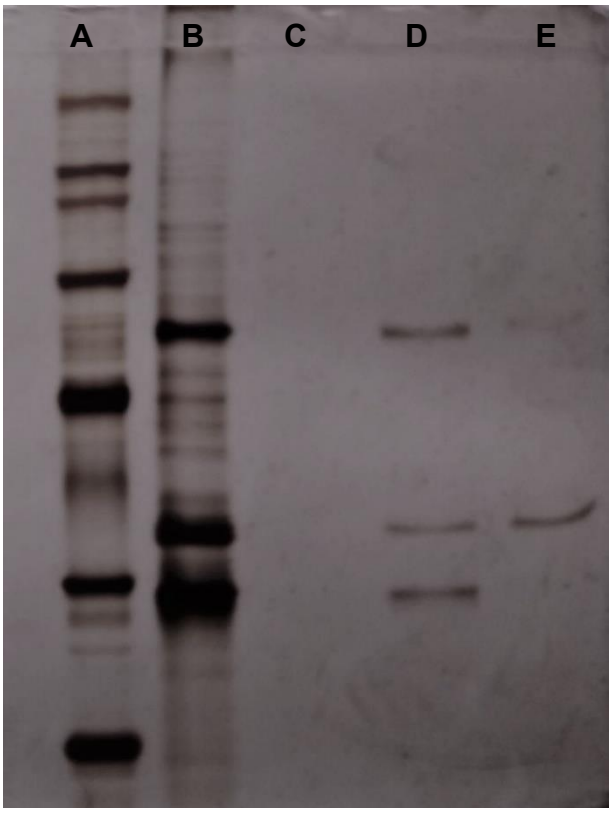
The lowest yield was found at pH 3.0 (67%), possibly due to the fact that β-glucosidase is positively charged at this pH, as are most of the molecules on the adsorbent surface. However, because the charges on the protein surface are distributed asymmetrically, binding can also occur close to the pI even when the overall charge is the same as that on the ion exchanger. Also, despite electrostatic repulsion, the adsorption process may occur due to hydrophobic interactions (Lin et al., 2001). At pH 7.0, the enzyme has a negative charge, opposite to the exchanger, but this pH value is much closer to the point of zero charge, at which point the adsorbent molecules have the same charge as the enzyme.

The purification factor was not affected by the pH at a 95% significance level ($p=0.8030$). The values obtained are considered low for a chromatographic separation step. However, it should be noted that isocratic elution was the option used and so the β-glucosidases adsorbed were not separated from others proteins also adsorbed, contributing to low values of P_F.

An increase in a final P_F values could be obtained by changes in the purification process, such as the addition of other unit operation or a change in the environmental conditions. The use of a sequence of combined chromatography techniques will probably increase the P_F values, however around 10% of the enzyme can be lost during each purification step, leading to low recovery (Singhania et al., 2010). Thus, a limited number of separation steps is very important to increase recovery and reduce costs. Meanwhile, the downstream processing depends on the degree of purity required and hence the application of the enzyme. Besides the number of steps, a lot of experimental conditions, such as pH value, salt concentration and temperature can alter the P_F since they can interfere in the absorption of a specific enzyme to the detriment of others (Lin et al., 2001). Furthermore, as isocratic elution was not efficient in the separation process, the P_F could be increased by changes in the elution techniques, for example. The adoption of a gradient or stepwise elution usually results in better chromatographic separation. Elution in ion exchange separation of proteins is invariably by gradient elution, typically conducted by an increase in the ionic strength, by inclusion of new species or even by a change in the pH (Janson and Rydén, 2011; Lenhoff, 2016)

Figure 8 shows that, despite the values of purification factors, the crude extract was partially purified regardless of the pH, so the amount of proteins in the eluted sample were clearly lower. No dense band was verified at pH 3.0, three were verified at pH 5.0, and two bands were found at pH 7.0, in which only one was quite dense while the other was almost undetectable, thus indicating a high degree of purity of the sample. Further to this result of SDS-PAGE, the specific activity of β-glucosidase was higher at pH 7.0 (both crude extract and eluted fractions), suggesting that this pH is interesting to this purification process. The value of yield obtained at pH 7.0 was lower than at pH 5.0 but is also attractive in the case of only one separation step.

Figure 8. SDS-PAGE 10% (150 V running). (A) standard protein marker (kDa), (B) crude extract, (C) eluted pH 3.0, (D) eluted pH 5.0, (E) eluted pH 7.0.



Source: Prepared by the author.

Palma-Fernandez et al. (2002) also purified β-glucosidase from *Thermoascus aurantiacus* and after five purification steps obtained two enzymes with yield of 3.2 and 2.5% and molar masses of 175 and 157 kDa, respectively. Parry et al. (2001) obtained β-glucosidase from the same fungi and observed that the native enzyme had a molecular mass of approximately 360 kDa, and appeared to be a homotrimer. The data presented above suggests

that *Thermoascus aurantiacus* perhaps presents genetic variability, which results in different β-glucosidases.

The present work did not obtain a fraction with high specific activity, but as it only involved one separation step, the results can be considered satisfactory to direct future studies. Moreover, this result is very interesting from the industrial application point of view because, the smaller the number of steps of downstream processing, the lower the production costs, which can vary from 20 to 40%, for fermentation products (Straathof, 2011; Singhania et al., 2010).

4. Conclusion

This study deals with the capture of β-glucosidase from thermophilic fungi *Thermoascus aurantiacus*. The ion-exchange cryogel showed desirable properties such as high porosity, low dispersion, low flow resistance and high stability. Adsorption of the β-glucosidase as a function of the pH was tested using a chromatographic system. Taking into account the isoelectric point of the enzyme and the point of zero charge of the adsorbent, pH 5.0 presented higher recovery of the enzymatic activity. However, higher specific activity was obtained at pH 7.0 and the eluate at this pH presented only one dense band on the electrophoresis gel. The purification factor was low probably due to isocratic elution and so other proteins were not separated from the β-glucosidase by ionic strength. This suggests that gradient elution could be studied in the future. An increase in the number of purification steps implies a greater protein loss and greatly increases the cost of the process, which makes the industrial plants try to use as few downstream processes as possible. In this study, no previous stages of clarification have been carried out. The enzyme extract flowed through the adsorbent without any obstruction from the pores, so this adsorbent is an attractive support for the separation of biomolecules, reducing both time and costs.

CAPÍTULO III

Immobilization of β-glucosidase from crude extract on macroporous cryogel by hydrophobic interaction

Abstract

In the last decades, monolithic matrices produced under freezing conditions have gained interest as stationary phase in chromatography separation and immobilization of biomolecules such as proteins. In this work, polyacrylamide cryogel containing L-phenylalanine were prepared to be used in the adsorption of β-glucosidase from crude extract, aiming at its purification also immobilization. The enzyme was produced by solid state fermentation using corn cob as substrate. First, the adsorption was investigated as a function of the pH and the results of yield (Y, %) and purification factor (P_F) were determined. The P_F from eluate obtained at pH 3.0 was the highest. Then, the enzyme was immobilized on the cryogel via adsorption and the activity retained was determined as well as the amount of nitrophenol released from the cryogel samples containing enzymes. The effect of the temperature of adsorption and the presence of different saline solution were determined. As expected in hydrophobic interaction, salt type and ionic strength affected the amount and consequently the activity of the immobilized enzyme.

Keywords: β-glucosidase, adsorption, cryogel, L-phenylalanine, hydrophobic interaction

1. Introduction

β-glucosidase or β-D-glucoside glycohydrolase (EC 3.2. 1.21) is a ubiquitous enzyme found in a wide diversity of sources such as animals, plants and microorganisms, with various biotechnological applications, such as biofuel production, isoflavone hydrolysis, flavor enhancement, among others (Cairns and Esen, 2010). There is an increased demand of β-glucosidase production from microbial sources. For industrial utilization, microorganisms are considered the best choice since they grow rapidly, are easily to handle, require less space, can easily be manipulated by genetic engineering and produce enzymes with special characteristics such as temperature and pH tolerance (Ahmed et al., 2017).

However, the processes of recovery and final purification of products of biotechnological origin such as enzymes usually account for the highest percentage of the production costs, reaching up of more than 50% of the total cost. The cost of a downstream

process depends on the degree of purity required and the purpose of molecule use (Singhania et al., 2010). Normally, the high dilution of the biomolecules presents in fermentation broths and cell supernatants and the existence of chemical interferences that hinder the recovery make the purification even more expensive. According to Singhania et al. (2010), depending on the degree of purity required, the purification of an enzyme from a concentrated and clarified extract can involve at least five steps and about 10% of the enzyme can be lost in each step, resulting in low recovery. Thus, to reduce the cost of a purified enzyme, it is necessary to increase the yield of the process and also decrease the number of steps.

Chromatographic processes are far away the most common technique when products with high purity and high yield are desired (Janson and Rydén, 2011). Hydrophobic interaction chromatography (HIC) is a frequently method used in downstream protocols. The important factors that impact HIC are pH, salt type and concentration and temperature. Used as first step in downstream process, HIC serve as an effective step for containing dilute samples.

Adsorption is also a simple method widely used for immobilizing enzymes. The main advantages are low cost, ease and simplicity of the process. In addition, adsorption promotes little change in conformational structure of the enzyme, since the enzyme is spontaneously immobilized in an orientation that is preferred and energetically favorable. As disadvantages, there is the randomness of the enzyme-support interaction and the possibility of desorption and leaching of the enzyme due to variation in temperature, pH and ionic strength (Liese and Hilterhaus, 2013).

Many methodologies have been developed aiming the efficient immobilization of β-glucosidase (Ortega et al., 1998; Gómez et al., 2005; Su et al., 2010; González-Pombo et al., 2011; Khan et al.; 2012; Chen et al., 2013; Borges et al., 2014; Chang et al., 2013; Tsai and Meyer, 2014; Tan and Lee, 2015; Zhou et al., 2019; Venezia et al., 2020).

The success of purification and immobilization protocols also depends on the characteristics of the support. Cryogels are polymeric gels formed in frozen conditions and has been recognized in biotechnological and biomedical areas (Lozinsky, et al., 2001; Buchmeiser, 2007; Andrabi et al., 2016) These matrices have many advantages such as low cost, ease of preparation, chemical and mechanical stabilities, besides allowing the direct capture of proteins and other biomolecules from crude extracts or even from unprocessed fermentation media (Arvidisson et al., 2002; Mól et al., 2017). Few studies of immobilization of β-glucosidase on cryogels are found in the literature (Khan et al., 2012).

In this context, this work deals with the adsorption of β-glucosidase from a fermentative crude extract on a polyacrylamide cryogel containing L-phenylalanine via hydrophobic interaction. The effect of pH on the adsorption was assessed using a chromatographic system and then the effect of temperature and different saline solutions was investigated in terms of the activity retained in the cryogel samples.

2. Materials and methods

2.1. Materials

Acrylamide (AAm, 99%), N,N'-methylene-bis-acrylamide (MAAm, 99%), ammonium persulfate (APS), N,N,N',N'-tetramethyl-ethylene-diamine (TEMED, 99%), allyl glycidyl ether (AGE, 99%), L-phenylalanine were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade and Milli-Q System deionized water was used throughout the experiments (MilliporeSigma, Burlington, USA).

2.2. Cryogel preparation

The cryogels were prepared according to Mól et al. (2017, 2019). The monomers of AAm and MBAAm (6% m/v) and AGE (0.04 % v/v) were dissolved in utrapure water until total solubilization and the mixture was degassed. The polymerization reaction was started with TEMED and APS. The mixture was immediately poured into a glass column 10/50. The columns were sealed and immersed in a bath containing ethylene glycol at -12 °C for 24h. Afterwards, the frozen cryogels were thawed at refrigeration temperature, extensively washed with deionized water and dried at 40 °C for 48 h.

The immobilization of the ligand L-phenylalanine was performed via the glutaraldehyde method, according to De Oliveira et al. (2019) and Neves et al. (2020). First, the cryogel column was immersed in 1,6-hexanodiamine (0.5 mol/L) at 65 °C. After 10 h, the cryogel was thoroughly washed with deionized water. Then, the cryogel was immersed in phosphate buffer (0.1 mol/L; pH 5.0) for 30 min and subsequently immersed in glutaraldehyde (5 % v/v in 0.1 mol/L sodium phosphate, pH 5.0) for 4 h. The amino acid binding was performed by immersing the columns in L-phenylalanine solution (5 mg/mL in sodium carbonate 0.2 mol/L, pH 9.5). Lastly, the cryogels were submerged in 50 mL of sodium borohydride 0.1 mol/L (in sodium carbonate 0.2 mol/L, pH 9.5) for 30 min. The column was finally washed with distilled water

to remove any residual monomers. The cryogel samples were properly stored at room temperature until the moment of use.

2.3. β-glucosidase production

2.3.1. Microorganism

Fungal β-glucosidase was produced from *Thermoascus aurantiacus*, as described in Mól et al. (2019). The fungus was cultivated in 250 mL erlenmeyers flasks containing 40 mL of Sabouraud dextrose agar slope at 50°C for 48 h.

2.3.2. Solid state fermentation

First, 25 mL of mineral solution (0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4.7H_2O$ and 0.1% NH_4NO_3, w/v) was added to Erlenmeyers flasks and the culture medium was gently scrapped in order to obtain the inoculum. Then, 3 mL of the microorganism suspension were transferred to polypropylene packages containing 5 g of corn cob mixed with 8 of mL mineral solution (Figure 1). Solid-state fermentation was carried out at 50°C for 96 h. All the materials were sterilized at 120 °C for 20 min.

Figure 1. Systems where the solid state fermentation was developed.

Source: Prepared by the author.

2.3.3. Enzymatic extract preparation

The enzyme present in each package was extracted with 50 mL distiller water. The whole content of crude extract was reunited and properly prepared according to Mól et al (2019).

2.4. Adsorption studies

2.4.1. Effect of pH

The adsorption of β-glucosidase by the tailor made cryogel was then investigated as a function of the pH of the adsorption (3.0, 5.0, 7.0 and 9.0). The cryogel column was placed into a Tricorn 10/50 glass column (GE Healthcare, Chicago, USA) and was equilibrated with 4 column volumes (CV) of sodium phosphate buffer solution (0.02 mol/L; variable pH). Then, 1 mL of crude enzymatic extract was injected into the column at a flow rate of 1.0 mL/min. The column was washed with 4 CV of the equilibrium buffer and isocratic elution were performed with 3 CV of acetic acid solution (2% v/v). The removal of the elution solution and the column re-equilibration was performed with 4 CV of sodium phosphate buffer. The adsorption and elution profiles were monitored at 280 nm. The peak corresponding to the elution was collected and stored for subsequent analysis. The experiments were done in duplicate using an Äkta Pure chromatographic system (GE Healthcare Life-Sciences, Uppsala, Sweden) at room temperature.

2.4.2. Effect of temperature

The effect of temperature on the adsorption of β-glucosidase was investigated batchwise. A mass of approximately 0.02 g of dried cryogel was previously incubated with 1.0 mL of sodium acetate buffer (0.1 mol/L; pH 5.0) for 30 min. Then, 1 mL of the crude extract was added and the tubes containing the system were incubated for 24 h at different temperatures (25, 35 and 45 °C). The experiments were conducted in quadruplicate. The activity of β-glucosidase of the supernatant and of the crude extract were measured according to Section 2.6. The amount of activity remained in each cryogel was determined by (Eq. 1):

$$q = \frac{A_1 V_1 - A_2 V_2}{m_d} \quad (1)$$

where q (U/g) is the activity of β-glucosidase in the solid phase, A_1 (U) is the initial activity of the crude extract, V_1 (mL) is the volume of crude extract added, A_2 (U) is the activity in the

liquid solution after 24 h, V_2 (mL) is the final volume of the liquid solution and M (g) is the cryogel weight.

2.4.3. Effect of buffer and ionic strength

The effect of different molar concentrations of NaCl (0, 0.5 and 1.0 mol/L) on the physical adsorption of β-glucosidase was tested, using five different saline buffers (potassium sulfate, sodium acetate, sodium citrate, sodium phosphate and sodium sulfate; all of them 0.02 mol/L and pH 3.0). The adsorptions were conducted batchwise at room temperature, according to the methodology described in section 2.4.2, with at least three repetitions. The adsorption yield (%) was determined as the ratio between the adsorbed activity and the total activity left in contact with the adsorbent.

Besides the activity adsorbed on the solid phase and the yield, the amount of nitrophenol released per mass of the cryogels containing the adsorbed enzymes were determined.

2.4.4. Operational stability

The operational stability of the immobilized β-glucosidase was evaluated for seven successive cycles of pNPG hydrolysis, at room temperature. First, the cryogel column was placed into a glass column and equilibrated with sodium phosphate buffer (0.02 mol/L; pH 5.0) at a flow rate of 1 mL/min, as described in 2.4.1. After complete column hydration, the adsorption of β-glucosidase was performed by passing 10 mL of the crude extract solution at the same flow and then 10 mL of buffer to remove the non-adsorbed proteins. In each hydrolysis cycle, 5 mL of substrate solution were percolated through the column. After each cycle, the cryogel column was rinsed with 5 mL of sodium phosphate buffer and then used for a new run of operation. The solutions were collected for analysis and the amount of nitrophenol released were determined by the standard curve. The column was then used in a new run with fresh solution of substrate.

2.5. Quantification of total protein

Protein concentration in the enzymatic samples was determined by Bradford (1976), using bovine serum albumin as the reference protein.

2.6. β-glucosidase activity

The activity of β-glucosidase (U) was assessed by measuring the amount of nitrophenol released from the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate. For that, 50 µL of the enzymatic extract was added to 250 µL of acetate buffer (0.1 mol/L; pH 5.0) and 250 µL of *p*NPG 4 mmol/L. After incubation at 60 °C for 10 min, the enzymatic reaction was stopped by the addition of 2 mL of sodium carbonate 2 mol/L (Leite et al., 2008). The absorbance of the mixture was measured at 410 nm and the concentration of nitrophenol was determined using a standard curve. One unit of enzymatic activity was defined as the quantity of enzyme needed to release 1 µmol of nitrophenol per minute of reaction, under the described conditions.

2.7. Determination of kinetic parameters

The Michaelis-Menten constant (K_M) and maximum reaction velocities (V_{max}) for substrate hydrolysis by free and immobilized enzyme were calculated by Lineawever-Burk, Eadie-Hofstee, Hanes-Woolf plots and also by non-linear equation. The concentrations of pNPG varied from 0.3 to 5 mM.

2.8. Characterization of the prepared cryogel

2.8.1. Swelling capacity and expansion degree: The swelling capacity of the cryogel (*S*, g water/g dried cryogel) was determined according to Eq. (2) (Savina et al., 2005). Samples of dried cryogels (m_d) were immersed (m_d) in 50 mL of distilled water for 24 h. The excess of water was then gently removed from the cryogel and the weight of the dried sample (m_w) was measured.

$$S = \frac{m_w - m_d}{m_d} \quad (2)$$

The degree of expansion (*ED*, mL water/g dried cryogel) was calculated according to Eq. (3). The same samples used above, after being saturated with water for 24 h, were transferred to a graduate cylinder containing a known volume of distilled water (V_1) and the final volume was determined (V_2).

$$ED = \frac{V_2 - V_1}{m_d} \quad (3)$$

2.8.2. Point of zero charge: The point of zero charge (pH_{pzc}) was determined based on the methodology described by Vieira et al (2011). For that, samples of 0.03 g of dried cryogels were added to centrifuge tubes containing 25 mL of aqueous solution at variable pH (1.0 to 12.0) and left under mild agitation. After 24 h, the final pH of each solution was measured. The point of zero charge was determined in the experimental condition where the pH variation was zero.

2.8.3. Porosity and axial dispersion: The residence time distribution (RTD) was determined according to the method described by Yao et al. (2006a, 2006b) with modifications. The cryogel column was placed in a Tricorn 10/50 glass column (GE Healthcare, Uppsala, Sweden) was connected in a preparative chromatographic system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Deionized water was pumped through the column at a constant rate and a pulse of a non-reactive tracer (100 µL of acetone at 10% v/v) was injected into the column. The corresponding response was measured by a UV detector at 280 nm. The RTD curves was measured at various fluids velocities (0.6 cm/min to 6.4 cm/min) and the assays were conducted in duplicate.

The momentum method was used to determine the mean residence time ($\boldsymbol{t_R}$) and variance ($\boldsymbol{\sigma_t^2}$) using the RTD curves. Then, porosity ($\boldsymbol{\varepsilon_T}$) was determined by linear regression of Eq. (4) (Furusawa, 1976):

$$t_R = \varepsilon_T . \frac{L}{U} \tag{4}$$

where L (m) is the column length and U (m/s) is the superficial fluid velocity.

Then, and axial dispersion coefficients were determined by non-linear regression of Eq. (5) (Chang et al., 1996; Levenspiel, 1999) and height equivalent to theoretical plate (HETP) by Eq. (6) (Guiochon, 2006):

$$\boldsymbol{\frac{\sigma_t^2}{t_R^2} = 2\left(\frac{D_{ax}}{uL}\right) - 2\left(\frac{D_{ax}}{uL}\right)^2 \left[1 - exp\left(-\frac{uL}{D_{ax}}\right)\right]} \tag{5}$$

where D_{ax} is the axial dispersion coefficient and u is the interstitial fluid velocity through the column ($u = U/\varepsilon_T$).

$$HETP = L\frac{\sigma_t^2}{t_R^2} \tag{6}$$

2.8.4. Flow resistance: The resistance to flow provided by the cryogel column was measured using deionized was mobile phase and different flow velocities (0.6 cm/min to 6.4 cm/min). The pressure drop through the column for each flow rate of the mobile phase was recorded and the hydraulic permeability was obtained by linear regression of the Darcy´s equation (Yao et al., 2006a).

$$\frac{\Delta P_w}{L} = -\frac{\mu_w}{K_w}.U \qquad (7)$$

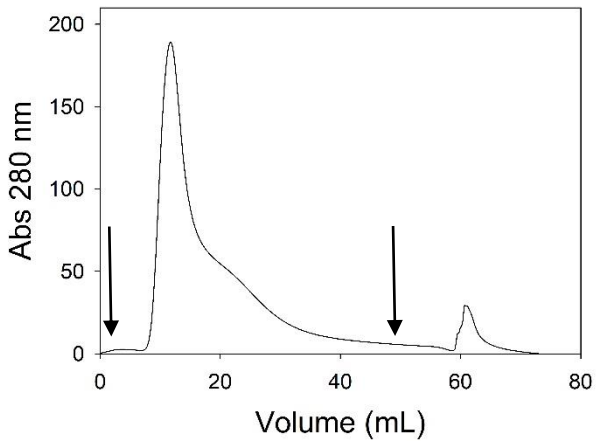
where ΔP_w is the pressure drop through the column (Pa), μ_w is water viscosity (Pa·s) and K_w is hydraulic permeability (m^2).

3. Results and discussion

3.1. Effect of pH

Figure 2 illustrates the chromatographic profile obtained in the capture of β-glucosidase from the crude extraction, highlighting the injection of the enzymatic solution (first arrow) and the isocratic elution with acetic acid solution (second arrow).

Figure 2. Adsorption profile of the capture of β-glucosidase from crude extract, with the main steps of injection and elution.



Source: Prepared by the author.

The adsorption of β-glucosidase in the hydrophobic cryogel was investigated under different pH values (3.0, 5.0, 7.0 and 9.0). Purification factors and yield parameters are shown in Table 1. These experimental results were subjected to analysis of variance at 95% of significance level.

Table 1. Capture of β-glucosidase from fermentation crude extract using the prepared cryogel: effect of pH. Results of total protein content (P_T, mg), total activity (A_T, U), specific activity (A_S, U/mg), purification factor (P_F) and yield (Y, %) of the crude extract and the eluted fractions.

Sample	P_T (mg)	A_T (U)	A_S (U/mg)	P_F	Y (%)
Extract	0.11	2.11	18.83	1	100
Eluted pH 3.0	0.02	1.92±0.09	96.70±4.70	5.13±0.24[a]	91.20±4.46[a]
Eluted pH 5.0	0.06	1.80±0.02	29.56±2.80	1.57±0.14[b]	85.41±1.03[a]
Eluted pH 7.0	0.05	1.91±0.14	42.83±1.85	2.27±0.10[b]	90.39±6.54[a]
Eluted pH 9.0	0.04	1.35±0.10	34.06±0.85	1.81±0.05[b]	64.19±4.72[b]

*Mean values followed by the same lower case do not statistically differ from each other ($p<0.05$).

Source: Prepared by the author.

Since each protein could have different behavior in distinct pH values, the optimization of this parameter is essential for protein separation by HIC (Queiroz et al., 2001). In this case, purification fold was affected by pH ($p < 0.0001$), with the highest value occurring at pH 3.0. In general, an increase in pH weakens hydrophobic interactions, probably as a result of increased titration of charged groups, thereby leading to an increase in the hydrophilicity of the proteins. On the other hand, a decrease in the pH results in an apparent increase in hydrophobic interactions (Queiroz et al., 2001; Janson and Rydén, 2011). This is in accordance with the findings obtained in the present work, suggesting that pH is an important parameter in tassays separation via HIC. Beyond hydrophobic interactions, other types of weak interactions such as Van der Waals interactions, weak ion exchange interactions and hydrogen bonds are involved in HIC and may contribute to the results obtained, as also observed by De Oliveira et al. (2009).

According to Table 1, the eluate obtained at pH 3.0 was 2.26 to 3.27 purer than the others, in relation to β-glucosidase content. Electrophoresis would be indispensable to conclude these results, however carrying out this experiment has not been possible yet. The values obtained are considered low for a chromatographic separation step. However, it should be noted that isocratic elution was the option used and so the β-glucosidases adsorbed were not separated from others proteins also adsorbed, contributing to low values of P_F (Mól et al., 2019).

Neves et al. (2020) studied the adsorption proteins from ora-pro-nobis extract on cryogels functionalized with L-phenylalanine and with L-tryptophan as a function of pH (4.0, 5.5 and 7.0) and observed that, for both adsorbents, higher adsorption capacities were observed as the pH increased. On the other hand, De Oliveira et al. (2019) verified that the adsorptive

capacity of pure lysozyme on cryogels containing L-tryptophan was slighter higher at pH 7.0 than at pH 5.0 or 9.0

The yields values obtained in this work, except the one found at pH 9.0, were higher than those obtained by Mól et al. (2019). The same crude extract was applied to an ion exchange cryogel aiming β-glucosidase capture and found that pH 5.0 showed the highest yield of adsorption (82%) and pH 3.0 showed the lowest yield (67%). They also observed that yield was affected by pH variation, but purification fold was not influenced by this parameter at 95% significance level. In both cases, it is worth emphasizing that only one separation step was applied. However, comparing these two process of β-glucosidase adsorption, the results suggest that hydrophobic interaction enable a more selective adsorption of the enzymes from the crude extract, which may require fewer steps in a broader purification process.

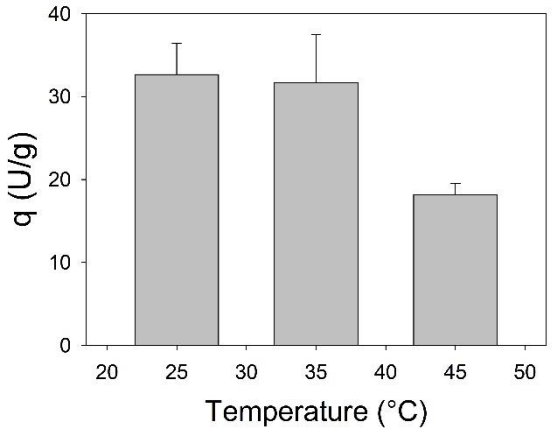
The chromatographic step performed may still be improved through more elaborate studies in order to optimize other parameters, such as type and concentration of the running and elution buffers. Besides that, changes in the elution step may be essential to further improve the proteins separation, since isocratic elution is not the most recommended in this case. However, it was used because the main objective was the evaluation of the adsorption yield as a function of the pH.

3.2. Effect of temperature

The adsorption of β-glucosidase on the cryogel via hydrophobic interaction as a function of temperature is shown in Figure 3. The highest activity retained on the solid phase was obtained at 25 °C, which was quite similar at the one found at 35 °C. However, the activity of β-glucosidase adsorbed considerably decreased at 45 °C.

In general, as temperature increases, the strength of hydrophobic interaction also increases (Janson and Rydén, 2011). However, an opposite behavior can occur on protein retention due to temperature effect on the conformational state of different proteins (Queiroz et al., 2001). It can be noted that in this case the higher temperature was the least efficient for β-glucosidase adsorption. This could also be associated with the thermal stability of the enzyme. Leite et al. (2007) observed that β-glucosidase were stable at 40 °C and 50 °C for 1 h, but the adsorptive time used in this experiment was 24 h, which may have been long enough for the enzyme to begin loss activity.

Figure 3. Effect of temperature on the adsorbed of β-glucosidase on the prepared cryogel.



Source: Prepared by the author.

3.3. Effect of buffer and ionic strength

The influence of salts on HIC is commonly found on the literature (Queiroz et al., 2001; Janson and Rydén, 2011; Hackemann and Hasse, 2017; De Oliveira et al., 2019; Neves et al., 2020). However, the choice of salt type and concentration is strongly dependent of the protein-adsorbent system (Hackemann and Hasse, 2017). The effect of NaCl concentration within different saline buffer on the adsorption of β-glucosidase was evaluated and the experimental results of the adsorbed activity, yield and production of nitrophenol per mass of cryogel containing the adsorbed enzyme are shown in Table 2.

According to Table 2, the adsorption of β-glucosidase by HIC was stronger with higher ionic strengths, using sodium acetate and sodium phosphate as buffer. This is quite expected, since salts promote protein binding on the hydrophobic materials (Queiroz et al., 2011; Janson and Rydén, 2011; De Oliveira et al., 2009).

However, when sodium sulfate and sodium citrate was used as a buffer, it was observed that increasing the molar concentration of NaCl was unfavorable to the adsorption of β-glucosidase on the hydrophobic cryogel. De Oliveira et al. (2019) also observed this behavior in the adsorption of lysozyme on a similar hydrophobic cryogel immobilized with L-tryptophan. They observed that increasing the molar concentration of NaCl was detrimental to the adsorption of lysozyme on the hydrophobic cryogel.

The influence of different anions on hydrophobic interaction follows the Hofmeister series (Janson and Rydén, 2011). Anions that promote hydrophobic interaction most are to the left in the series.

$$SO_4^{2-} > HPO_4^{-2} > CH_3COO^- > Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > SCN^-$$

Also, cations influence the strength of the interaction:

$$Mg^{2+} > Li^{+} > Na^{+} > K^{+} > NH_4^{+}$$

Table 2. Adsorption of β-glucosidase from fermentation crude extracts using the prepared cryogel: effect of ionic strength and salt type.

Salt type	Ionic strength (M)	Q (U/mg)	Yield (%)	Q (μmol pNP/mg)
Sodium acetate	0	5.03±1.29	16.03±3.37	14.58±1.55
	0.5	8.81±1.69	29.86±1.91	15.29±2.15
	1.0	12.81±1.75	38.44±3.92	16.02±3.46
Sodium citrate	0	8.99±0.16	37.23±2.61	37.99±3.56
	0.5	8.57±0.89	45.97±6.29	37.44±0.92
	1.0	6.48±0.82	29.19±4.51	28.02±3.71
Sodium sulfate	0	14.04±1.57	35.67±8.01	21.94±2.12
	0.5	4.42±0.67	13.33±0.73	12.83±0.75
	1.0	3.50±1.31	9.23±2.93	10.96±0.34
Sodium phosphate	0	1.65±0.28	6.95±0.67	11.0±2.61
	0.5	3.62±0	14.45±0	9.81±0.60
	1.0	5.24±0.98	19.71±4.80	10.47±0.22
Potassium sulfate	0	7.81±1.28	39.61±10.76	6.15±0.42
	0.5	4.10±1.22	28.14±0.17	4.59±0.82
	1.0	5.00±0.25	21.47±3.68	6.15±0.74

Source: Prepared by the author.

Analyzing the last column of Table 2, which shows the amount of nitrophenol released by the immobilized β-glucosidase, we verified that sodium sulfate (SO_4^{2-}) possibly contributes more to β-glucosidase adsorption and consequently immobilization than sodium phosphate (HPO_4^{-2}). Meanwhile, the results obtained with sodium acetate (CH_3COO^{-}) were better than those of phosphate. Neves et al. (2020) also verified that an increase in the ora-pro-nobis protein interactions with cryogel containing L-phenylalanine follows the Hofmeister series, as the sodium sulfate contributes more to adsorptive capacity increase than sodium phosphate.

According to Melander and Horvath (1977), the effectiveness of different salts in promoting hydrophobic interaction can be explained by their contribution to the surface tension of the solution. The formation of a cavity in water with a salt that gives a high surface tension

needs a bigger input of energy than does the formation of a cavity with the same surface area in water containing a salt giving lower surface tension. The higher the salt concentration, the stronger the interaction. It is the salts that increase the surface tension most that give the strongest hydrophobic interaction. Factors others than surface tension, such as protein hydration and specific interactions between the protein and the salt ions, can also influence the strength of the interaction.

From the above results, it was observed that the effect of different electrolytes of Hofmeister series on the adsorption process depends on the nature and concentration of the electrolyte. The results of β-glucosidase adsorption using sodium citrate were almost twice as high which indicates that this salt should be used in further studies. Also, the results of the pH experiments could have been even better if citrate had been used.

The results of specific activity (U/mg) and yield (%) were higher than those obtained by Khan et al. (2012), who also used polyacrylamide cryogels as supports. The specific activity of the biocatalyst ranged from 1.1 to 16.9 U/mg, depending on the temperature and on the reactive group of the support, and the maximum immobilization efficiency was 0.7%, even with the addition of glutaraldehyde. However, the values obtained in this work, especially those of yield, are still low in relation to studies involving other supports (Chen et al., 2014; Nishida et al., 2018) and may be improved through the optimization of other parameters, as already mentioned.

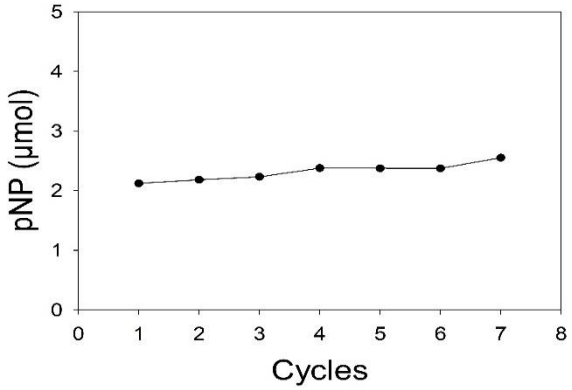
3.4. Operational stability

One of the greatest advantages of immobilized enzymes over free enzymes is the repetitive and continuous use of the biocatalyst, since the reusability is important for economic viability of bioprocess (Chang et al., 2007; Da Silva et al., 2014). The cryogel column containing adsorbed β-glucosidase was used for seven cycles of reaction measured with the synthetic substrate pNPG as substrate without activity loss (Figure 4).

Da Silva et al. (2014) immobilized β-glucosidase from *Aspergillus japonicus* by ionic interactions and observed that the enzyme presented about 50-60% residual activity after five cycles. Enzyme leaching is commonly observed in immobilization procedures by adsorption, resulting in relative low stability (Guo et al., 2019; Irfan et al., 2019). However, this behavior was not observed in this work. On other hand, Chang et al. (2007) immobilized the enzyme on chitosan-clay composite and observed that the activity is lower at the beginning of the recycling, and increases when the beads were repeatedly used, reaching a plateau after the eight use. The

operational stability was also evaluated by Khan et al. (2012) when immobilizing two variants of β-glucosidase on Eupergit® C250L and the biocatalyst lost 25-50% of the original activity after 10 rounds. Chen et al. (2014) showed that the their immobilized β-glucosidase on magnetic nanoparticles retained more than 90% of its original activity after 15 batches of 10 min each. All the studies presented also used pNPG as substrate.

Figure 4. Operational stability of immobilized β-glucosidase using pNPG as substrate.

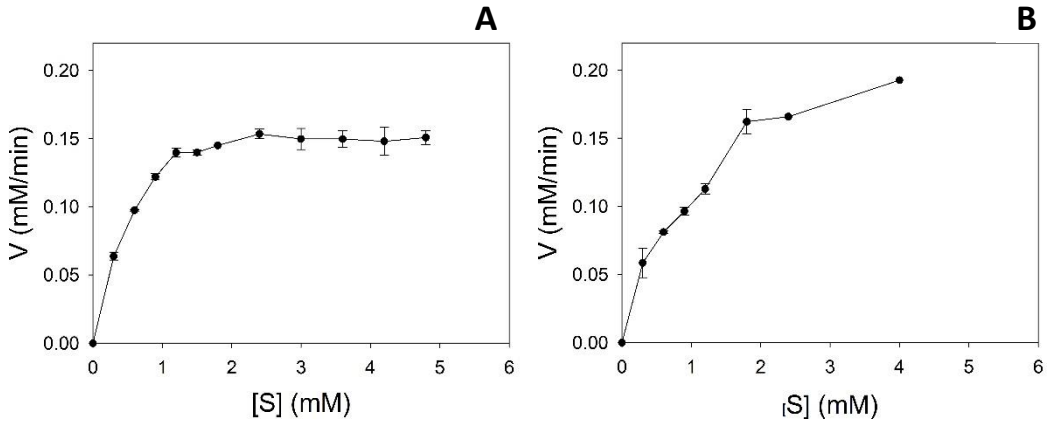


Source: Prepared by the author.

3.5. Kinetics of free and adsorbed enzyme

The Michaelis-Menten curves for free and immobilized β-glucosidase reaction are show in Figure 5. The kinetic parameters K_M and V_{max} were calculated using four different models (Table 3).

Figure 5. Michaelis-Menten plots of free (A) and immobilized (B) enzymes.



Source: Prepared by the author.

Table 3. Kinetic parameters of β-glucosidase from Thermoascus aurantiacus.

Parameter/ Model	Enzyme	K_M	V_{max}	R^2
Lineweaver-Burk	Free	0.65	0.20	0.99
	Immobilized	0.80	0.20	0.95
Eadie-Hofstee	Free	1.57	0.32	0.95
	Immobilized	0.88	0.21	0.83
Hanes-Woolf	Free	0.29	0.16	0.99
	Immobilized	1.24	0.25	0.98
Non-linear equation	Free	0.39	0.17	0.98
	Immobilized	1.32	0.26	0.98

Source: Prepared by the author.

Although all models had fitted well, the behavior of the kinetic parameters as a function of the immobilization process was different. According to Eadie-Hofstee adjustment, K_m and V_{max} decreased after immobilization. On the other hand, according to Lineweaver-Burk and Hanes-Woolf adjustment, the parameters increased. The increase of K_M might be due to the limited accessibility of substrate molecules to active sites of the immobilized enzyme (Zhang et al., 2014) and it was observed by many authors. Also, the decrease in the maximum reaction rate may be observed and attributed to reduction of active sites and limitation of the enzyme flexibility (Sui et al., 2019). However, some authors reported an increase in V_{max}, which may be to enhanced stability (Liang et al., 2012; Çelik et al., 2016). The linearization of kinetic equations is still a widely established practice for determining these parameters, however these methods contain certain drawbacks that may result in misleading estimation of the enzyme parameters (Marasovic et al., 2017). Because of this, the kinetic parameters were also determined by non-linear regression and the results obtained were very similar to those obtained by the Hanes-Woolf method, which indicates that this is probably the most reliable method based on Michaelis-Menten equation.

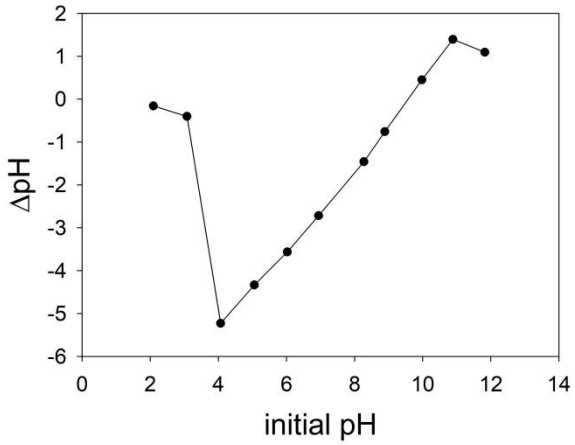
3.6. Characterization of the cryogel

The swelling capacity of the adsorbent was determined as 10.10±0.64 kg/kg and the degree of expansion as 12.21±0.61 L/kg. The first parameter expresses the amount of water absorbed by dry cryogel and the second relates its dehydrated mass with the hydrated volume.

The point of zero charge was determined and the value obtained was 10 (Figure 6). The pH affects the dissociation of the active sites present on the adsorbent surface. Although hydrophobic interaction is the objective, it has already been mentioned that other weak interactions can interfere. The surface is protonated in solutions at pH below the point of zero charge, favoring the adsorption of negatively charged molecules. However, at pH values above the point of zero charge, the surface is deprotonated, which favors the adsorption of positively charged compounds. At pH equal to the point of zero charge, the adsorbent acts as a buffer (Vieira et al., 2010; Mól et al., 2019).

The isoelectric point of β-glucosidase found in the literature ranges from 3.9 to 4.5 (Bedino et al., 1985; Kalogeris et al., 2003). Above this range value up the point of zero charge, electrostatic interaction between enzyme and the adsorbent may occur, contributing to the adsorption. However, the best pH value obtained was 3.0, where both enzyme and adsorbent have positive charge.

Figure 6. Determination of the point of zero charge of the prepared cryogel.



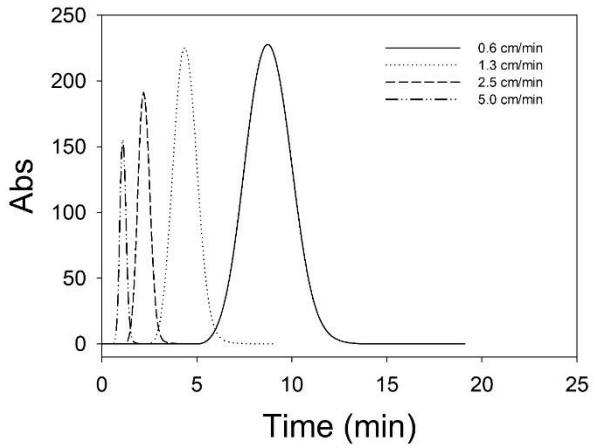
Source: Prepared by the author.

The residence time distribution at different flow velocities of the mobile phase was determined by a tracer pulse method. Figure 7 shows the RTD curves of some water flow velocities. The mean residence time and variance were determined for each flow velocity using the momentum method. The cryogel monolith porosity was determined (Eq. 4) as 0.96.

The axial dispersion coefficients at flow velocities ranging from 0.6 cm/min to 6.4 cm/min was calculated according to Eq. (5) and varied from 10^{-7} to 10^{-6} m^2/s. The values of HETP were also calculated and varied from 1.1 to 1.2 mm (Figure 8). These values are close to those found by other authors (Mól et al., 2017, Mól et al., 2019, Neves et al., 2020). These

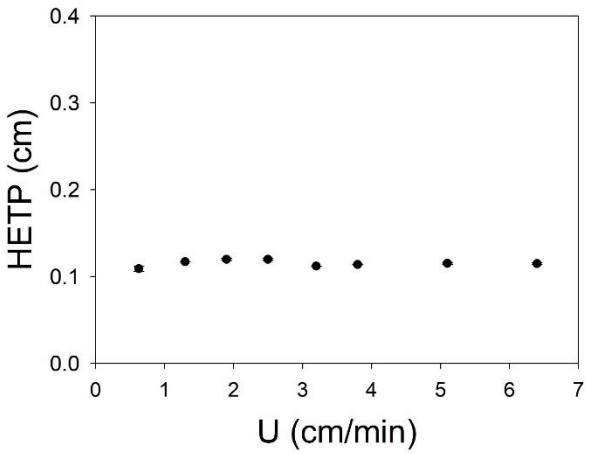
results indicate small dispersion, contributing to high column efficiency and consequently high yields (Mól et al., 2019).

Figure 7. Residence time distribution curves as a function of fluid velocities.



Source: Prepared by the author.

Figure 8. Eight equivalent to a theoretical plate (HETP) for the cryogel as a function of the mobile phase velocities.

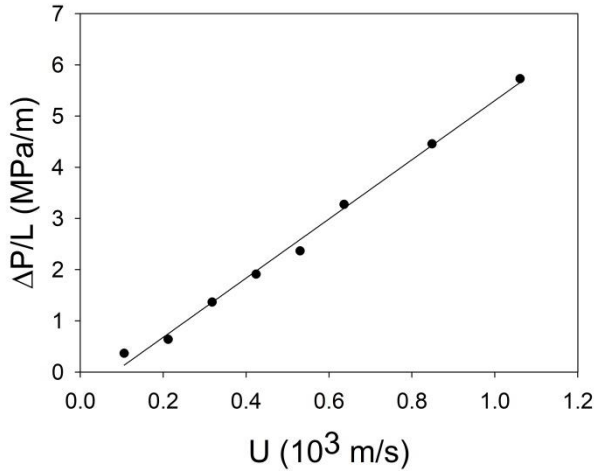


Source: Prepared by the author.

Hydraulic permeability is related to the resistance to flow through the cryogel column. Experimental data for flow resistance is shown in Figure 9. The value determined by the Darcy-Weisbach equation was 1.7 x 10^{-13} m^2, indicating that flow resistance within the cryogel is low. The value is similar to others found for polyacrylamide cryogels (Mól et al., 2017; Veríssimo et al., 2018; Mól et al., 2019) The highly porous structure of the cryogels and the presence of

large pores allow the unobstructed passage of solutes and, consequently, the low flow resistance normally observed.

Figure 9. Experimental data of pressure drop as a function of the water flow rates (●) and the adjusted equation to determine permeability (-).



Source: Prepared by the author.

4. Conclusion

The results obtained so far suggest that the cryogel functionalized with L-phenylalanine can be used as support for purification and immobilization by the principle of hydrophobic interaction. Until that moment, it was verified that pH 3.0 appeared to be the best in relation to the separation of β-glucosidase from the crude extract, as it was the one that presented the highest purification factor. In addition to this chromatography study, β-glucosidase was immobilized on the cryogel via adsorption, as a function of the temperature and after as a function of salt type and concentration. The cryogel containing immobilized enzymes showed hydrolytic activity, demonstrating its probable use as biocatalyst, which was reused for seven cycles without loss of activity. The properties of the support produced that were evaluated were similar to other produced cryogels. To conclude, the work should be improved through studies involving potential applications of the cryogel containing immobilized β-glucosidase.

5. CONCLUSÃO GERAL

A β-glicosidase foi produzida por fermentação em estado sólido, objetivando-se a utilização de subprodutos geralmente descartados.

Os criogéis utilizados como suporte foram sintetizados no laboratório e tiveram suas principais características morfológicas e hidrodinâmicas avaliadas, e os resultados experimentais obtidos indicaram a sua adequabilidade para utilização em ensaios de purificação e imobilização da β-glicosidase produzida.

A avaliação geral dos resultados obtidos mostrou que os criogéis produzidos possuem potencial de aplicação na purificação e imobilização da β-glicosidase, uma vez que a enzima manteve sua atividade catalítica após a etapa de adsorção, o que é essencial para a continuação dos estudos destes processos. Por meio da cromatografia de troca iônica, foi possível observar purificação parcial do extrato bruto utilizado.

Entretanto, mais estudos precisam ser desenvolvidos visando otimizar a interação da enzima com o suporte e consequentemente melhorar parâmetros como eficiência de separação, eficiência de imobilização, fator de purificação, entre outros.

Além disso, por meio da revisão de literatura realizada, foi possível concluir que embora grande parte dos resultados obtidos com a imobilização de β-glucosidase sejam significativos, o número de enzimas imobilizadas realmente implantando no mercado ainda é baixo, o que requer constantemente a pesquisa pelo método, tipo de suporte e fonte de enzima que seja o mais adequado possível para a aplicação industrial.

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ANEXO A – Direitos do uso do artigo do periódico Process Biochemistry em tese