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MURILO AMARAL FONSECA

SACARIFICAÇÃO E ISOMERIZAÇÃO SIMULTÂNEAS DE DEXTRINA NA PRODUÇÃO DE XAROPE DE FRUTOSE POR AÇÃO SINÉRGICA DE CLEAs MAGNÉTICOS DE AMILOGLICOSIDASE E SWEETZYME®

SÃO CARLOS-SP
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FEDERAL UNIVERSITY OF SÃO CARLOS
CENTER OF EXACT SCIENCES AND TECHNOLOGY
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SIMULTANEOUS SACCHARIFICATION AND ISOMERIZATION OF DEXTRIN IN THE PRODUCTION OF FRUCTOSE SYRUP BY SYNERGIC ACTION OF MAGNETIC CLEAs OF AMYLOGLUCOSIDASE AND SWEETZYME®

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Thesis submitted to Postgraduate Program in Chemical Engineering at the Federal University of São Carlos in partial fulfillment of the requirements for the Doctor of Philosophy Degree in Chemical Engineering, area of concentration in Chemical Process Research and Development.

Advisor: Prof. Dr. Paulo Waldir Tardioli

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DEDICATÓRIA

Dedico este trabalho a minha família, meus pais José Alfredo e Eloisa, meu irmão Bruno e aos meus avós, Jonas e Lúcia (*In memoriam*), Walter (*In memoriam*) e Zélia.

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“One who does his best does all that can be asked” – Helena P. Blavatsky.

“Man Plans, and God Laughs” – Yiddish adage.

RESUMO

O xarope rico em frutose é um adoçante amplamente utilizado como substituto da sacarose pela indústria de alimentos e bebidas, com muitas vantagens, como doçura relativa superior à sacarose, alta solubilidade, resistência à cristalização e ação umectante. Sua produção industrial atual é via enzimática, ocorrendo basicamente em três processos: liquefação, sacarificação e isomerização, pela ação das enzimas α-amilase, amiloglicosidase (AMG) e glicose isomerase (GI), respectivamente. Devido ao fato de que essas enzimas operam em diferentes condições de pH e temperaturas, os três processos são realizados de maneira sequencial, demandando tempo, equipamentos e reagentes para o ajuste do pH. Além disso, as enzimas α-amilase e amiloglicosidase são aplicadas na forma solúvel, limitando seu uso a operações em bateladas. A glicose isomerase, por ser de grande interesse industrial, é comercializada em sua forma imobilizada. Na busca por alternativas para aumentar a produtividade e eficiência do processo com menores custos operacionais, este trabalho teve como objetivo estudar a aplicação de um processo simultâneo de sacarificação e isomerização, que pudesse ser operado repetidamente, reutilizando os biocatalisadores empregados. Para desenvolver esse processo multi-enzimático simultâneo, seria inicialmente necessário realizar a imobilização da AMG, com o intuito de torna-la insolúvel e operacionalmente mais estável. O preparo de agregados enzimáticos entrecruzados, conhecida como CLEA (do inglês cross-linked enzyme aggregate), é uma técnica simples, custo-efetiva e sem o uso de suporte, capaz de gerar biocatalisadores insolúveis com alta atividade volumétrica e com estabilidade melhorada. Seu preparo consiste na agregação da enzima, por precipitação, e sua posterior reticulação, com um agente bifuncional. Neste trabalho, os CLEAs de amiloglicosidase foram preparados co-agregados na presença de polietilenoimina (PEI) e/ou amido, com nanopartículas magnéticas aminadas (MNPs) ou albumina de soro bovino (BSA), com o intuito de melhorar as propriedades do catalisador. Os CLEAs preparados apenas com MNPs em diferentes concentrações de glutaraldeído forneceram atividade recuperada de cerca de 20%. A adição de amido ou PEI aumentaram a atividade recuperada para em torno duas vezes (40%). Além disso, sob as mesmas condições, a AMG co-agregada com BSA também foi sintetizada, produzindo CLEAs com atividades recuperadas muito semelhantes. Ambos os CLEAs (co-agregados com PEI e MNPs ou BSA) foram quatro vezes mais estáveis que a enzima solúvel. Estes CLEAs também foram avaliados na hidrólise do amido a 35% (m/v), alcançando mais de 95% de conversão de amido em glicose, medida como Dextrose Equivalente (DE). Além disso, os dois tipos de CLEAs puderam ser reutilizados por cinco ciclos, mantendo uma DE em torno cerca de 90%. Embora ambos CLEAs tivessem boas propriedades, os CLEAs magnéticos foram mais atraentes devido à sua fácil separação por um campo magnético externo. Com os biocatalisadores imobilizados (CLEA de AMG e a GI) foi possível trabalhar em uma janela de operação mais ampla, permitindo a aplicação de um planejamento fatorial com delineamento composto central rotacional, que foi capaz de definir uma condição ótima de pH e temperatura do processo, bem como a melhor relação entre as duas enzimas. A sacarificação e isomerização simultâneas a partir de uma solução de dextrina a 35% (m/v) atingiram uma DE acima de 95%, com rendimentos de conversão em torno de 48% de frutose ao final de 30 h de reação. Além disso, os catalisadores puderam ser reutilizados por seis ciclos consecutivos, mantendo a conversão em torno de 47% de frutose sem perda de atividade e com fácil recuperação dos biocatalisadores. Além disso, por serem de naturezas diferentes (CLEA magnético de AMG e pellets de GI), caso haja a inativação de um dos biocatalisadores, estes poderiam ser facilmente separados e recarregados individualmente.

Palavras-chave: Xarope rico em frutose. Sacarificação e isomerização simultânea. Agregados de enzima entrecruzados. Amiloglicosidase. Glicose isomerase.

ABSTRACT

High fructose syrup is a sweetener widely used as a substitute for sucrose by the food and beverage industry. It has many advantages such as relative sweetness superior to sucrose, high solubility, crystallization resistance and humectant action. Its current industrial production is by enzymatic route basically occurring in three processes: liquefaction, saccharification and isomerization, by the action of the enzymes α-amylase, amyloglucosidase (AMG) and glucose isomerase (GI), respectively. Due to the fact that these enzymes are conducted under different conditions, all three processes are sequential, requiring time, equipment, and reagents for pH adjustment. Moreover, the enzymes α-amylase and amyloglucosidase are applied in a soluble form, limiting their use to batch operations. Due to its considerable industrial interest, glucose isomerase is marketed in its immobilized form. In the search for alternatives to increase productivity and efficiency of processes with lower operating costs, the aim of this research is to study the application of a simultaneous saccharification and isomerization process that could be operated repeatedly, reusing the biocatalysts employed. In order to develop this simultaneous multi-enzymatic process, initially it would be necessary to immobilize the AMG in order to make it insoluble and operationally more stable. The preparation of cross-linked enzyme aggregates (CLEA) is a simple, cost-effective and carrier-free technique capable of generating insoluble biocatalysts with high volumetric activity and improved stability. Its preparation consists of enzyme aggregation by precipitation and its subsequent cross-linking with a bifunctional agent. In this study, the CLEAs of AMG were prepared co-aggregated in the presence of polyethyleneimine (PEI) and/or starch, with aminated magnetic nanoparticles (MNPs) or bovine serum albumin (BSA), in order to improve the properties of the catalyst. The CLEAs prepared only with MNPs at different glutaraldehyde concentrations yielded a recovered activity of around 20%. The addition of starch or PEI increased the recovered activity around twofold (40%). Moreover, under the same conditions, AMG co-aggregated with BSA was also synthesized, yielding CLEAs with very similar recovered activity. Both CLEAs (co-aggregated with MNPs or BSA) were four times more stable than the soluble enzyme. These CLEAs were also evaluated in the hydrolysis of starch at 35% (w/v), achieving more than 95% starch-to-glucose conversion measured as Dextrose Equivalent (DE). Besides, both CLEAs could be reused for five cycles maintaining a DE of around 90%. Although both CLEAs had good properties, magnetic CLEAs could be more attractive because of their easy separation by an external magnetic field. Having the immobilized biocatalysts (AMG CLEA and GI) it was possible to work in a wider operational window, allowing the application of a factorial design with a central composite rotatable design, which was able to define an optimum pH and process temperature condition, as well as the best relation between the two enzymes. Simultaneous saccharification and isomerization from a dextrin solution 35% (w/v) reached a DE above 95%, with conversion yields around 48% of fructose at the end of 30 h of reaction. In addition, the catalysts could be reused for six consecutive cycles, maintaining conversion yields around 47% of fructose without loss of activity and with easy recovery of the biocatalysts. Furthermore, because they are of different natures (magnetic CLEA of amyloglucosidase and pellets of GI), if there is any inactivation of one of the biocatalysts, they could be easily separated and recharged individually.

Keywords: High fructose syrup. Simultaneous saccharification and isomerization. Cross-linked enzyme aggregate. Amyloglucosidase. Glucose isomerase.

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1 INTRODUCTION

Many of the key components of the modern diet were sugars. Natural, refined, artificial, caloric or not (WHITE, 2014). Sugar consumption is growing every year worldwide, showing evidence of a high market demand, in which the most important products are sucrose and high fructose syrup.

High fructose syrup is a sweetener formed by free monosaccharides of glucose and fructose. In the United States of America, the main producer and consumer of this sweetener, it is produced from corn starch know as High Fructose Corn Syrup (HFCS), a sweetener with a relative sweetness similar to sucrose or even higher (according to the concentration of fructose present). Other advantages of HFCS are high solubility, crystallization resistance and humectant action (PARKER; SALAS; NWOSU, 2010; WHITE, 2014).

Widely applied in the food industry, HFCS is produced by an industrial process that basically includes three main steps (CRABB; MITCHINSON, 1997; MARSHALL; KOOI, 1957).

a) liquefaction: hydrolyzes the starch polymer to oligosaccharides and glucose with hydrochloric acid and/or α-amylase (EC 3.2.1.1);
b) saccharification: hydrolyzes the oligosaccharides to glucose with amyloglucosidase (AMG) (EC 3.2.1.3, glucoamylase);
c) isomerization: isomerizes glucose to fructose by glucose isomerase (GI) (EC 5.3.1.5, xylose isomerase).

These sequential processes occur in various separate steps due to the fact that these enzymes are operated under different conditions, requiring time, equipment, and reagents to adjust the pH, which generates a cost and a large amount of salts (HOBBS, 2009; KANEKO; TAKAHASHI; SAITO, 2000).

Nevertheless, as it is an enzymatic process, fructose production has advantages, such as the high selectivity and specificity of the enzymes, avoiding the formation of by-products, and mild operational conditions of temperature, pressure and pH (LEHNINGER; NELSON; COX, 1992).

However, enzymes are relatively expensive and unstable catalysts that can be

easily inactivated and/or denatured when used in the soluble form, which also makes their recovery and reuses impracticable, and also limits their use to batch operations with catalytic activity limited by the medium (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013; GUISAN, 2006a). As an alternative to overcome such drawbacks, enzymatic immobilization techniques can be applied to produce an insoluble biocatalyst, allowing its recovery and reuse in the process, as well as allowing its use in continuous operations and with greater operational stability (HOMAEI et al., 2013; KENNEDY; CABRAL, 1987; MATEO et al., 2007).

Among the existing immobilization methods, the cross-linked of enzyme aggregate (CLEA) is a simple, cost-effective and carrier-free technique which basically consists of enzyme aggregation by precipitation, followed by cross-linking with a bifunctional agent (CAO; VAN RANTWIJK; SHELDON, 2000; SCHOEVAART et al., 2004; SHELDON, 2007a). The CLEA may also be prepared co-immobilized in the presence of additives, such as bovine serum albumin, magnetic nanoparticles, polyethyleneimine, etc., in order to improve the final properties of the biocatalyst (SHELDON, 2019).

CLEAs of many different enzymes have been reported: penicillin G acylase, invertase, lipase, laccase, galactose oxidase, trypsin, glucose oxidase, β-galactosidase, alcohol dehydrogenase, catalase, β-amylase, cyclodextrin glucosyltransferase, etc. (ARAUJO-SILVA et al., 2018; MAFRA et al., 2016, 2018; RAMOS et al., 2018; ROJAS et al., 2019; SCHOEVAART et al., 2004; TALEKAR et al., 2010; WILSON et al., 2004), including amyloglucosidase (GUPTA et al., 2013; NADAR; RATHOD, 2016). However, these studies have not yet validated the amyloglucosidase performance with high starch concentration (typical industrial conditions).

On the other hand, glucose isomerase is commonly marketed in its immobilized form by companies such as Genencor/DuPont and Novozymes A/S (DICOSIMO et al., 2013; JØRGENSEN et al., 1988).

In this context, the aim of this study was to evaluate the simultaneous saccharification and isomerization of dextrin to fructose syrup catalyzed by immobilized amyloglucosidase and glucose isomerase. As the latter is marketed in the immobilized form it was necessary to immobilize the former using the CLEA technique with magnetic nanoparticles to allow the easy separation of the two biocatalysts when one of them is inactivated. Also, a factorial design was performed to define an optimum

operational condition for both immobilized enzymes in the selected reaction.

The specific objectives of this work were the following:

a) immobilize amyloglucosidase by the CLEA technique, evaluating precipitating and crosslinking agents, and optimize the catalyst, evaluating the co-aggregation in the presence of additives such as magnetic nanoparticles, polyethyleneimine, starch, and bovine serum albumin, aiming at high retention of activity and thermal stability;
b) evaluate the main parameters for the action of amyloglucosidase CLEA on the hydrolysis of commercial starch under industrial conditions and the operational stability of the biocatalyst, performing recovery and reuse assays;
c) select the optimum conditions of pH, temperature, and the ratio between the amyloglucosidase and glucose isomerase enzymes, applying the factorial design method and then validating the parameters obtained;
d) evaluate the process of simultaneous saccharification and isomerization in the production of high fructose syrup using high concentrations of substrate and evaluate the operational stability of the biocatalysts by conducting recovery and reuse tests.

This thesis is structured in chapters as follows:

Chapter 2: "Literature review", addresses the main topics concerning high fructose syrup production (starch, amylolytic enzymes, production of starch hydrolysates and high fructose syrup), multi-enzymatic systems and enzyme immobilization (binding to the support, entrapment and cross-linked enzyme aggregates);

Chapter 3: “Preparation of magnetic cross-linked amyloglucosidase aggregates: solving some activity problems”, reports the immobilization of amyloglucosidase by the CLEA technique in an article format published in Catalysts 2018, Volume 8, Issue 11, 496 (DOI: 10.3390/catal8110496);

Chapter 4: “Optimization of simultaneous saccharification and isomerization of dextrin to high fructose syrup using a mixture of immobilized amyloglucosidase and glucose isomerase by design of experiments”, reports in article format, selecting

optimal conditions for high fructose syrup production in a simultaneous saccharification and isomerization process;

Chapter 5: “General conclusions”, a compilation of the main results obtained from this work.

2 LITERATURE REVIEW

2.1 STARCH

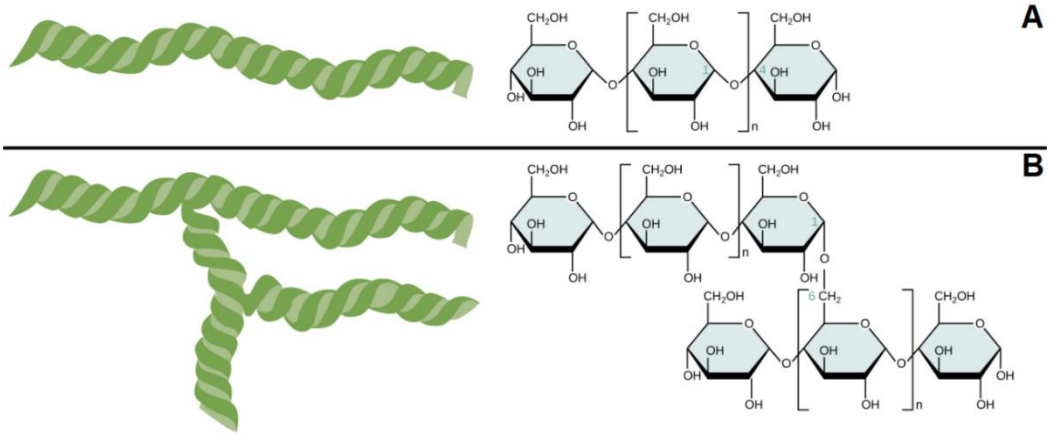
Starch is the main energy reserve of higher plants and it is a polysaccharide composed of glucose units (CEREDA, 2001). This carbohydrate is the second most synthesized by plants adopting the photosynthesis process, after cellulose (GUZMÁN-MALDONADO; PAREDES-LÓPEZ; BILIADERIS, 1995). The synthesis and storage of starch granules are performed by amyloplast, an intracellular organelle that occurs in a wide range of plant tissues. The synthesized starch granules vary according to the botanical origin, presenting different characteristics of size (1-100 μm in diameter), shape (round, lenticular, polygonal), distribution (uni- or bi-modal) and composition (α-glucan, lipid, moisture, protein and mineral content), and are thus able to present simple or compound association (granular clusters) (TESTER; KARKALAS; QI, 2004).

The starch granules are composed by an association of two polysaccharides, amylose and amylopectin and the ratio of these two components varies according to the plant source.

The amylose (Figure 2.1 a) is formed by long chains of glucose units (about 200-700 units) predominantly (99%) formed by linear glycosidic bonds α (1→4), arranged in a helix format, containing six units of glucose at each turn (TESTER; KARKALAS; QI, 2004; ZOBEL, 1988). Due to its tightly packed structure with intramolecular hydrogen bonds the amylose is insoluble in cold water (GREEN; BLANKENHORN; HART, 2009).

Amylopectin (Figure 2.1 b) is a larger molecule than amylose, formed by 95% of α (1→4) glycosidic bonds. These linear bonds form the backbone, ranging in size from 35-100 glucose units and the other 5% is formed by α (1→6) glycosidic bonds in the branches, containing about 15-35 glucose units (TESTER; KARKALAS; QI, 2004; ZOBEL, 1988). By having an open and branched structure, the amylopectin molecule can be easily accessed by the solvent molecules, making it soluble in cold water (GREEN; BLANKENHORN; HART, 2009).

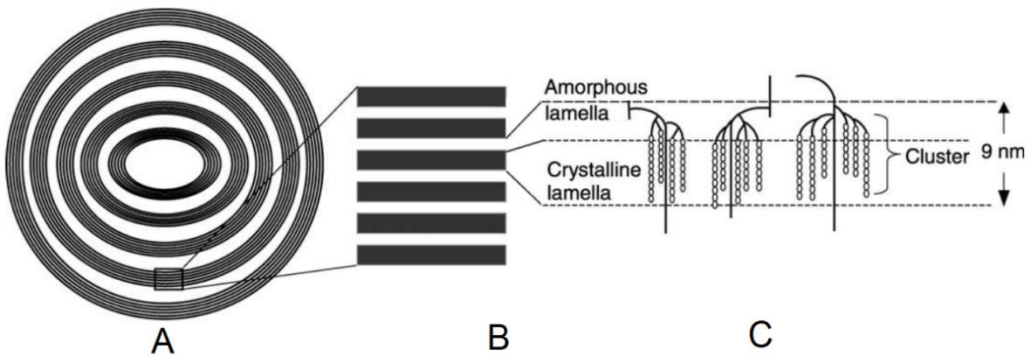
Figure 2.1 — Chemical structure and model of amylose (a) and amylopectin (b).



Source: Adapted from CNX OpenStax (2016).

The ratio between amylose and amylopectin and its organization within the semi-crystalline ultrastructure of starch granules is complicated and also depends on its botanical origin (BULÉON et al., 1998). Starches with high indices of amylopectin are subject to forming double helices of interlacing of amylopectin adjacent chains, forming clusters called crystalline lamellae, which alternate between amorphous lamellae that contain the branching points. This radial arrangement of layers comprises multiple rings in a model called "growth rings", originally proposed by French (1972), and is widely accepted today (Figure 2.2) (BULÉON et al., 1998; TESTER; KARKALAS; QI, 2004).

Figure 2.2 — Representation of starch granule and lamellar structure. (A) Microcrystalline lamellae separated by amorphous growth rings (A); Magnified view of the amorphous and crystalline regions (B); Double helice structures and branching points.



Source: Adapted from TESTER et al. (2004). Reprinted from Journal of Cereal Science, Vol 39, Richard F. Tester,John Karkalas,Xin Qi, Starch—composition, fine structure and architecture, 151-165, Copyright (2019), with permission from Elsevier

Starch is widely used as a food and this polysaccharide is essential in the human diet, representing around 70 to 80% of the calories consumed (CEREDA, 2001). The most common sources of starch are cereal grains (containing 40 to 90% starch), legumes (30 to 70%) and tubers (65 to 85%). Starch production worldwide is mainly extracted from maize and potatoes (GUZMÁN-MALDONADO; PAREDES-LÓPEZ; BILIADERIS, 1995).

Besides being used as a food source, starch has several applications and is used in textile, pharmaceutical, chemical, and especially in the food and beverage industry. In the food industry, starch is applied in its native, modified and hydrolyzed form (CEREDA, 2001).

2.2 AMYLOLYTIC ENZYMES

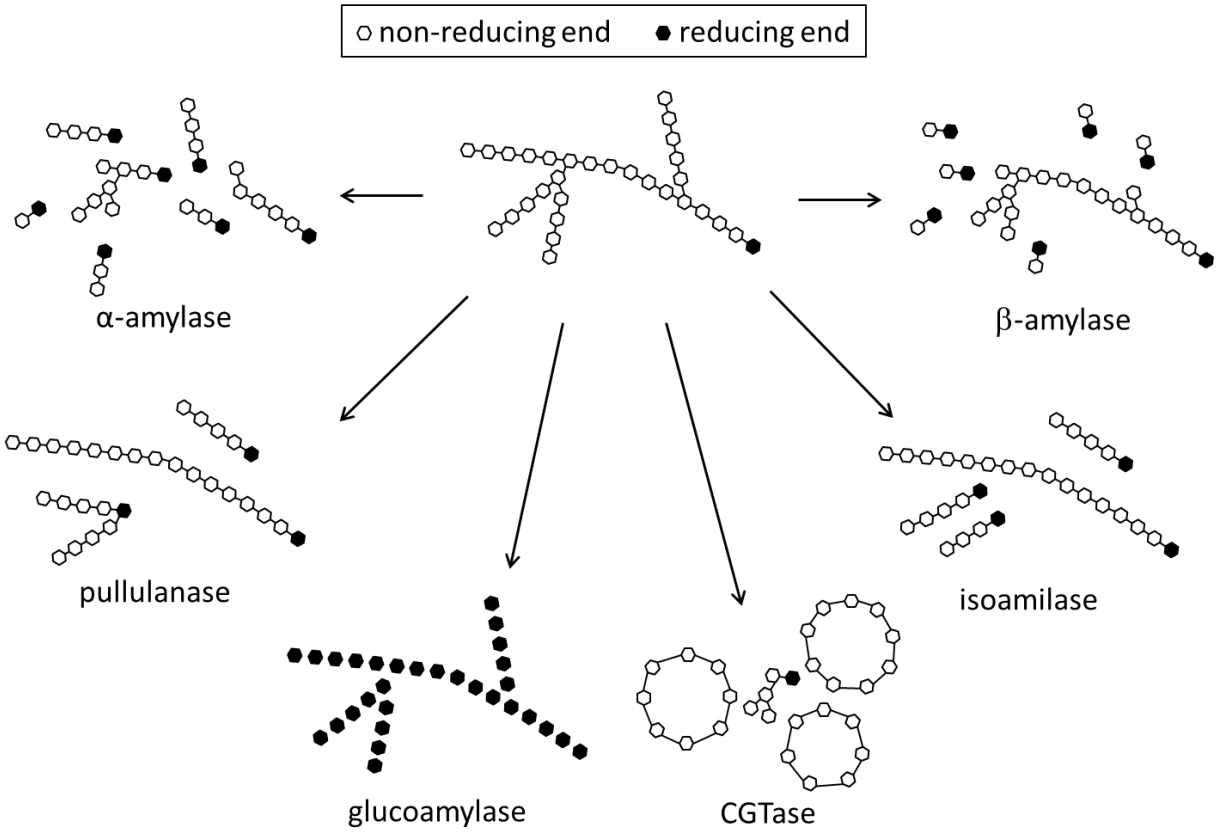
Various products of industrial interest are obtained from total or partial starch hydrolysis. In order to achieve only the desired products, avoiding undesirable reaction, the enzymatic route is preferential, due to its selectivity, and also to its high specificity, reacting with specific substrates. Another interesting aspect of using enzymes is that they catalyze reactions under mild pH and temperature conditions, avoiding "darkening", which is very important in starch hydrolysis (CEREDA, 2001; NIGAM; SINGH, 1995).

Enzymes specialized in catalyzing reactions using starch as substrate are known as amylolytic enzymes. Amylolytic enzymes or amylases are found in plant, animal, and microbial sources. These enzymes can be classified according to the mechanism of action and according to the action.

By classifying them according to the mechanism of action, they are divided into two groups: endoamylases, which catalyze the hydrolysis inside the starch molecule randomly; and exoamylases, which perform hydrolysis at the non-reducing ends of the starch molecule (CEREDA, 2001; FOGARTY; KELLY, 1990; GUPTA et al., 2003; NIGAM; SINGH, 1995; TOMASIK; HORTON, 2012).

The main ones classified according to the action are (Figure 2.3):

Figure 2.3 — Amylolytic enzymes and catalyzed reactions.



Source: Adapted from (TOMASIK; HORTON, 2012).

a) α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase): an endo-enzyme that catalyzes the hydrolysis of α (1→ 4) bonds, releasing dextrins and oligosaccharides. It is widely used industrially in starch liquefaction, drastically reducing the viscosity of the gelatinized starch;
b) β-amylase (EC 3.2.1.2; 1,4-α-D-glucan maltohydrolase): exo-enzyme that hydrolyzes α (1→4) bonds, releasing maltose only. Associated with α-amylase, it produces maltose and dextrins of different molecular weights since the hydrolysis of α (1→6) bonds does not occur.
c) glucoamylase (EC 3.2.1.3; 1,4-α-D-glucan glucohydrolase or amyloglucosidase): this enzyme selectively hydrolyzes the α (1→4) bonds from a non-reducing end, also capable of hydrolyzing α (1→6) bonds more slowly. It is used in industry to liquefy and saccharify the starch completely into glucose molecules.

d) debranching enzymes: Isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41) are the most common endo-enzymes capable of hydrolyzing only the α (1→6) bonds of the starch branches. Among them, only pullulanase is able to hydrolyze the pullulan.
e) cyclodextrin glucosyl transferase (EC 2.4.1.19; CGTase) are endo-enzymes capable of hydrolyzing dextrins of 7, 8 and 9 glucose units and cyclizing these dextrins by ligand binding α (1 → 4).

2.3 STARCH HYDROLYSATE PRODUCTION

Among the catalysts presented previously, two enzymes are particularly important in the industrial scenario: α-amylase and amyloglucosidase, which are enzymes required in the two basic stages of starch conversion, liquefaction and saccharification.

In the industrial processing of starch, the first step of the process consists of starch gelatinization. A solution of starch granules (30-40%, w/v) is heated in a jet cooker at temperatures between 90-110 °C. Under these conditions, the starch granules begin to hydrate, disrupting the hydrogen bonds of the double helices and collapsing the crystalline structure. This swelling of granules solubilizes the starch and makes the solution very viscous, forming a gel. To minimize the high viscosity of the solution, some amounts of thermostable α-amylase are added at the beginning of the process, liquefying part of the solution until they are thermally inactivated (CRABB; MITCHINSON, 1997; GUZMÁN-MALDONADO; PAREDES-LÓPEZ; BILIADERIS, 1995; POLAINA et al., 2007; TESTER; QI; KARKALAS, 2006).

After the gelatinization process, the starch polymer is ready for the enzymatic action and the liquefaction process can be initiated. At this stage, the temperature is maintained between 90-105 °C, pH 6.0, and a new amount of α-amylase is added to the solution. At the end of the process, maltooligosaccharides are formed and the solution is completely liquefied (CRABB; MITCHINSON, 1997; TOMASIK; HORTON, 2012).

The processing time and type of α-amylase used may vary according to the expected product. The saccharifying α-amylases are capable of hydrolyzing 50-60%

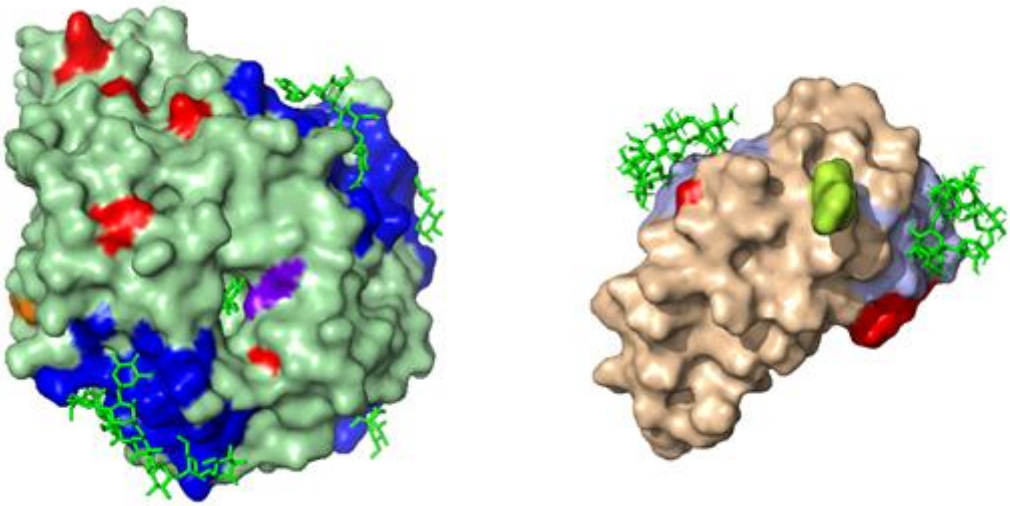
of the starch and are used in the production of maltodextrins of a lower degree of polymerization. The liquefying α-amylases can hydrolyze about 30-40% of the starch and are used for the formation of dextrins. They are used in the following saccharification process (GUZMÁN-MALDONADO; PAREDES-LÓPEZ; BILIADERIS, 1995; TOMASIK; HORTON, 2012; VIHINEN; MANTSIILA, 1989).

The saccharification process occurs after starch liquefaction. At this stage, the oligosaccharides and dextrins previously obtained are hydrolyzed in glucose units, producing high glucose syrup, that have a content of 96-98% glucose. To perform the saccharification process, the enzyme amyloglucosidase (AMG; 1,4-α-D-glucan hydrolase; EC 3.2.1.3) is applied, catalyzing the release of glucose from the non-reducing ends, selectively hydrolyzing the α (1→4) bonds and also the glycosidic α (1→6)bonds slowly (CRABB; MITCHINSON, 1997; PARKIN, 2007). Unlike the previous processes, this stage occurs at mild temperatures (40-60 °C) and at pH 4.5-6.0.

The AMG can be obtained from bacteria, yeast and fungi, and fungal amyloglucosidases are widely used in industry, such as those produced by *Aspergillus niger* (LEE; PAETZEL, 2011; SORIMACHI et al., 1997; SVENSSON et al., 1983a). The *A. niger* produces two isoforms of AMG with different shapes and molecular weights: the G1 isoform, which corresponds to a protein having a catalytic domain (structure on the left in Figure 2.4), and a starch-binding domain (structure on the right in Figure 2.4) with a total of 640 amino acid residues and Mw around 68 kDa, and G2 isoform, which contains only the catalytic domain with 470 amino acid residues and Mw around 50 kDa (LEE; PAETZEL, 2011; SORIMACHI et al., 1997). Both isoforms are highly glycosylated by both N-linked and O-linked carbohydrates. These isoforms are derived from the same genetic material but differ because of a different RNA splicing after transcription (TOMASIK; HORTON, 2012).

The glucose syrup produced is applied in the food, beverage and fermentation industry and is also widely used as a substrate to produce others syrups, such as high fructose syrup (CRABB; MITCHINSON, 1997; PAZUR; ANDO, 1959; TOMASIK; HORTON, 2012).

Figure 2.4 — Three-dimensional structures of *A. niger* amyloglucosidase, showing the catalytic domain on the left and the starch-binding domain on the right. Color patterns: red (lysine residues), purple (active site), orange (N-terminal residue), lemon (C-terminal residue), blue and light blue (glycosylated regions in the catalytic and starch binding domains, respectively).



Source: Adapted from 3eqa (left) and 1ac0 (right) PDB structures (LEE; PAETZEL, 2011; SORIMACHI et al., 1997), respectively). The figures were generated using PyMol (The PyMol Molecular Graphics System; Version 2.1.0; Schrödinger, LLC).

2.4 HIGH FRUCTOSE SYRUP

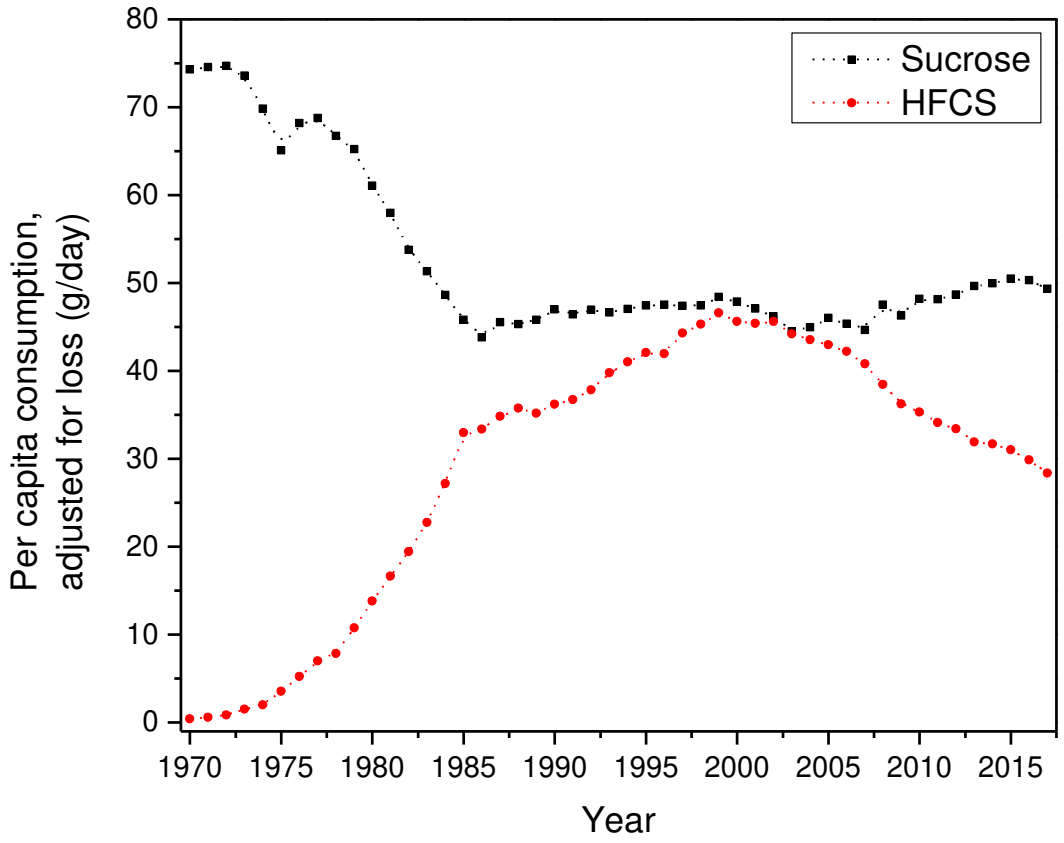
High Fructose Syrup (HFS) is a liquid sweetener produced from the starch hydrolysate (glucose syrup), which passes through the enzymatic process of isomerization, converting the glucose into fructose. In the USA, this syrup is produced mostly from corn starch, known as High Fructose Corn Syrup (HFCS), a sweetener widely used in the food, beverage and pharmaceutical industries (PARKER; SALAS; NWOSU, 2010; WHITE, 2008, 2014).

The development of the HFCS production process began in 1957 with studies by MARSHALL & KOOI showing the ability of the xylose isomerase enzyme produced by *Pseudomonas hydrophila* to isomerize D-glucose in D-fructose. In 1961, TSUMURA & SATO investigated the parameters that influenced the formation of fructose from glucose through the *Aerobacter cloacae* microorganism. However, only in 1972 was it possible to scale up the production of HFCS with 42% fructose in an economically

viable process. This was done through a joint venture between the Japanese Agency of Industrial Science and Technology (AIST), holder of the heat-stable enzyme xylose isomerase from *Streptomyces sp.* isolated by the scientist Yoshiyuki Takasaki, and the North American company Clinton Corn Processing Company, which immobilized the enzyme and applied it in a process of continuous production (PARKER; SALAS; NWOSU, 2010; WHITE, 2014).

In the USA, corn (maize) is the most used crop to obtain starch. As its cultivation is highly subsidized by the US government, corn starch is an abundant and renewable raw material for HFCS production. Furthermore, fluctuations in the availability and price of sucrose stimulated the adoption of HFCS as a substitute for sucrose by the US industry, rising to a peak in 1984 when it was adopted as an integral substitute in products such as Coca-Cola and Pepsi. After developing and consolidating the glucose isomerization process, HFCS consumption increased exponentially until 1985 and continued to increase until 1998, when it reached values close to sucrose consumption, as shown in Figure 2.5 (WHITE, 2014).

Figure 2.5 — Per capita consumption of sucrose and HFCS.



Source: Adapted from UNITED STATES DEPARTMENT OF AGRICULTURE (2018).

High fructose syrup is a very attractive food ingredient for the food and beverages industry. One of its main advantages is the relative sweetness superior to sucrose (reference sugar, 100), according to the concentration of fructose. Corn syrups (most common) are presented in three concentrations of fructose (HOBBS, 2009; PARKER; SALAS; NWOSU, 2010; WHITE, 2008):

a) HFCS 42% (42% fructose, 58% glucose): it has an average relative sweetness of 92, promotes a sweetness that does not mask the natural flavor. It is used in sauces, canned fruit, baked goods, soups, condiments and processed foods;
b) HFCS 55% (55% fructose, 45% glucose): it has an average relative sweetness of 105 and sweetness superior to sucrose. It is obtained from the blend between HFCS 42% and 90% and is used widely in the beverage industry to sweeten soft drinks;
c) HFCS 90% (90% fructose, 10% glucose): it has an average relative sweetness of 140 and is obtained from HFCS 42% after liquid chromatography on a cation exchange resin, enriching the fructose concentrations; it is mainly used in a blend with the HFCS 42% for the production of HFCS 55%, and also for the production of crystalline fructose.

Other advantages of HFS on sucrose are: high solubility, fructose is four times more soluble than glucose and twice as soluble as sucrose; resistance to crystallization, the ability to remain in solution facilitates the transport and storage while retaining the original characteristics, different from sucrose that under certain conditions recrystallizes; stability in acidic foods, it does not undergo auto-hydrolysis, which causes alterations in aroma and taste (PARKER; SALAS; NWOSU, 2010; WHITE, 2014).

Syrups with a higher concentration of fructose, such as HFCS 90% and pure fructose (crystalline or liquid), can be used as alternative sweeteners for diabetics. Fructose is slowly reabsorbed through the stomach and intestinal tract and does not trigger the production of insulin by the pancreas. The proviso that excessive consumption of any type of sweetener can cause damage should always be remembered (CRAPO; KOLTERMAN; OLEFSKY, 1981; MOORE et al., 2000).

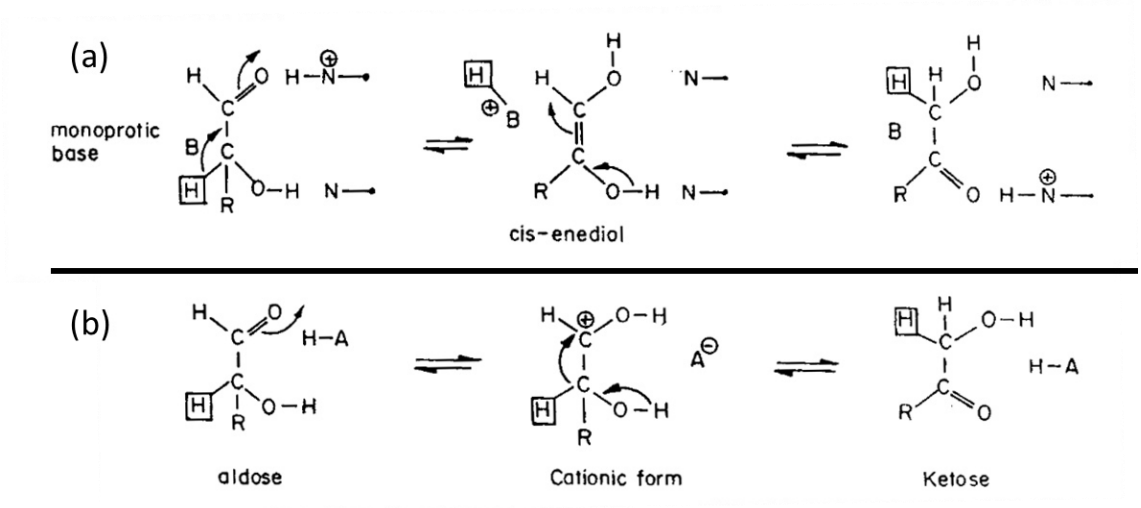
As previously stated, fructose syrup is produced by the isomerization of glucose (obtained at the saccharification stage) in an *in vitro* reaction catalyzed by xylose

isomerase enzyme (also known as glucose isomerase), which *in vivo* catalyzes the isomerization of xylose in xylulose. The isomerization process occurs at temperatures of 40-80 °C and pH 6.0-9.0, requiring the presence of divalent cations, such as Co^{2+}, Mg^{2+} or Mn^{2+}, in which Co^{2+} and Mg^{2+} are indispensable for activation and stabilization of enzymatic activity (BHOSALE; RAO; DESHPANDE, 1996; HOBBS, 2009).

Isomerization is a reversible first order reaction, and equilibrium is reached with the conversion of 42-45% to fructose. Higher fructose conversions can be achieved by increasing the temperature. Although it is industrially attractive, high-temperature conditions cause thermal inactivation of the enzyme and the degradation of monosaccharides (BHOSALE; RAO; DESHPANDE, 1996; CRABB; MITCHINSON, 1997; HOBBS, 2009).

Different catalytic mechanisms are attributed to glucose isomerase. Initially, the *cis*-enediol mechanism was assumed (Figure 2.6 a), similarly to triose-phosphate isomerase. Currently, the hydride shift mechanism is the most indicated for the functioning of glucose isomerase (Figure 2.6 b). Basically, the mechanism involves opening of the cyclic form, followed by the displacement and reorganization of the hydrogen protons, guided essentially by a histidine and by a carboxylic residue as aspartic acid, resulting in the formation of a ketose followed by ring closure (BHOSALE; RAO; DESHPANDE, 1996; RASMUSSEN et al., 1994).

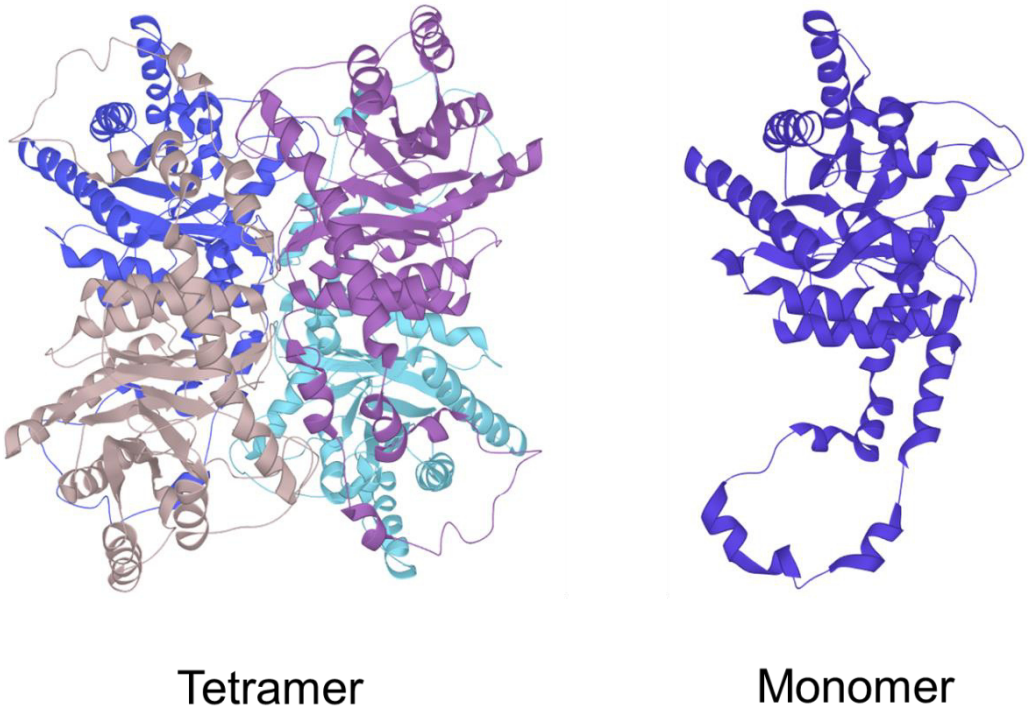
Figure 2.6 — Mechanism of action of GI. (a) *cis*-enediol and (b) hydride shift. Boxes indicate the hydrogen atoms that are transferred stereospecifically.



Source: Adapted from BHOSALE, RAO and DESHPANDE (1996)

The glucose isomerase is produced by prokaryotic organisms and has a relatively expensive production cost. The GI produced by *Streptomyces spp.* presents a three-dimensional tetramer structure with four active sites (Figure 2.7), formed by monomer structures of 43 kDa. Two monomers form a tight dimmer and two dimmers connected with non-covalent bonds form a tight tetramer (BHOSALE; RAO; DESHPANDE, 1996; RASMUSSEN et al., 1994).

Figure 2.7 — Three-dimensional structure of *Streptomyces spp* glucose isomerase, showing the tetramer on the left, composed of four identical subunits associated (monomer on the right).



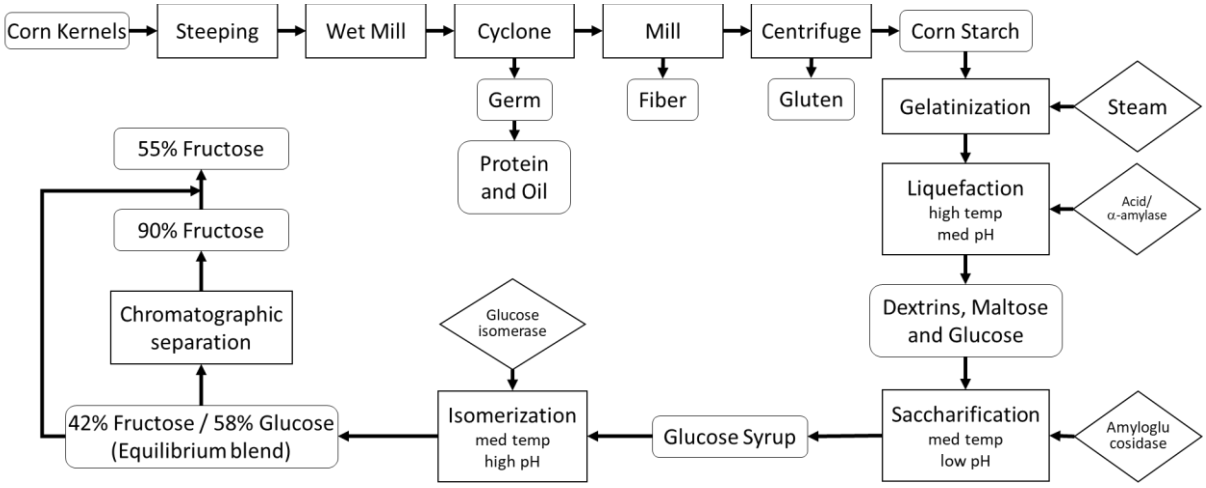
Source: Adapted from 1dxi (left) and 4j4k (right) PDB structures (RASMUSSEN et al., 1994). The figures were obtained from LiteMol at Protein Data Bank in Europe (website).

2.5 MULTIENZYME SYSTEM

As described above, the high fructose syrup production process generally occurs in several separate steps, as show the flowchart in Figure 2.8. This occurs due to the different temperature and pH conditions of each enzyme applied. In liquefaction, the conditions are adjusted around 90 °C, pH 6.0, saccharification 55 °C, pH 4.5, and

for isomerization at 60 °C and pH 8.0. To fulfill each condition, time and reagents are consumed for the pH adjustment, which incurs a cost and a large amount of salts in the subsequent purification steps (HOBBS, 2009; KANEKO; TAKAHASHI; SAITO, 2000).

Figure 2.8 — Flowchart of high fructose corn syrup production process



Source: Adapted from Hobbs (2009)

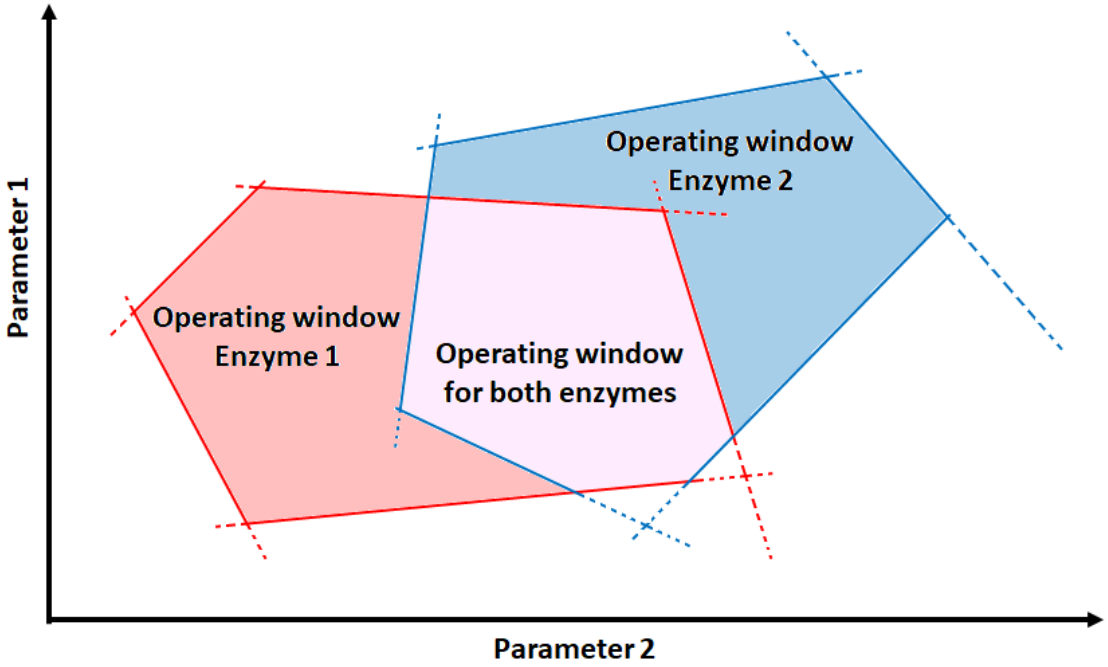
Inspired by the functioning of cells, which are capable of performing different metabolic pathways in cascade reactions catalyzed by several enzymes, *in vitro*, a multi-enzymatic system composed of two or more enzymes was be able to perform simultaneous reactions, mimicking the cellular concept (SANTACOLOMA et al., 2011).

Simultaneous multi-enzymatic processes have a great advantage over those catalyzed in series reactors. In the simultaneous cascade reactions, the product of the first enzymatic reaction is the substrate for another reaction, thus being able to accelerate and shift the balance of reaction favoring the formation of the final product. Moreover, the process occurs in a single reactor, potentially saving equipment, time and energy (HUI et al., 1992; SANTACOLOMA et al., 2011), and also dispenses intermediate purification steps. These advantages make this one-pot process of great industrial interest, in order to increase productivity and process efficiency with lower operating costs (SANTACOLOMA et al., 2011).

However, developing and implementing of a single-step biocatalytic process has limitations, since each reaction has its proper conditions (such as pH, temperature and cofactors). Thus, the process must be balanced between the operating windows of each enzyme applied, as illustrated in Figure 2.9, aiming to optimize productivity in

an integrated way (XUE; WOODLEY, 2012).

Figure 2.9 — Operating window for two enzymes.



Source: Adapted from Xue & Woodley (2012). Reprinted from Bioresource Technology, Vol 115, Rui Xue,John M. Woodley, Process technology for multi-enzymatic reaction systems, 183-195, Copyright (2019), with permission from Elsevier

Immobilized enzymes can be applied in multi-enzymatic processes with the purpose of expanding the operating window, since they have greater operational stability compared to the soluble form. They can be easily separated and recovered at the end of the process and can be reused several times.

Due to the industrial interest in increasing production and reducing costs, studies reporting the use of amyloglucosidase and glucose isomerase enzymes in simultaneous reactions, applied in their soluble, immobilized or co-immobilized forms, are presented in Table 2.1.

Table 2.1 — Comparison of studies of simultaneous saccharification and isomerization.

Enzyme	Method	Conditions	Enzyme loading	Conversion yield/ Productivity	Reference
α-amylase, AMG and GI	Covalent coupling of the enzymes mixture of Cyanogen bromide (CNBr)-activated Sepharose-6MB, followed by blocking of unreactive groups with ethanolamine. Simultaneous hydrolysis, saccharification and isomerization in a reactor jacketed column.	60 °C, pH 6.0, 6 h, soluble starch (5%, w/v).	98 $U_{\alpha\text{-amylase}}$ 28 U_{AMG} 80 U_{GI} /g starch	40% fructose 3.33 g/L.h	KATWA; RAO, 1983
AMG, pullulanase and GI	Coimmobilized via adsorption on Biobone™ (granular chicken bone).	55 °C, pH 6.5, 5 h, glycogen (12%, w/v).	0.7 U_{AMG}, 10 $U_{pullulanase}$ 22.3 U_{GI}	20% fructose 4.8 g/L.h	SCHAFHAGSER; STOREY, 1992
		55 °C, pH 6.0 (1 h) and pH 7.2 (1 h), glycogen (12%, w/v).		25% fructose 15 g/L.h	
AMG and GI	AMG immobilized on anilinosulphonic polystyrene (ASPS) and GI immobilized on trimethylamine polystyrene (TMPS) with CNBr as activating agent. Simultaneous saccharification and isomerization in tubular reactor.	60 °C, pH 7.0, 10 h, dextrin (MW 1500 at 10%, w/v).	350 U_{AMG} 250 U_{GI} /g dry gel	45% fructose 4.5 g/L.h	HUI et al., 1992
AMG and GI	Adsorption of GI on macroporous TMPS followed by trimethylammonium iodine treatment and adsorption of AMG, finishing by crosslinking with glutaraldehyde.	60 °C, pH 6.0, 24 h, dextrin-27 (5%, w/v).	110 U_{AMG} 304 U_{GI}	42% fructose 0.875 g/L.h	GE et al., 1999
AMG and GI	Soluble AMG with soluble acid-stable Glucose isomerase from *S. olivaceoviridis* E-86.	60 °C, pH 5.5 (AMG from *A. niger*) or 58°C, pH 6.0 (AMG from *R. niveus*), 12 h, liquefied starch (30% w/v).	4 U_{AMG} 40 U_{GI} /g starch	50% fructose 12.5 g/L.h	KANEKO; TAKAHASHI; SAITO, 2000
AMG, urease and GI	GI immobilized on gelatin-cellulose with glutaraldehyde followed by co-immobilization with urease by impregnation. Gelatin-cellulose matrix (GI and urease) packed in a column, connected to a flask containing soluble AMG.	50 °C, pH 5.0, 4 h, starch (1%, w/v), 20 mM urea.	Data not provided	40% fructose 1.0 g/L.h	MISHRA et al., 2002

2.6 ENZYMES IMMOBILIZATION

As described earlier, enzymes are excellent biological catalysts, capable of performing the most diverse reactions, both in diversity and complexity. Enzymes act in a highly selective and specific manner, avoiding the formation of undesirable products, catalyzing reactions that in their absence would not occur or would occur very slowly (LEHNINGER; NELSON; COX, 1992). It is also well known that these biocatalysts are mostly of a protein nature and are synthesized by animal cells, plants, and microorganisms to act intra- or extracellularly under mild conditions (ambient temperature, physiological pH and atmospheric pressure). They are also environmentally friendly and more sustainable (LEHNINGER; NELSON; COX, 1992).

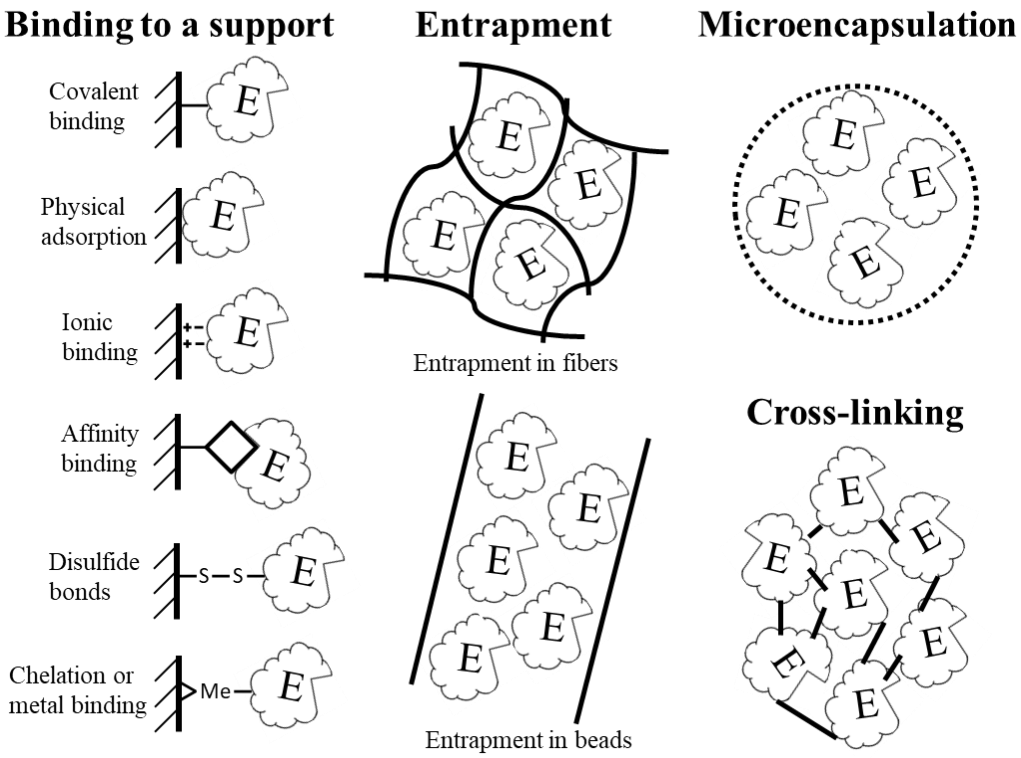
In addition to being limited to specific conditions of use, enzymes are soluble and relatively expensive and unstable catalysts can be easily inactivated and/or denatured due to changes in the medium, *e.g.*, pH and temperature, which technically hamper long-term operations, recovery and reuse of the enzyme in the active form in the reaction mixture after using it (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013; GUISAN, 2006a).

Therefore, these drawbacks can be overcome by using enzyme immobilization techniques that produce an insoluble biocatalyst, allowing its easy recovery, reuse and often improvements in operational stability. They can also be used in different configurations of reactors (batch, semi-continuous and continuous operations) without protein contamination (HOMAEI et al., 2013; KENNEDY; CABRAL, 1987; MATEO et al., 2007). These advantages could help to reduce process costs and, therefore, the final price of the product.

Several studies have reported different protocols of enzyme immobilization for industrial applications. However, for these catalysts to be viable, they must fulfill certain factors. The functional properties of immobilized catalysts must be improved (to justify the process), have a simple and inexpensive preparation procedure (to be cost-effective) and no toxicity (to avoid the contamination of the product) (GUISAN, 2006a).

Among the existing immobilization methods, they can be divided into three main categories: binding to a support (carrier); entrapment or microencapsulation; and cross-linking, as shown in Figure 2.10.

Figure 2.10 — Different techniques for enzyme immobilization.



Source: Author (2019).

2.6.1 Binding to a Support

The binding of an enzyme to a support can occur in several ways: covalent binding (*e.g.*, covalent coupling of aminoglutaraldehyde, epoxy and glyoxyl groups on support with lysines of enzyme), physical adsorption (*e.g.*, hydrophobic interactions, van der Waals forces or hydrogen bonding), ionic binding (salt linkages), affinity binding (enzyme affinity for a ligand precoupled) , disulfide bonds (*e.g.*, reversible covalent coupling between pyridyldisulfide group on support with thiol groups of enzyme) and chelation or metal binding (chelate formation between the metal salts on the surface of the support and the amino acids of the enzyme) (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013; GUISAN, 2006b).

Each of these immobilization techniques has advantages and disadvantages intrinsic to the method, and the main factor of choice is the objective expected by the immobilization.

Immobilizations by physical adsorption and ionic binding are procedures that can be highlighted due to their ease and simplicity, preserving the structure and

catalytic activity of the enzyme. However, interactions with the support are relatively weak and the enzyme can leak due to changes in pH, ionic strength, temperature or polarity of the solvent, thus the catalyst must be operated under very specific conditions. These two methods, as affinity binding, disulfide bonds and chelation, are regarded as reversible and are attractive from an economic point of view, because when the activity decays the support can be regenerated and re-loaded with fresh enzymes (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013).

Immobilizations by covalent bonds are among the most used and are preferred for use in industrial processes, because they promote a stable bond between the enzyme and the carrier, preventing the leaching of enzymes into the reaction medium (SHELDON; VAN PELT, 2013).

Typical supports made of polysaccharides, proteins, carbon, synthetic polymers, and natural or processed minerals (such as silica and glass), can be activated by various reactive groups. The carriers activated with epoxy, aminoglutaraldehyde and glyoxyl groups are able to react with lysines on the surface of the enzyme, and thiol and maleimide groups can react with the cysteine, amino groups on the support with carboxylate, and divinyl sulfone groups react with amino, thiol, and hydroxyl (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013; HOARAU; BADIEYAN; MARSH, 2017).

Covalent immobilization can be single or multipoint attachment, promoting the stabilization of the tertiary structure of the immobilized enzyme, and consequently increasing the stability of the catalytic activity against high temperatures, extreme pH and in the presence of solvents. However, strong stiffening of the enzyme structure can lead to a loss of activity and inactivation (GUISAN, 2006a). Moreover, unlike reversible methods, covalent immobilization does not allow regeneration and re-load of the support and must be discarded with the enzyme after inactivation which can make the process costly (SHELDON; VAN PELT, 2013).

2.6.2 Entrapment

Immobilization by entrapment is a technique that is of particular interest due to the fact that it is simple to prepare and does not require sophisticated equipment. It

can also be extended to any type of enzyme (CAMPÁS; MARTY, 2006). The method consists of the occlusion of the enzyme within a polymer network, which is formed around the enzyme, as a gel or membrane, or a pre-formed polymer where the enzyme is introduced and subsequently confined (O'DRISCOLL, 1976).

The most popular matrices used for such immobilization may be of organic or inorganic origin (*e.g.*, alginate, silica, chitosan, polyacrylamide, gelatin, cellulose triacetate, carrageenan, polyurethane, polyvinyl alcohol, and agar) (ILLANES et al., 2012; SHELDON, 2007b).

Unlike the previously described techniques, in the entrapment, there is no covalent association between the enzyme and support, where the enzyme remains free within the fiber, bead, membrane or microcapsule, and is permeable only to the transport of substrate and product (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013; CAMPÁS; MARTY, 2006; O'DRISCOLL, 1976).

However, entrapment may exhibit mass transfer limitations, and is more suitable for reactions with low molecular weight compounds, even though the porosity of the material is tuneable by matrix concentration or by chemical modifications (CAMPÁS; MARTY, 2006).

2.6.3 Cross-linking

Different from the conventional immobilization methods described above, the cross-linking of enzymes, enzyme crystals and enzyme aggregates (CLE, CLEC, and CLEA, respectively) are enzymatic immobilization methods which do not use any carrier or matrix. In this method, immobilization occurs by multipoint attachment between enzyme molecules (intermolecular) with the aid of a bifunctional or polyfunctional reagent, forming an insoluble catalyst (GUISAN, 2006a; SHELDON, 2007b).

The discovery of the cross-linking process occurred around 1960, when the formation of insoluble cross-linked enzymes (CLEs) was observed through a reaction between the NH2 groups (on the surface of the enzymes) with a bifunctional reagent (such as glutaraldehyde), in studies of solid protein chemistry. However, the insoluble enzyme produced by this method was not functional and had several drawbacks: low activity, reproducibility and stability, as well as the difficulty in handling gelatinous material (SCHOEVAART et al., 2004).

The first authors to describe the cross-linking of an enzyme crystal (CLECs) were Quiocho and Richards (1964), who used glutaraldehyde to stabilize the enzyme crystals in X-ray diffraction studies, and also observed that the catalytic activity was retained (SHELDON, 2007b).

The CLEC immobilization method is applicable to a broad range of enzymes, producing catalysts with a controllable particle size. It is highly active and has improved stability to pH, temperature, organic solvent and proteolysis, as well as operational robustness, capable of being recovered and reused. In view of this, in the early 1990s, CLECs were successfully commercialized as industrial biocatalysts. On the other hand, the formation of CLECs requires the enzyme in the crystallized form, which demands a laborious and costly procedure requiring an enzyme with high purity (SCHOEVAART et al., 2004; SHELDON, 2007b).

The requirement for high purity enzymes and crystallization processes was replaced by the simpler and less costly enzyme precipitation process (CLEAs), and therefore the CLEC process is no longer commercially available (SHELDON, 2019).

Protein precipitation in aqueous solutions is a well-known purification methodology in which protein aggregates are formed after adding salts (ammonium sulfate or sodium sulfate), water-miscible organic solvents (acetone, ethanol, methanol, isopropyl alcohol) or non-ionic polymers (such as polyethylene glycol), without disturbing the three-dimensional structures. In light of this, Cao et al. (2000) demonstrated that a simple process of physical aggregation by precipitation followed by cross-linking with glutaraldehyde led to the formation of highly active and stable insoluble catalysts, more efficient than CLECs produced with the same enzyme (penicillin G acylase) and with activities similar to CLEC in organic solvents, developing a new class of immobilized enzymes, the cross-linked enzyme aggregates (CLEAs) (CAO; VAN RANTWIJK; SHELDON, 2000; SHELDON, 2007b).

Currently, CLEAs of many different enzymes have been reported: invertase, lipase, laccase, galactose oxidase, trypsin, glucose oxidase, β-galactosidase, alcohol dehydrogenase, catalase, etc. (MAFRA et al., 2016, 2018; RAMOS et al., 2018; SCHOEVAART et al., 2004; TALEKAR et al., 2010; WILSON et al., 2004), including amylolytic enzymes, *e.g.*, β-amylase (ARAUJO-SILVA et al., 2018) and amyloglucosidase (AMARAL-FONSECA et al., 2018; GUPTA et al., 2013; NADAR;

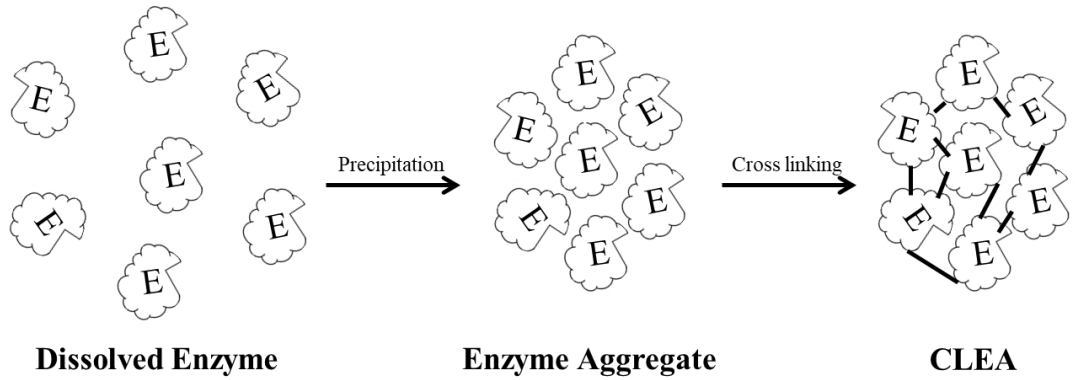
RATHOD, 2016).

2.7 CROSS-LINKED ENZYME AGGREGATES (CLEAs)

As described above, the cross-linked enzyme aggregates are a simple, cost-effective and carrier-free technique for enzyme immobilization proposed by Sheldon. The biocatalysts formed have a better stability (compared to the soluble enzyme) and as the CLECs, have high volumetric activity, since the mass of the catalyst is mainly formed by enzymes, in contrast to the methods that use support, in which 90 to 99% of the mass of the final catalyst is assigned to the carrier, which leads to "dilution of activity" with a non-catalytic mass and an additional cost (CAO; VAN RANTWIJK; SHELDON, 2000; SCHOEVAART et al., 2004; SHELDON, 2007a).

The standard CLEA preparation consists of the aggregation of the enzyme by precipitation with the addition of precipitating agents (*e.g.*, salts, organic solvents or nonionic polymers), where the enzymes associate as insoluble aggregates that are held together by noncovalent bonding (TALEKAR et al., 2013). Thereafter, the formed aggregates are cross-linked using bifunctional (usually glutaraldehyde) or polyfunctionals (dextran polyaldehyde, for example) agents, which react with reactive groups on the enzyme surface (*e.g.*, free amino groups of lysine residues) (MATEO et al., 2004; SHELDON, 2011), as shown in Figure 2.11.

Figure 2.11 — Preparation of cross-linked enzyme aggregates.



Source: Author (2019).

In the precipitation stage, screening is necessary to select the best precipitant agent, nature and concentration that can directly influence the catalyst generated.

Higher precipitation yields (precipitated protein) and high precipitated activities (determined by measuring the activity of the redissolved aggregate in a buffer) do not mean that the final CLEA will retain all of the precipitated activity, this is because the formed aggregates can retain the enzyme in a conformation unfavorable, which is not maintained after resuspension, but remains with the cross-linking (SHELDON, 2011).

Analogous to the precipitant, the crosslinking agent should also be evaluated, because each enzyme has a unique surface structure. Very low ratios of crosslinkers may be insufficient to stabilize intermolecular bonds, which may leach enzymes during the application of CLEAs, whereas very high ratios may excessively crosslink, stiffening the tertiary structure of the enzyme and inactivating it (SHELDON, 2011). Moreover, glutaraldehyde, the main crosslinker agent used, is a relatively small molecule that can penetrate into the protein and react with amino acids essential to catalytic activity, which is a problem that does not occur when using polyaldehydes such as polyaldehyde dextran (SHELDON, 2019).

Another important factor to be evaluated in the CLEA process is the pH of the solution due to its direct influence on the free amine groups reactive on the surface of the enzyme and on the behavior of the crosslinker. Glutaraldehyde has complex chemistry and is not fully understood. It is widely used under pH conditions ranging from 7.0 to 9.0, wherein it is observed that at pH acid to neutral its aldehyde groups react with the NH_2 groups of the lysines to form Schiff bases. At alkaline pH conditions, the glutaraldehyde molecule undergoes condensation, polymerizing, and can thus react with the enzymes by the Schiff base formation mechanism or by Michael reaction (Michael-type 1.4 addition), generating different catalysts, due to the greater spacing of the binding (BARBOSA et al., 2014).

However, some enzymes have a low content of lysine groups on the enzyme surface to permit effective crosslinking with glutaraldehyde. This problem has been solved using Lys-rich proteins as protein feeders (ARAUJO-SILVA et al., 2018; MAFRA et al., 2018; RAMOS et al., 2018; SHAH; SHARMA; GUPTA, 2006), co-immobilizing enzymes and a primary amino rich polymer (*e.g.*, polyethylenimine, PEI) (WILSON et al., 2006) or even enriching the enzyme in primary amino groups via chemical modification (GALVIS et al., 2012). AMG has 13 Lys, but only 9 are exposed to the medium (SORIMACHI et al., 1997; SVENSSON et al., 1983a). Two of them are

located in the starch-binding domain and one close to the active site (see Figure 4). Because of this, the enzyme crosslinking with bifunctional agents may be very poor, resulting in a CLEA with low mechanical stability and allowing leaching of the enzyme in the reaction medium (LÓPEZ-GALLEGO et al., 2005; SHELDON, 2011; VIRGEN-ORTÍZ et al., 2017; ZHENG et al., 2014).

In some instances, crosslinking with glutaraldehyde may lead to enzyme inactivation by altering the active center. In these cases, large aldehyde polymers (*e.g.*, aldehyde dextran) or other crosslinkers have been proposed (MATEO et al., 2004; VELASCO-LOZANO et al., 2014).

CLEAs also have some problems. First, CLEAs are mechanically fragile, complicating their recovery. Second, the pore sizes may be small and this can lead to high diffusional limitations (GARCIA-GALAN et al., 2011a).

The first problem may be solved using magnetic nanoparticles (MNPs) that are co-aggregated with the enzyme to permit magnetic handling of the final CLEAs (TALEKAR et al., 2012b). The size, and functionalization of the MNPs can determine the final properties of the magnetic CLEA (KOPP et al., 2015). Immobilization of different amylases related enzymes using CLEA technology associated with MNPs functionalized with 3-aminopropyltriethoxysilane (APTES) has already been reported, showing that its application confers mechanical stability and efficient magnetic separation of CLEAs (GUPTA et al., 2013; NADAR; RATHOD, 2016; PANEK; PIETROW; SYNOWIECKI, 2012).

The second problem may be reduced if strategies to enlarge the pores of the CLEA are utilized. The co-aggregation of PEI with the enzyme can significantly improve the cross-linking efficiency, preventing leakage and promoting the generation of a hydrophilic microenvironment that protects the enzyme from organic solvents (LÓPEZ-GALLEGO et al., 2005; MATEO et al., 2000; TALEKAR et al., 2013; VIRGEN-ORTÍZ et al., 2017; ZHENG et al., 2014), but that also may result in enlarged pore sizes. Similarly, the use of polymers such as starch during the aggregation and crosslinking steps may facilitate the formation of large pores during the CLEA production. As at the final CLEA preparation, the starch is degraded by α-amylase and washed away from the CLEA, this strategy reduces the internal mass-transfer limitations and increases the catalytic efficiency (TALEKAR et al., 2012a; WANG et al., 2011).

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3 PREPARATION OF MAGNETIC CROSS-LINKED AMYLOGLUCOSIDASE AGGREGATES: SOLVING SOME ACTIVITY PROBLEMS

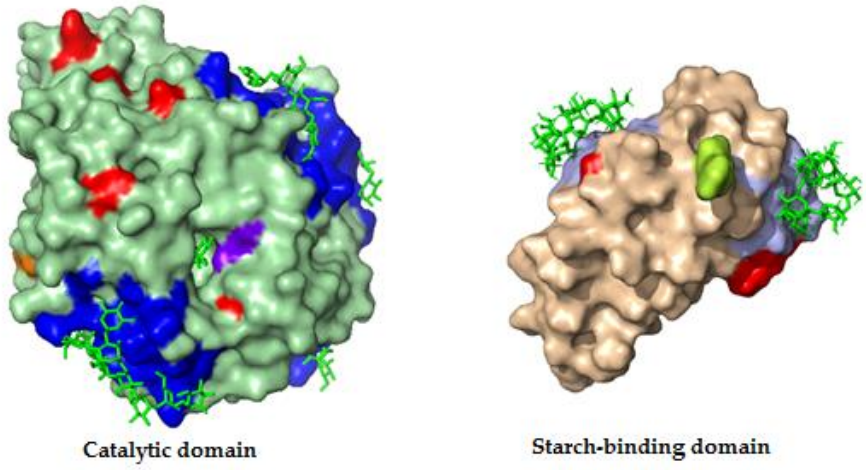
Abstract: The preparation of Cross-Linked Enzyme Aggregates (CLEAs) is a simple and cost-effective technique capable of generating insoluble biocatalysts with high volumetric activity and with improved stabilities. The standard CLEA preparation consists of the aggregation of the enzyme and its further crosslinking, usually with glutaraldehyde. However, some enzymes have a too low content of surface lysine groups to permit effective crosslink with glutaraldehyde, requiring co-aggregation with feeders rich in amino groups to aid the formation of CLEAs. The coaggregation with magnetic particles makes their handling easier. In this work, CLEAs of a commercial amyloglucosidase (AMG) produced by *Aspergillus niger* were prepared by co-aggregation in the presence of polyethyleneimine (PEI) or starch with aminated magnetic nanoparticles (MNPs) or bovine serum albumin (BSA). Firstly, CLEAs were prepared only with MNPs at different glutaraldehyde concentrations, yielding a recovered activity of around 20%. The addition of starch during precipitation and crosslinking steps increased the recovered activity around twofold. Similar recovered activity (around 40%) was achieved changing starch by PEI. Moreover, under the same conditions, AMG co-aggregated with BSA was also synthesized, yielding CLEAs with very similar recovered activity. Both CLEAs (co-aggregated with MNPs or BSA) were four times more stable than the soluble enzyme. These CLEAs were evaluated in the hydrolysis of starch at typical industrial conditions, achieving more than 95% starch-to-glucose conversion measured as Dextrose Equivalent (DE). Besides, both CLEAS could be reused for five cycles maintaining a DE of around 90%. Although both CLEAs had good properties, magnetic CLEAs could be more attractive for industrial purposes because of their easy separation by an external magnetic field, avoiding the formation of clusters during the usually used filtration or centrifugation recovery methods.

Keywords: Cross-linked enzyme aggregate. Amyloglucosidase. Magnetic nanoparticles. Bovine serum albumin. Polyethyleneimine. Starch hydrolysis.

3.1 INTRODUCTION

Amyloglucosidase (1,4-α-D-glucan hydrolase, EC 3.2.1.3) is an enzyme that catalyzes the release of glucose from the non-reducing ends of glucose polysaccharides. In addition to selectively hydrolyzing α-1,4-glycosidic bonds, it is also capable to hydrolyze starch branches (α-1,6-glycosidic bonds), but in a slower manner (CRABB; MITCHINSON, 1997; PARKIN, 2007).

Figure 3.1 — Three-dimensional structures of *A. niger* amyloglucosidase, showing the catalytic domain on the left and the starch-binding domain on the right. Color patterns: red (lysine residues), purple (active site), orange (N-terminal residue), lemon (C-terminal residue), blue and light blue (glycosylated regions in the catalytic and starch binding domains, respectively).



Source: Adapted from 3eqa (left) and 1ac0 (right) PDB structures (LEE; PAETZEL, 2011; SORIMACHI et al., 1997), respectively). The figures were generated using PyMol (The PyMol Molecular Graphics System; Version 2.1.0; Schrödinger, LLC).

Fungal amyloglucosidases, like the ones produced by *Aspergillus niger* (AMG) may present more than one form, with different molecular weights (LEE; PAETZEL, 2011; SORIMACHI et al., 1997; SVENSSON et al., 1983b). *Aspergillus niger* produces two isoforms, namely: G1 isoform, which corresponds to a protein having a catalytic domain (structure on the left in Figure 3.1) and a starch-binding domain (structure on the right in Figure 3.1) with a total of 640 amino acid residues and Mw around 68 kDa, and G2 isoform, which contains only the catalytic domain with 470 amino acid residues and Mw around 50 kDa (LEE; PAETZEL, 2011; SORIMACHI et al., 1997). Both

isoforms are highly glycosylated by both N-linked and O-linked carbohydrates. These isoforms are derived from the same genetic material, but differ because of a different RNA splicing after transcription (TOMASIK; HORTON, 2012).

AMG from *A. niger* is extensively used in industrial starch saccharification, almost completely hydrolyzing maltodextrins, amylose, and amylopectins to produce glucose syrup, which serves as a substrate in the production of other syrups for application in the beverage and food industries (CRABB; MITCHINSON, 1997; PAZUR; ANDO, 1959; TOMASIK; HORTON, 2012).

The saccharification of the starch is usually carried out by the enzyme in the soluble form. Because of this, the enzyme is neither recovered nor reused (PARKER; SALAS; NWOSU, 2010). This drawback can be overcome by the use of enzyme immobilization techniques that allow enzyme recovery, reuse, and often improvement of the operational stability (HOMAEI et al., 2013; KENNEDY; CABRAL, 1987; MATEO et al., 2007). These advantages could contribute to reduced processing costs and, therefore, a lower final price of the product. Several studies reported the immobilization of amyloglucosidase by different protocols (BRYJAK, 2003; SHAH; SELLAPPAN; MADAMWAR, 2000; SILVA; ASQUIERI; FERNANDES, 2005; TARDIOLI et al., 2011a; WANG et al., 2013).

An alternative to the immobilization of enzymes on solid supports is carrier-free immobilization, such as the crosslinked enzyme aggregates (CLEAs) proposed by Sheldon (CAO; VAN RANTWIJK; SHELDON, 2000; SCHOEVAART et al., 2004; SHELDON, 2007a). This methodology involves the precipitation of the proteins by the addition of precipitating agents (*e.g.*, salts, organic solvents or nonionic polymers), where the enzymes associate as insoluble aggregates (TALEKAR et al., 2013). Thereafter, the formed aggregates are cross-linked using bifunctional (usually glutaraldehyde) or polyfunctionals (dextran polyaldehyde, for example) agents (MATEO et al., 2004). CLEAs of many different enzymes have been reported (MAFRA et al., 2016, 2018; RAMOS et al., 2018; SCHOEVAART et al., 2004; TALEKAR et al., 2010; WILSON et al., 2004), including amylolytic enzymes, *e.g.*, β-amylase (ARAUJO-SILVA et al., 2018) and amyloglucosidases (GUPTA et al., 2013; NADAR; RATHOD, 2016).

The preparation of CLEAs may lead to some problems. At first glance, it should be relatively simple to find a precipitant that permits the recovery of high levels of enzyme activity. However, the crosslinking step may be problematic if the enzyme is

poor in external free primary amino groups. This problem has been solved using Lys-rich proteins as protein feeders (ARAUJO-SILVA et al., 2018; MAFRA et al., 2018; RAMOS et al., 2018; SHAH; SHARMA; GUPTA, 2006), co-immobilizing enzymes and a primary amino rich polymer (*e.g.*, polyethylenimine, PEI) (WILSON et al., 2006) or even enriching the enzyme in primary amino groups via chemical modification (GALVIS et al., 2012). AMG has 13 Lys, but only nine are exposed to the medium (SORIMACHI et al., 1997; SVENSSON et al., 1983b), two of which are located in the starch-binding domain and one close to the active site (see Figure 3.1). Because of this, the crosslinking of the enzyme with bifunctional agents may be poor, resulting in a CLEA with low mechanical stability and allowing leaching of the enzyme in the reaction medium (LÓPEZ-GALLEGO et al., 2005; SHELDON, 2011; VIRGEN-ORTÍZ et al., 2017; ZHENG et al., 2014). In some instances, the crosslinking with glutaraldehyde may lead to enzyme inactivation by altering the active center; in these cases large aldehyde polymers (*e.g.*, aldehyde dextran) or other crosslinkers have been proposed (MATEO et al., 2004; VELASCO-LOZANO et al., 2014).

The use of CLEAs also poses some problems. First, CLEAs are mechanically fragile, complicating their recovery. Second, pore sizes may be small and this can lead to high diffusional limitations (GARCIA-GALAN et al., 2011b).

The first problem may be solved using magnetic nanoparticles (MNPs) that are co-aggregated with the enzyme to permit magnetic handling of the final CLEAs. (TALEKAR et al., 2012b). The size, and functionalization of the MNPs can determine the final properties of the magnetic CLEA (KOPP et al., 2015). Immobilization of different amylases-related enzymes using CLEA technology associated with MNPs functionalized with 3-aminopropyltriethoxysilane (APTES) has already been reported, showing that its application confers mechanical stability and efficient magnetic separation of CLEAs (GUPTA et al., 2013; NADAR; RATHOD, 2016; PANEK; PIETROW; SYNOWIECKI, 2012).

The second problem may be reduced if strategies to enlarge the pores of the CLEA are utilized. The co-aggregation of PEI with the enzyme can significantly improve the crosslinking efficiency, preventing leakage and promoting the generation of a hydrophilic microenvironment that protects the enzyme from organic solvents (LÓPEZ-GALLEGO et al., 2005; MATEO et al., 2000; TALEKAR et al., 2013; VIRGEN-

ORTÍZ et al., 2017; ZHENG et al., 2014), but that also may result in enlarged pore sizes. Similarly, the use of polymers like starch during the aggregation and crosslinking steps may facilitate the formation of large pores during the CLEA production (and perhaps, as it is a substrate of the enzyme, it may protect the active center of the enzyme during the different steps of the CLEA production). As at the final CLEA preparation the starch is degraded by α-amylase and washed away from the CLEA, this strategy may reduce internal mass-transfer limitations and, thus, increase the catalytic efficiency (TALEKAR et al., 2012a; WANG et al., 2011).

In this context, this paper intends the co-aggregation of commercial AMG with MNPs to get a magnetic CLEA. A set of parameters was evaluated to prepare CLEAs of AMG with high activity, reduced leaching, and high thermal and operational stabilities, such as type and amount of precipitants, concentration of glutaraldehyde, time of glutaraldehyde treatment, stirring speed, use of PEI and starch during precipitation, and crosslinking steps to generate larger pores and, perhaps, to protect the active center of the enzyme. The addition of starch or PEI has been studied as a strategy for the formation of CLEAs with larger pores (among other likely effects). The catalytic properties of the most active and stable CLEAs were characterized (optimal pH and temperature for enzyme activity, thermal stability and performance in the hydrolysis of starch at high starch concentration (typical industrial conditions)).

3.2 MATERIALS AND METHODS

3.2.1 Materials

AMG 300L from *Aspergillus niger* (EC 3.2.1.3; glucoamylase; amyloglucosidase) and α-amylase BAN 480L were from Novozymes A/S (Bagsværd, Denmark), dextrin 10, bovine serum albumin (BSA) and polyethylenimine (oligomer mixture, Mn ~423) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Soluble starch and glutaraldehyde 25% (v/v) aqueous solution from Vetec (Duque de Caxias, RJ, Brazil). Mono reagent colorimetric enzymatic (GOD-POD) obtained from Gold Analisa (Belo Horizonte, MG, Brazil). The magnetic nanoparticles were purchased from Kopp Technologies (São Carlos, SP, Brazil). All other chemicals (analytical grade) were purchased from Synth (Diadema, SP, Brazil) and Vetec (Duque de

Caxias, RJ, Brazil).

3.2.2 Precipitant screening

The precipitation of amyloglucosidase was carried out by adding the protein precipitant (acetone, ethanol, isopropanol or saturated ammonium sulfate solution) to an enzyme solution (protein concentration of 20 mg/mL in 50 mM sodium citrate buffer pH 4.5) in a volume ratio of 9:1. The mixture was incubated in shaker under 150 rpm at 4 °C for 60 min, the precipitate was recovered by centrifugation (1500 x g at 4 °C for 5 min) and resuspended in 1 ml of 50 mM sodium citrate buffer pH 4.5. Enzyme activity and protein content were measured in the supernatant and in the re-suspended precipitate as described in sections 3.2.7 and 3.2.8, respectively. For the precipitant chosen based on the highest yields of activity and protein, the volume ratio of precipitant and enzyme solution was also evaluated, but at static conditions in an ice bath for 30 min. Within the volume ratios evaluated, the better one was chosen for further assays.

3.2.3 General cross-linking procedure using glutaraldehyde

Ethanol was added to an enzyme solution prepared in 100 mM carbonate buffer pH 10.0 to a volume ratio of 9:1 (ethanol/enzyme). After 30 min precipitation in an ice bath, volumes of glutaraldehyde 25% (v/v) to final concentrations of 100, 300, 500 and 700 mM were added to the precipitated enzyme (crosslinking step). The aggregates suspension was homogenized and incubated statically in a refrigerator at 4 °C for 16 h or under gently stirring in a three-dimensional laboratory agitator (KASVI, K45-4020) at 4 °C for 4 or 16 h. After the incubation period, the suspension was centrifuged (1500 x g for 5 min at 4 °C) and the precipitate was washed twice with 50 mM sodium citrate buffer pH 4.5, and finally re-suspended in the same buffer. Then, the activity recovery was calculated by Equation (3.1):

$$Activity\ recovery(\%) = \frac{Total\ activity\ in\ the\ CLEA\ suspension}{Total\ activity\ offered\ initially} \times 100 \qquad \text{Equation (3.1)}$$

The same procedure described above was used for evaluation of co-aggregation of AMG with magnetic nanoparticles (MNPs) (characteristics described in Table 3.1) or bovine serum albumin (BSA) as additive or co-feeder, respectively. In this case, the MNPs or BSA was added to the enzyme solution (in 100 mM carbonate buffer pH 10) to a final protein/MNPs (or BSA) mass ratio of 1:1 (20 mg total/mL). When BSA was used, the CLEAs were separated by centrifugation (1500 x g for 5 min at 4 °C); on the other hand, when MNPs were used, the CLEAs were separated by applying an external magnetic field.

Starch was also evaluated as protector additive of the enzyme active site. In this case, a 1% (w/v) starch solution was prepared in 100 mM carbonate buffer pH 10 and was used to prepare the mixture of enzyme and other additives in the CLEA preparation. The other steps remained unaltered.

Polyethyleneimine (PEI) was also evaluated as crosslinking aid. In this case, an aqueous solution of PEI was prepared and the pH was adjusted to 7.0. This solution was mixed with the enzyme solution (protein concentration of 20 mg/mL in 100 mM sodium phosphate buffer pH 7.0) to a protein/PEI mass ratio of 1:1, as described by López-Gallego et al. (2005). After 30 min stirring in a tridimensional laboratory agitator at room temperature, the precipitant was added, and the protocol followed the procedure described above.

3.2.4 Characterization of the biocatalysts

The activity of soluble and immobilized AMG was measured as a function of the pH (at 55 °C) in the range from 3.0 to 7.0, and as function of the temperature (at pH 4.5) in the range from 35 to 85 °C. For pH values from 3.0 to 5.5, 50 mM sodium citrate and acetate buffers were used, and for pH values from 5.5 to 7.0 a 50 mM sodium phosphate buffer was used.

Thermal inactivation assays were carried out at 55 °C and pH 4.5 (50 mM sodium citrate buffer). Enzyme activity was measured at regular time intervals until 24 h. The model of Sadana & Henley (1987) was fitted to the experimental data to determine the half-life time. The stabilization factor (SF) was calculated as the ratio between the half-life of the immobilized enzyme and that of the free enzyme.

3.2.5 Hydrolysis of starch

A solution of starch (35%, w/v) was prepared in 50 mM citrate buffer (pH 4.5). The starch was pre-hydrolyzed with α -amylase (BAN 480L, 3 mL/kg starch) at 60 °C for 20 min. The temperature was fitted to 55 °C and soluble AMG (2 mL/kg starch) or CLEAs of AMG (37820 U/kg starch, equivalent to the amount of soluble enzyme) was added. The reaction was carried out for 24 h in a batch reactor (8.5 x 3.33 cm (length x diameter) under 900 rpm stirring using a cylindrical impeller without blades (8.4 x 0.80 cm (length x diameter) to prevent loss of biocatalyst by shearing. Samples of the reaction medium were withdrawn at regular time intervals to measure reducing sugars by DNS method (MILLER, 1959). Dextrose Equivalent (DE) (KEARSLEY; DZIEDZIC, 1995) was calculated by Equation (3.2) to construct the hydrolysis time profile.

$$Dextrose\ Equivalent = \frac{Amount\ of\ reducing\ sugar\ (expressed\ as\ glucose)}{Starch\ dry\ mass} \times 100$$

Equation (3.2)

In the selection of CLEAs, their performance in the hydrolysis of starch was evaluated using 3% (w/v) starch. The hydrolysis reactions were performed at 55 °C and pH 4.5 for 6 h under 300 rpm stirring. Reducing sugars and Dextrose Equivalent were determined as described above, only at the final stage of the reaction.

3.2.6 Reuse assays

The reusability assays of amyloglucosidase CLEA were performed at 45 °C under 300 rpm shaking with a solution of pre-hydrolyzed starch (35%, w/v) prepared in 50 mM citrate buffer pH 4.5. After each cycle of 6 h reaction time, the conversion of starch (DE) was determined and the CLEA was recovered by applying an external magnetic field and/or by centrifugation (1500 x g for 5 min at 4 °C), washed with citrate buffer (50 mM, pH 4.5) and re-suspended in a fresh substrate solution.

3.2.7 Enzymatic activity assay

Enzymatic activity of amyloglucosidase (free and immobilized as CLEAs) was determined by calculating the initial velocity of glucose formation catalyzed by a known amount of enzyme. The standard substrate was dextrin 1% (w/v, in 50 mM sodium citrate buffer at pH 4.5). The reaction was carried out at 55 °C for 10 min under 900 rpm stirring. Samples were withdrawn every 2.5 min, the reaction was quenched with 1 M HCl, and glucose was measured as described in section 3.2.8. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol of glucose per minute under the conditions above.

3.2.8 Determination of glucose and protein concentration

Glucose was determined spectrophotometrically at 505 nm by glucose oxidase and peroxidase colorimetric enzymatic test (GOD-POD). The GOD-POP solution (1 mL) was added to the sample (10 µL) and incubated at 37 °C for 10 min (TRINDER, 1969). The concentration of glucose was quantified using a glucose concentration vs. absorbance curve constructed with glucose as standard.

The protein content was determined spectrophotometrically at 595 nm by the Bradford method, using bovine serum albumin as standard protein (BRADFORD, 1976).

3.2.9 SDS-PAGE electrophoresis

The commercial AMG was characterized by polyacrylamide gel electrophoresis (10% SDS-PAGE) using Coomassie Brilliant Blue for staining (LAEMMLI, 1970).

3.3 RESULTS AND DISCUSSION

3.3.1 Precipitation selection

Because the biochemical and structural properties differ from one enzyme to another, a screen of precipitants should be performed for the preparation of CLEAs of

a particular enzyme (CUI; JIA, 2015; TALEKAR et al., 2013). Thus, in this work, a screen of five precipitants (acetone, ethanol, iso-propanol, ammonium sulfate and PEG) was carried out aiming to full protein precipitation and high recovered activity of the re-dissolved precipitate. Commercial AMG 300L is relatively pure (Figure 3.2), exhibiting two main bands (around 70 and 100 kDa), probably corresponding to the G1 and G2 isoforms (SVENSSON et al., 1983b).

Figure 3.2 — Electrophoresis gel (10% SDS-PAGE) of commercial amyloglucosidase (AMG, 300L).

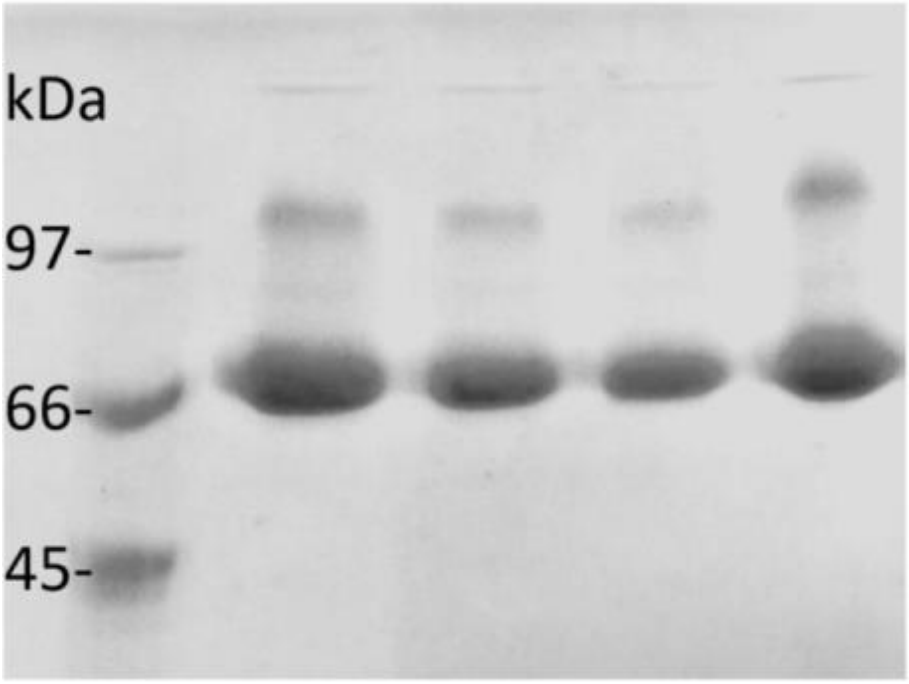
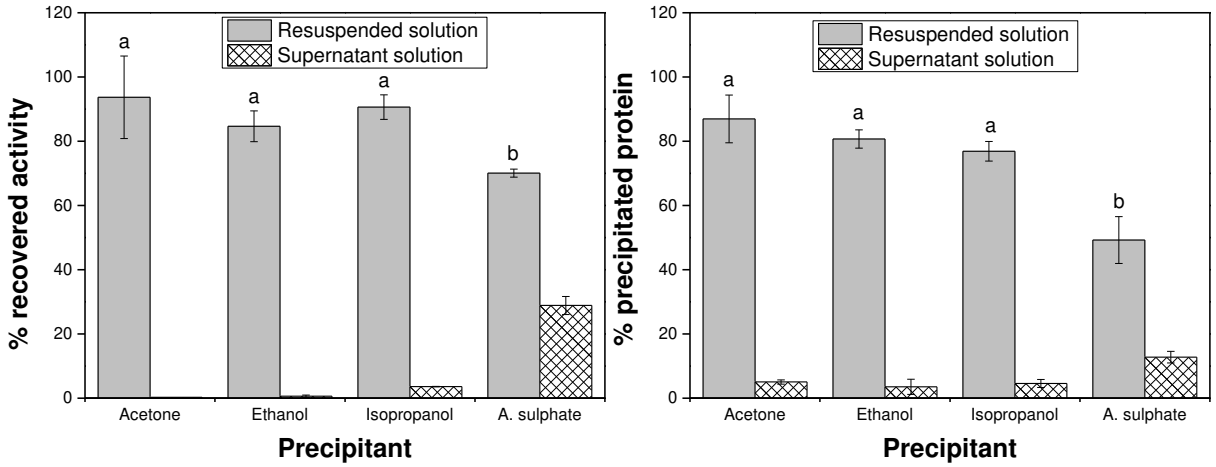


Figure 3.3 shows that acetone, ethanol, and isopropanol were capable of precipitating around 80% of proteins, retaining high activity of the re-dissolved precipitates (around 90%), while ammonium sulfate precipitated only around 50% of the proteins and polyethylene glycol (PEG) did not have precipitation action on AMG (data not shown). The different precipitation yields may be explained by the different mechanisms of aggregation of each precipitant (changes in the hydration state of the molecules, or changes in the dielectric constant of the solution) (CUI; JIA, 2015).

Figure 3.3 — Screen of precipitants for amyloglucosidase (AMG 300L), the percentage of recovered activity (a) and the percentage of precipitated protein (b).



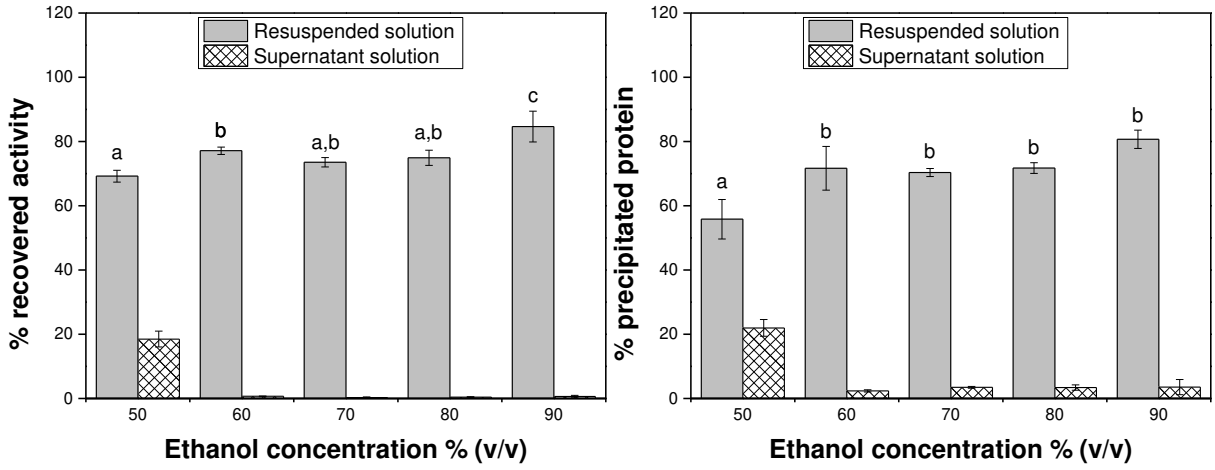
(**a**) (**b**)

Precipitation conditions: precipitant/enzyme solution volume ratio of 9:1, at 4 °C, 60 min precipitation under 150 rpm shaking, and enzyme solution prepared in 50 mM sodium citrate buffer pH 4.5. Note: Values are shown as the mean of triplicate measurements ± s.d. Means followed by the same letter are not statistically different by Tukey's test ($p < 0.05$). Percentage of protein was calculated taken the initial protein as 100%.

Ethanol exhibited good performance at precipitating AMG and is economically and environmentally more friendly (low-cost and -toxicity, and renewable) than the other precipitants evaluated; therefore, it was selected as a precipitant of AMG for the preparation of CLEAs.

Figure 3.4 shows the influence of the precipitant/enzyme solution volume ratio on the protein precipitation yields and recovered activity of AMG in the re-dissolved precipitate. It can be observed that for ethanol concentrations from 60% to 90% (v/v), protein precipitation yields were not statistically different. In terms of recovered activity, 90% (v/v) ethanol exhibited a small improvement (around 85% recovered activity). Thus, a volume ratio of 9:1 (precipitant/enzyme solution) was selected for the further assays.

Figure 3.4 — Influence of the ethanol concentration (vol %) on the (a) percentage of recovered activity, and (b) percentage of precipitated protein of amyloglucosidase (AMG 300L).



(**a**) (**b**)

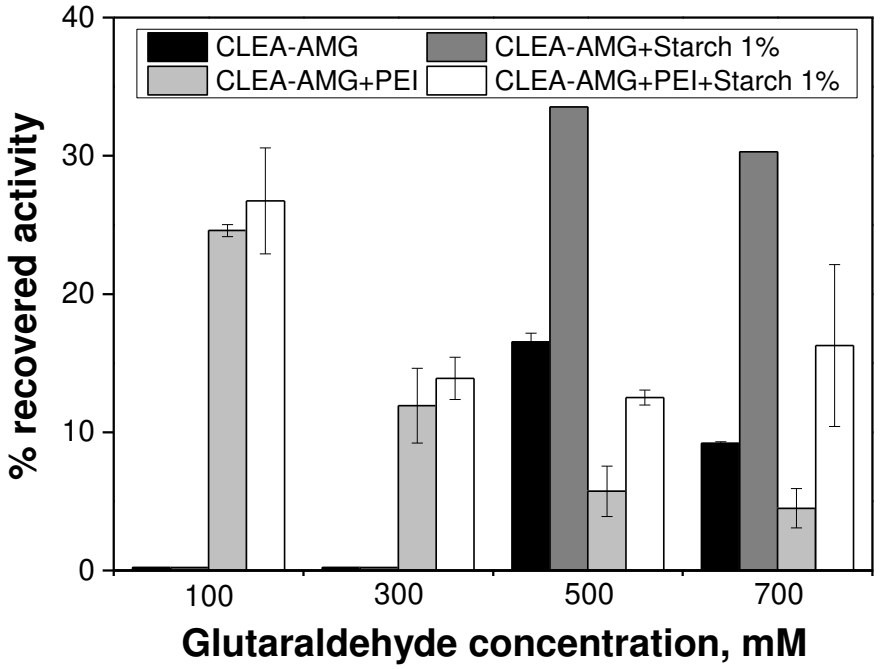
Assay conditions: 30 min precipitation under static conditions in an ice bath. AMG solution prepared in 50 mM sodium citrate buffer pH 4.5. Note: Values are shown as the mean of triplicate experiments ± s.d. Means followed by the same letter are not statistically different by Tukey's test ($p < 0.05$). Percentage of protein was calculated taken the initial protein as 100%.

3.3.2 Preparation of CLEAs

Initially, CLEAs were prepared without co-feeders or any other aid. Figure 3.5 shows that CLEAs could be only formed using 500 mM glutaraldehyde in the crosslinking step. But the recovered activity was lower than 20%. Using 100 or 300 mM glutaraldehyde, the aggregates were re-dissolved due to inefficient crosslinking.

The co-aggregation with PEI allowed the formation of CLEAs at 100 mM glutaraldehyde (recovered activity around 25%). The increase in the glutaraldehyde concentration led to CLEAs with lower recovered activity, probably due to excessive enzyme modification. The co-aggregation with starch produced better results (recovered activity around 35%), but required higher glutaraldehyde concentration in the crosslinking step. The combined use of PEI and starch did not improve the recovered activity.

Figure 3.5 — CLEAs of amyloglucosidase (AMG 300L) prepared using only enzyme, co-aggregation with polyethyleneimine (protein/PEI mass ratio of 1:1) and/or starch (1%, w/v).



Assay conditions: ethanol as precipitant (volume ratio of 1:9, enzyme solution pH 7.0 or 10.0/ethanol), 30 min precipitation in an ice bath, glutaraldehyde as a cross-linker (100–700 mM), 16 h crosslinking under gently stirring at 4 °C. Note: Values are shown as the mean of duplicate experiments ± s.d.

3.3.3 Preparation of magnetic AMG CLEAs

In order to prepare easily manageable CLEAs, MNPs were co-aggregated with AMG. A set of commercial MNPs functionalized with amino or amino/hydrophobic groups (Table 3.1) was evaluated.

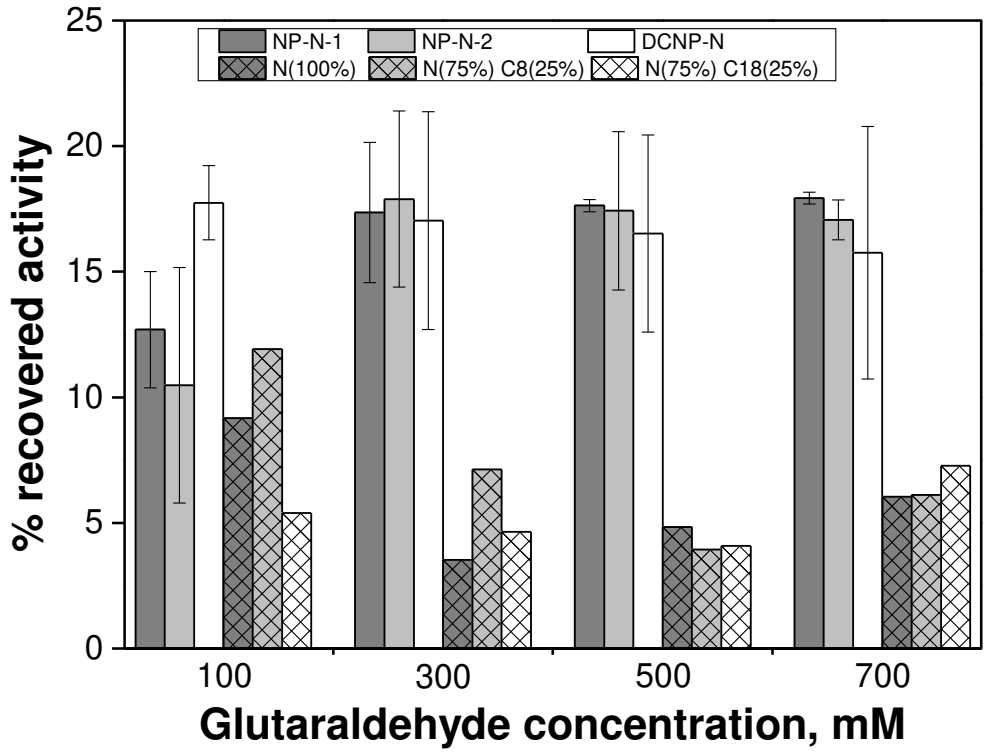
Table 3.1 — Content of amino and hydrophobic (octyl or octadecyl) groups in the magnetic nanoparticles supplied by Koop Technologies (São Carlos, SP, Brazil)

Magnetic Nanoparticle	-NH_2 content (µmol/g)	-C8 or -C18 (µmol/g)
NP-N-1	1013.1 [1]	Not applicable
NP-N-2	265.5 [1]	
DCNP-N	522.1 [1]	
N (100%)	275.7 [1]	348 ± 15 [2]
N (75%) C8 (25%)		136 ± 10 [2]	282 ± 19 [2]
N (75%) C18 (25%)		310 ± 39 [2]	479 ± 21 [2]

[1] Amino content quantified according to TNBS method (SNYDER; SOBOCINSKI, 1975). [2] Amino content quantified according to CNHS method. Note: Column 1 lists the names of commercial magnetic nanoparticles (MNPs) as provided by the manufacturer. N, C8 and C18 indicate chemicals used for the synthesis of the MNPs, such as (3-aminopropyl)triethoxysilane, triethoxy(octyl)silane and n-octadecyltriethoxysilane, respectively.

Figure 3.6 shows the recovered activity of CLEAs prepared by co-aggregation with MNPs in a AMG/MNPs mass ratio of 1:1 (TALEKAR et al., 2012b). As described above, using a glutaraldehyde concentration below 500 mM did not form CLEAs using only AMG, which was fully leached after resuspension in buffer or washing steps. However, using MNPs CLEAs were formed for all glutaraldehyde concentrations evaluated, despite the low recovered activity (less than 20%). The magnetic nanoparticle DCNP-N, containing 522 µmol of amino groups per gram, did give better results even at the lowest glutaraldehyde concentration; therefore, it was selected for further experiments.

Figure 3.6 — Effect of different commercial aminated magnetic nanoparticles (NP-N-1, NP-N-2, N(100%), N(75%)-C8(25%), N(75%)-C18(25%)) on the recovered activity of CLEAs of amyloglucosidase (AMG 300L).



Synthesis conditions: enzyme/magnetic nanoparticle mass ratio of 1:1, ethanol/enzyme solution volume ratio of 9:1 (in 100 mM carbonate buffer pH 10.0), glutaraldehyde concentration ranging from 100 to 700 mM, precipitation and crosslinking under static conditions in an ice bath. Activity of AMG CLEAs was measured with 1% (w/v) dextrin in 50 mM sodium citrate buffer pH 4.5–10 min reaction at 55 °C under 900 rpm stirring. Values are shown as the mean of triplicate experiments ± s.d.

3.3.4 Evaluation of glutaraldehyde effect on enzyme activity

In order to investigate the probable deleterious effect of glutaraldehyde on the AMG, the activity of free AMG was measured after incubation of the enzyme with glutaraldehyde at different concentrations in the same conditions used in the preparation of CLEAs (16 h at 4 °C), but in the absence of ethanol. Starch was also added to the enzyme/glutaraldehyde solutions to evaluate whether the active site would be protected in the presence of a natural substrate. Table 3.2 shows that more than 75% of activity was lost when free AMG (without starch) was incubated in the presence of glutaraldehyde. On the other hand, when starch was added to the enzyme solution, slight protection was observed. The interaction of the starch with the amino acid residues at the active site could avoid distorting changes in the tertiary structure of AMG promoted by excessive cross-links with glutaraldehyde and/or could prevent the establishment of a covalent link between glutaraldehyde and the ε-amino group from the lysine residue located close to the active site. The increase of the starch concentration from 1% to 5% (w/v) did not cause an improvement in the residual activity, probably due to the high viscosity of the medium at low temperature (i.e., 4 °C), preventing the formation of a homogeneous mixture (WANG et al., 2011).

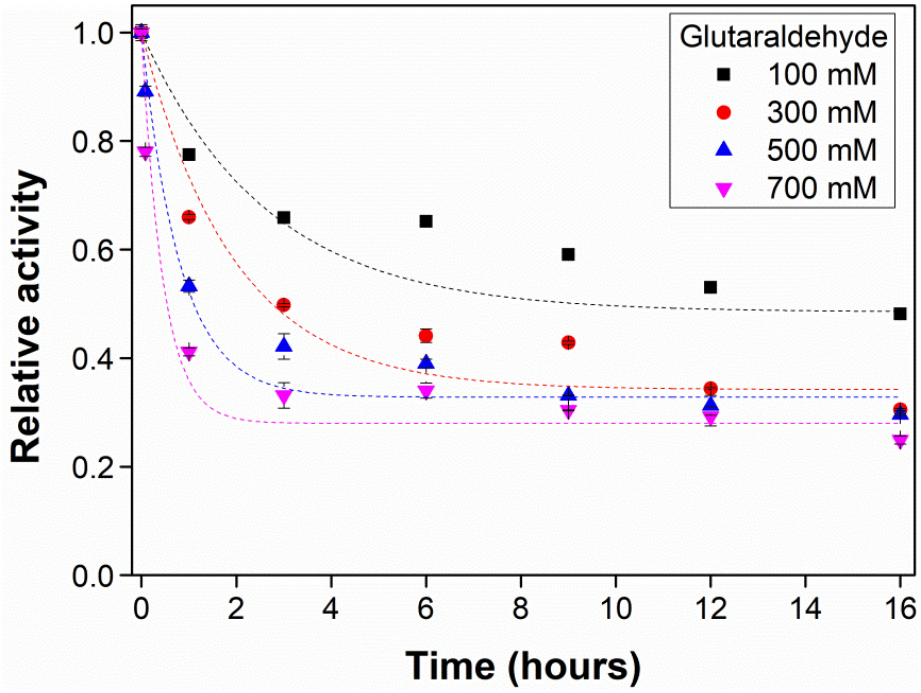
Table 3.2 — Effect of glutaraldehyde on the free amyloglucosidase activity in the presence or absence of starch. Residual activity was measured after 16 h incubation at 4 °C and the initial activity was taken to be 100%

Glutaraldehyde concentration	**Residual activity (%)**			
	Without Starch	Starch 1%	Starch 2.5%	Starch 5%
100 mM	34.46	48.12	43.18	36.37
300 mM	20.25	30.53	24.25	23.22
500 mM	19.48	29.63	26.80	24.40
700 mM	17.33	24.92	25.73	20.29

Figure 3.7 shows that the inactivation of AMG is very fast in the presence of glutaraldehyde, mainly at high glutaraldehyde concentrations. For short time periods and low glutaraldehyde concentrations, the residual activity is high, but these conditions do not favor the formation of stable CLEAs (MIGNEAULT et al., 2004; NADAR; RATHOD, 2016; TALEKAR et al., 2014). Thus, 16 h was kept as the glutaraldehyde treatment time in the preparation of CLEAs in the presence of 1% (w/v) starch as a pore-making agent. In the CLEA preparation (at 4 °C), the starch hydrolysis

rate catalyzed by AMG is very low, thus preserving the starch molecules large enough to serve as pore-making agents.

Figure 3.7 — Profile of enzymatic inactivation as a function of glutaraldehyde concentration in the presence of 1% (w/v) starch.



Assay conditions: water/enzyme solution volume ratio of 9:1 (in 100 mM carbonate buffer pH 10.0), glutaraldehyde concentration ranging from 100 to 700 mM, incubated statically in a refrigerator at 4 °C. Activity of amyloglucosidase was measured with 1% (w/v) dextrin in 50 mM sodium citrate buffer pH 4.5, 10 min reaction at 55 °C under 900 rpm stirring. Values correspond to a single assay with activity values measured in duplicate.

Table 3.3 shows that CLEAs prepared in the presence of 1% (w/v) starch (with and without the aminated magnetic nanoparticles DCNP-N) retained 2-3 times more activity, suggesting that the control of the pore size may play an important role in the enhanced activity during CLEA preparation. The combination of starch and DCNP-N yielded CLEAs with around 40% recovered activity using 500 mM glutaraldehyde. Although in the absence of DCNP-N the recovered activity was only a little lower (around 30%), the co-aggregation of AMG with DCNP-N has the advantage of easy separation of CLEAs without the formation of large clusters.

Table 3.3 — Comparison of the effect of glutaraldehyde concentration on the recovered activity of CLEAs (without and with DCNP-N [a]) synthesized in the presence of 1% (w/v) starch.

Glutaraldehyde concentration	**Activity recovery (%)**			
	Without Starch	Starch 1%	DCNP-N	DCNP-N+Starch 1%
100 mM	-	-	17.74 ± 1.47	18.72 ± 0.29
300 mM	-	-	17.03 ± 4.34	18.81 ± 0.31
500 mM	16.53 ± 0.64	33.53	16.52 ± 3.93	39.40 ± 1.44
700 mM	9.20 ± 0.11	30.28	15.76 ± 5.03	29.75 ± 1.15

Synthesis conditions: 16 h precipitation/cross-linking under static conditions in an ice batch, mass ratio protein/DCNP-N of 1:1, volume ratio enzyme-DCNP-N suspension/ethanol of 1:9.

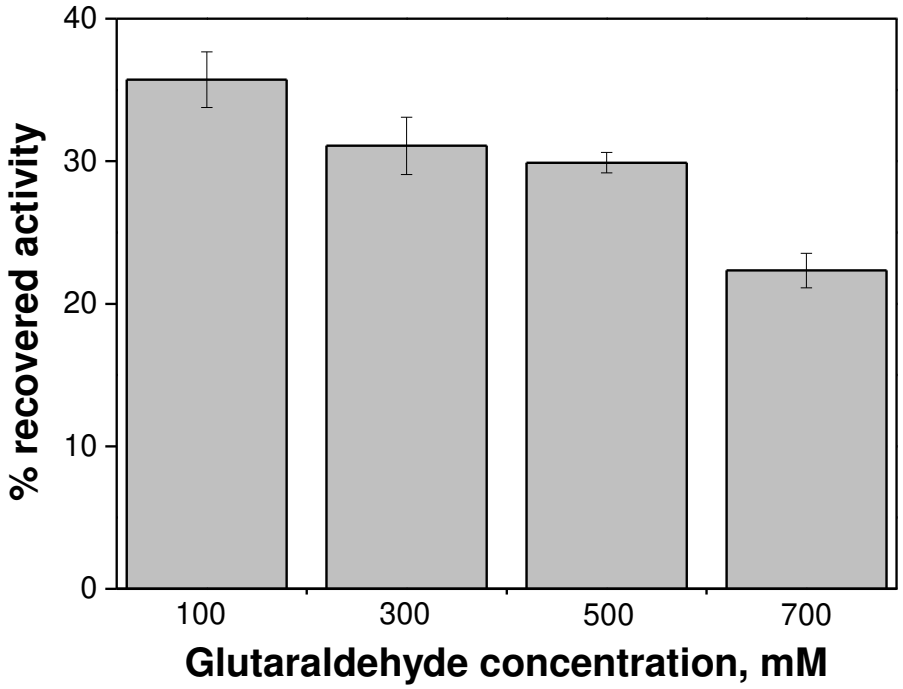
[a] DCNP-N refer to magnetic nanoparticles functionalized with amino groups.

3.3.5 Study of the co-aggregation of AMG and polyethyleneimine (PEI)

In order to improve the recovered activity of CLEAs of AMG, PEI was co-aggregated with the enzyme and the MNPs DCNP-N. Figure 3.8 shows that CLEAs could be formed even at a low glutaraldehyde concentration, yielding higher recovered activity compared to the CLEAs prepared without MNPs (Figure 3.5).

Despite the improvement obtained with the addition of PEI, the recovered activity of magnetic CLEAs (maximum around 35% in Figure 3.8) were lower than that achieved using starch as protective additive (maximum around 40% in Table 3.3). Therefore, the synthesis of CLEAs of AMG co-aggregated with the magnetic nanoparticles DCNP-N in the presence of both PEI and 1% (w/v) starch was also evaluated.

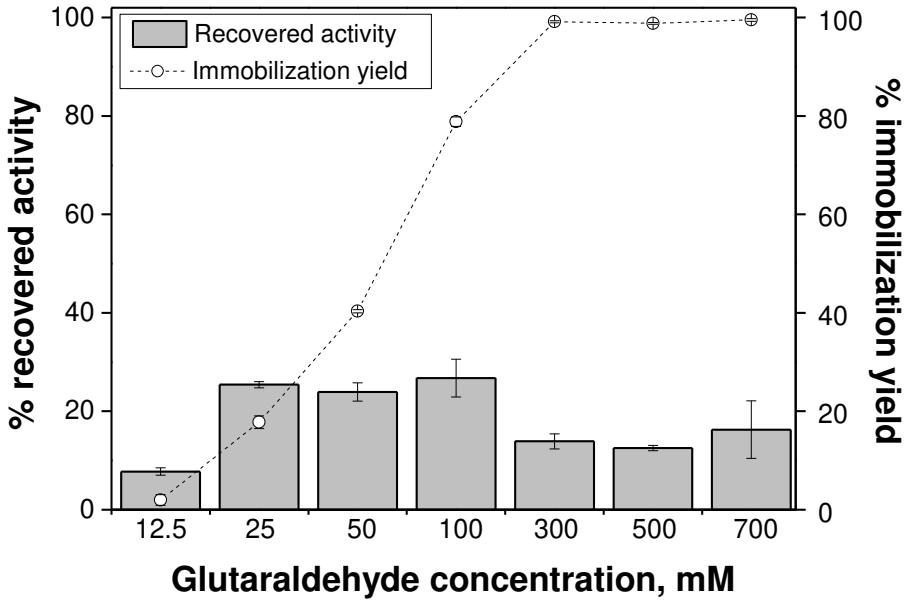
Figure 3.8 — Effect of glutaraldehyde concentration on the recovered activity of CLEAs synthesized in the presence of polyethyleneimine (PEI).



Synthesis conditions: PEI/enzyme mass ratio of 1:1 (30 min of gently stirring at 25 °C), addition of the magnetic nanoparticles DCNP-N to a DCNP-N/amyloglucosidase mass ratio of 1:1, precipitation with ethanol (volume ratio enzyme solution in 100 mM phosphate buffer pH 7.00 to ethanol of 1:9), crosslinking with glutaraldehyde concentrations ranging from 100 to 700 mM. Precipitation and crosslinking steps were performed under static conditions in an ice bath. Activity of immobilized amyloglucosidase was measured with 1% (w/v) dextrin in 50 mM sodium citrate buffer pH 4.5, 10 min reaction at 55 °C under 900 rpm stirring. Values are shown as the mean of duplicate experiments ± s.d.

Figure 3.9 shows that the presence of PEI and starch enabled the formation of CLEAs even at low concentrations of glutaraldehyde (12.5 mM). However, below 100 mM glutaraldehyde the immobilization yields were very low, probably because of insufficient cross-links to form stable structures (SHELDON, 2011; SHELDON; VAN PELT, 2013). On the other hand, when the glutaraldehyde concentrations ranged from 300 to 700 mM the immobilization yields were close to 100%, but the recovered activity was very low, probably due to the excessive crosslinking and/or deleterious effect of glutaraldehyde on the tertiary structure of AMG, as previously discussed. Therefore, a minimum concentration of 300 mM glutaraldehyde was selected for future experiments.

Figure 3.9 — Effect of glutaraldehyde concentration on CLEA of amyloglucosidase (AMG) synthesized in the presence of polyethyleneimine (PEI) and co-aggregated with the magnetic nanoparticles DCNP-N and starch 1% (w/v).



Synthesis conditions: treatment of AMG with PEI/enzyme mass ratio of 1:1 (30 min under gently stirring at 25 °C), co-aggregation with DCNP-N/enzyme mass ratio of 1:1, ethanol/enzyme solution (in 100 mM phosphate buffer pH 7.0) volume ratio of 9:1, crosslinking with glutaraldehyde concentrations ranging from 12.5 to 700 mM, precipitation and crosslinking under static conditions in an ice bath. Activity of immobilized AMG was measured with 1% (w/v) dextrin in 50 mM sodium citrate buffer pH 4.5, 10 min reaction at 55 °C under 900 rpm stirring. Values are shown as the mean of duplicate experiments ± s.d.

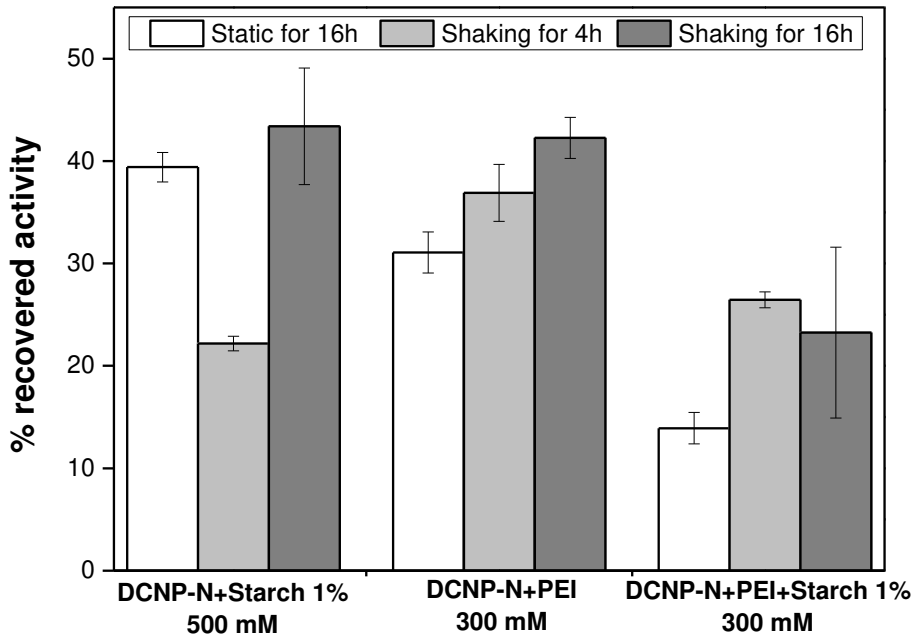
3.3.6 Influence of agitation, glutaraldehyde treatment time, cross-linker concentration, and co-feeders in the activity of AMG-CLEAs

It has been reported that at 4 °C, glutaraldehyde treatment time from 6 to 16 h is required to prepare stable CLEAs (MIGNEAULT et al., 2004; NADAR; RATHOD, 2016; TALEKAR et al., 2014). Thus, AMG CLEAs production was evaluated at 4 °C under stirring for 4 and 16 h, and under static conditions for 16 h.

Figure 3.10 shows that the glutaraldehyde treatment time was a key factor only for CLEAs prepared by co-aggregation with the magnetic nanoparticles DCNP-N and 1% (w/v) starch and crosslinking with 500 mM glutaraldehyde. Under these conditions, the immobilization yield increased from 45.3% to 70% and the recovered activity increased around twofold by increasing the time from 4 to 16 h. When PEI was added, no significant difference was observed. For the CLEAs prepared in the presence of PEI, the immobilization yields were higher than 95% for all conditions, but the recovered activity were higher when CLEAs were prepared under gently stirring in 3D

Platform Shaker. The gentle stirring promoted the formation of homogeneous CLEAs, which visibly reduced the particle size, thereby probably minimizing mass transfer problems (TALEKAR et al., 2013).

Figure 3.10 — Comparison of the effect of agitation and crosslinking time on the recovered activity of AMG CLEAs.



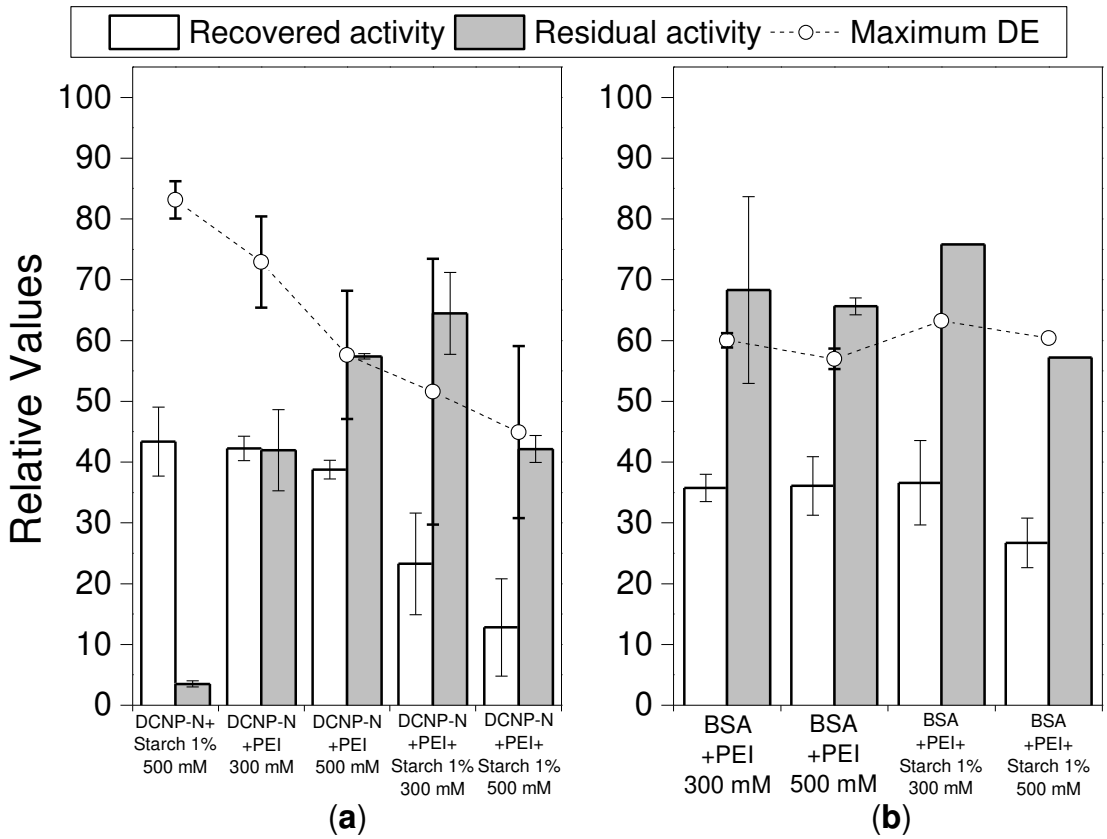
Synthesis conditions: co-aggregation of AMG with the magnetic nanoparticles DCNP-N and 1% (w/v) starch and crosslinking with 500 mM glutaraldehyde (DCNP-N+Starch 1%-500 mM); co-aggregation with the magnetic nanoparticles DCNP-N and polyethyleneimine (PEI) and crosslinking with 300 mM glutaraldehyde (DCNP-N+PEI-300 mM); co-aggregation with the magnetic nanoparticles DCNP-N, 1% (w/v) starch and PEI and crosslinking with 300 mM glutaraldehyde (DCNP-N+PEI+Starch 1%-300 mM). All CLEAs were prepared at 4 °C, mass ratio protein/DCNP-N of 1:1, precipitation with ethanol at volume ratio 1:9 (enzyme solution:ethanol). Values are shown as the mean of duplicate experiments ± s.d.

CLEAs previously prepared with 1% (w/v) starch, polyethyleneimine, and combinations thereof were synthesized again under stirring for 16 h at 4 °C; the co-aggregation with the aminated magnetic nanoparticles DCNP-N was compared with the most used co-feeder (bovine serum albumin), and two concentrations of glutaraldehyde (300 and 500 mM) were evaluated. These CLEAs were compared for their recovered activity as well as for their performance in the starch hydrolysis. The hydrolysis conditions were 55 °C under 300 rpm shaking for 6 h, using as a substrate 3% (w/v) starch prepared in 50 mM sodium citrate buffer pH 4.5 and pre-hydrolyzed

with α-amylase. At the end of the reaction, the Dextrose Equivalent (DE) of the hydrolyzed starch and residual activity of the CLEA were measured.

Figure 11(a) shows that magnetic CLEAs prepared in the presence of starch (DCNP-N+Starch 1%) yielded the highest DE (83.2), but its residual activity was very low (3.5%). Although the recovered activity was high (around 40%), these CLEAs were not sufficiently stable in starch hydrolysis conditions, even using a co-feeder rich in amino groups and high glutaraldehyde concentration in their preparation. On the other hand, magnetic CLEAs prepared in the presence of polyethyleneimine were also active in the starch hydrolyses (DE above 60) and stable (residual activiy around 40% and 60% for 300 and 500 mM glutaraldehyde, respectively). The addition of 1% (w/v) starch in the preparation of these CLEAs was not advantageous from a stability point of view.

Figure 3.11 — Recovered activity and performance of CLEAs of amyloglucosidase (AMG) in the hydrolysis of 3% (w/v) starch (Dextrose Equivalent and residual activity).



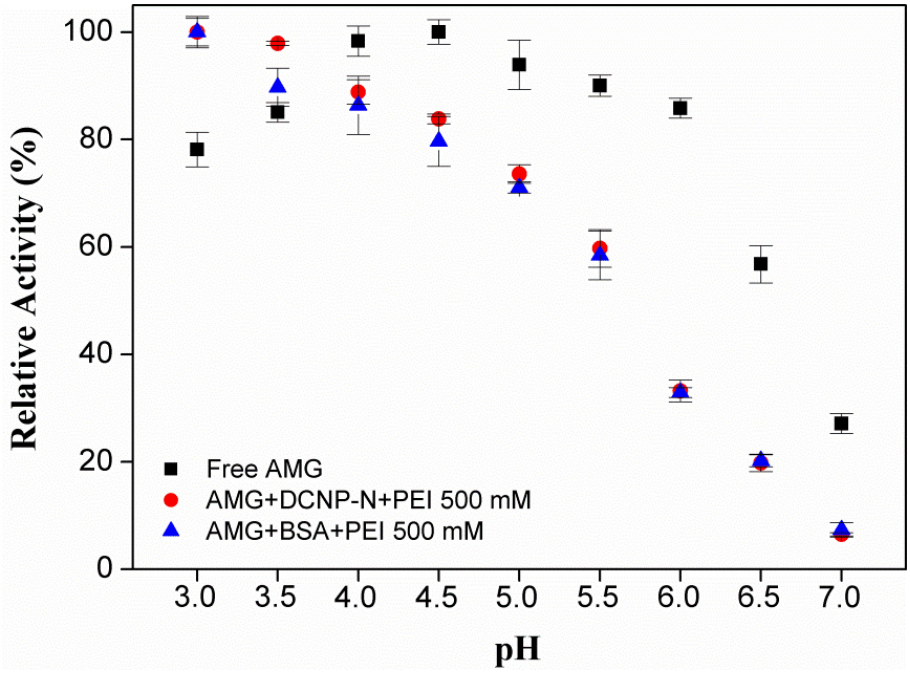
CLEAs were prepared by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA) in the presence of 1% (w/v) starch or polyethyleneimine (PEI) or both. A volume of 20 mL of starch (3%, w/v) solution was hydrolyzed by 20 U of AMG CLEAs at 55 °C and pH 4.5 for 6 h under 300 rpm stirring. Values are shown as the mean of duplicate experiments ± s.d.

CLEAs prepared under the same conditions, replacing magnetic nanoparticles with bovine serum albumin as the co-feeder, showed good performance (Figure 11b) regarding the recovered activity (around 35%), DE in the starch hydrolysis (around 60), and residual activity (above 60%). Although this co-feeder is widely used in the preparation of CLEAs (ARAUJO-SILVA et al., 2018; DAL MAGRO et al., 2016; MAFRA et al., 2016; SHAH; SHARMA; GUPTA, 2006; TALEKAR et al., 2013), the replacement by magnetic nanoparticles is advantageous because of the ease of capture by an external magnetic field (KOPP et al., 2015), avoiding the formation of clusters usually observed in the separation of CLEAs co-aggregated with bovine serum albumin by centrifugation (CUI; JIA, 2015). Moreover, magnetic CLEAs prepared in the presence of polyethyleneimine and crosslinking with 500 mM glutaraldehyde (CLEA DCNP-N+PEI) showed similar performance (recovered activity around 40%, DE in the starch hydrolysis around 60, and residual activity around 60%), so were selected to be kinetically characterized and used in the hydrolysis of starch under industrial conditions. For comparison, CLEAs of AMG prepared by co-aggregation with bovine serum albumin and crosslinking with 500 mM glutaraldehyde (CLEA BSA+PEI) were also selected.

3.3.7 Characterization of the CLEAs of AMG

The CLEAs AMG+DCNP-N+PEI and AMG+BSA+PEI were characterized regarding the activity as a function of pH and temperature, thermal stability at 55 °C and pH 4.5, and their performance in the hydrolysis of starch under industrial conditions (high starch concentration, i.e., 35%, w/v). Figure 3.12 shows that the activity profiles as a function of pH for CLEAs of AMG shifted the maximum activity from pH 4.5 to 3.0 compared to the free AMG. This can be associated with a higher stability of immobilized enzyme at this drastic pH.

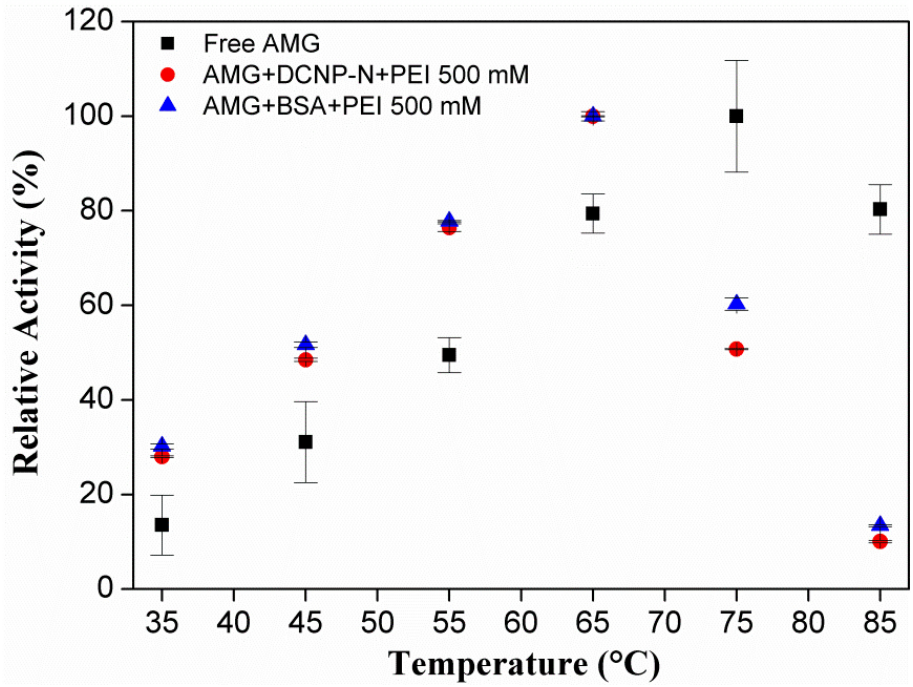
Figure 3.12 — Effect of pH on the activity of free amyloglucosidase (AMG) and AMG CLEAs prepared by enzyme treatment with polyethyleneimine (PEI) and by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA).



Activity assay conditions: 1% (w/v) dextrin solution in 50 mM buffer, 10 min reaction at 55 °C under 900 rpm stirring. Values are shown as the mean of triplicate experiments ± s.d.

Figure 3.13 shows that the maximum activity of the CLEAs was lowered from 75 °C (for free AMG) to 65 °C. This suggests that the immobilized enzyme was less stable at this high temperature, or that the CLEA structure may change and in that way alter the diffusional problems. However, at 55 °C and pH 4.5 the CLEA activity corresponds to around 80% of the maximum activity, while for soluble AMG the activity at these conditions corresponds to around 50% of the maximum value. Thus, the CLEAs of AMG could be more advantageous from an industrial point of view, because the typical industrial conditions of the saccharification of starch are pH 4.0–4.5 and temperature 55–60 °C (GUZMÁN-MALDONADO; PAREDES-LÓPEZ; BILIADERIS, 1995; HOBBS, 2009; SYNOWIECKI, 2007).

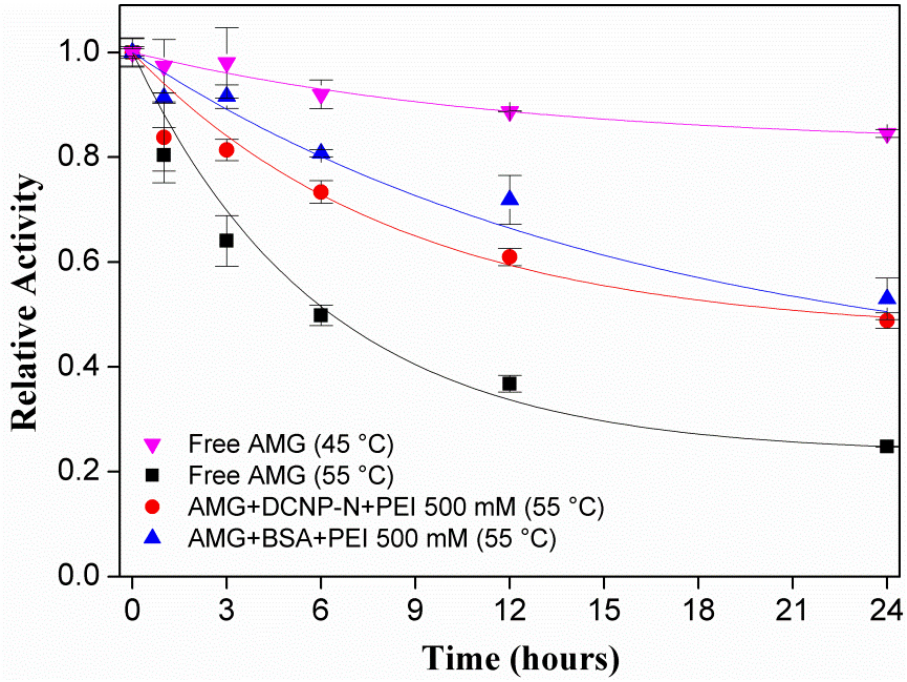
Figure 3.13 — Effect of the temperature on the activity of free amyloglucosidase (AMG) and AMG CLEAs prepared by enzyme treatment with polyethyleneimine (PEI) and co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA).



Activity assays conditions: 1% (w/v) dextrin solution in 50 mM sodium citrate buffer pH 4.5, 10 min reaction under 900 rpm stirring. Values are shown as the mean of triplicate experiments ± s.d.

Figure 3.14 shows the thermal inactivation profiles of free (45 and 55 °C and pH 4.5) and immobilized AMG at these typical industrial conditions (55 °C and pH 4.5) (HOBBS, 2009). Table 3.4 shows the parameters of the Sadana & Henley (1987) model fitted to the experimental data. It can be observed that CLEAs of AMG (with both magnetic nanoparticles and BSA as co-feeders) were around 4 times more stable than the free AMG at 55 °C, having a fraction of immobilized molecules more resistant to the inactivation as indicated by the parameter α (0.36–0.46 for CLEAs and 0.23 for free AMG). This higher stability is indicative of effective crosslinking with glutaraldehyde, aided by polyethyleneimine in the complex and rigid structure of the CLEAs (enzyme and co-feeders). It has been reported that the immobilization tends to restrict the conformational flexibility of the enzyme, which prevents conformational changes (GARCIA-GALAN et al., 2011b; MATEO et al., 2007; RODRIGUES et al., 2013).

Figure 3.14 — Profile of thermal inactivation of free amyloglucosidase (AMG) and AMG CLEAs prepared by enzyme treatment with polyethyleneimine (PEI) and by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA) at 45 and 55 °C and pH 4.5 (50 mM sodium citrate buffer).



Values are shown as the mean of triplicate experiments ± s.d.

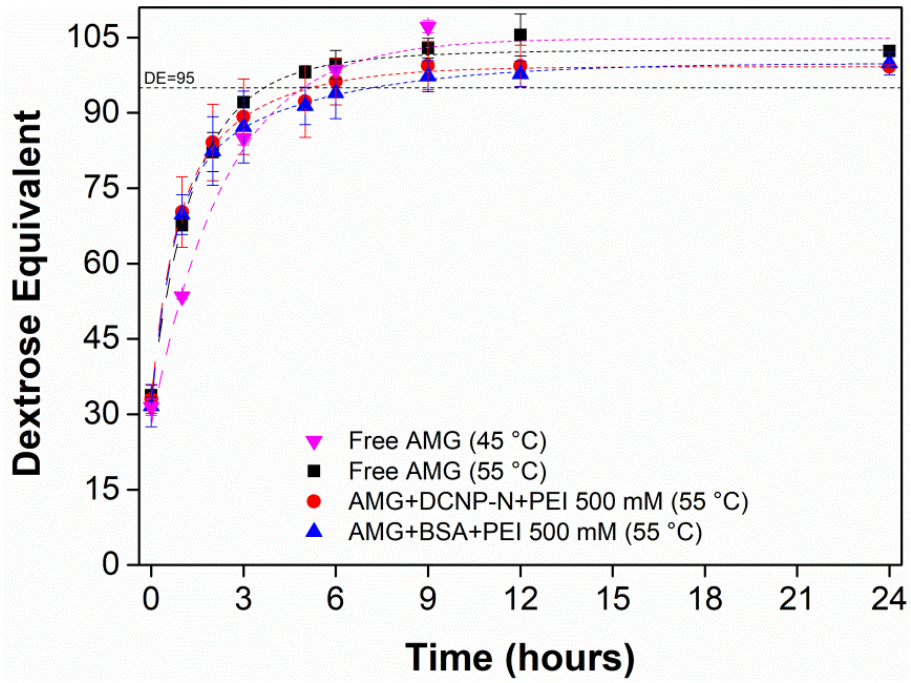
Table 3.4 — Half-life times ($t_{1/2}$) and stability factor (SF) for amyloglucosidase (free and immobilized AMG) at 55 °C and pH 4.5.

Biocatalyst	$t_{1/2}$ (hours)	SF	k_d	α	Adj. R^2
Free AMG	6.34	1.00	0.167 ± 0.014	0.234 ± 0.005	0.99
AMG+DCNP-N+PEI 500 mM	22.67	3.58	0.117 ± 0.016	0.462 ± 0.027	0.98
AMG+BSA+PEI 500 mM	24.60	3.88	0.062 ± 0.019	0.361 ± 0.119	0.95

Note: SF is the ratio between the half-life of the immobilized enzyme and of the free enzyme; the inactivation parameters (k_d and α) were estimated using the Sadana & Henley model; DCNP-N is magnetic nanoparticles functionalized with amino groups; PEI is polyethyleneimine; BSA is bovine serum albumin. All CLEAs were prepared by crosslinking with 500 mM glutaraldehyde.

Figure 3.15 shows the profiles of starch hydrolysis catalyzed by free and immobilized AMG at typical industrial conditions (35% starch solution and 2 mL of soluble AMG/kg of starch). When CLEA was used as the catalyst, the amount of CLEA was equivalent to 2 mL of soluble AMG in terms of activity. Because at 45 °C the free AMG is highly stable (more than 80% residual activity after 24 h incubation), starch was also hydrolyzed at 45 °C using free AMG, maintaining the other conditions.

Figure 3.15 — Profile of hydrolysis of 35% (w/v) starch (pre-hydrolyzed with α-amylase) catalyzed by free amyloglucosidase (AMG) and AMG CLEAs prepared by enzyme treatment with polyethyleneimine (PEI) and by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA) at 45 and 55 °C and pH 4.5 under 900 rpm stirring.



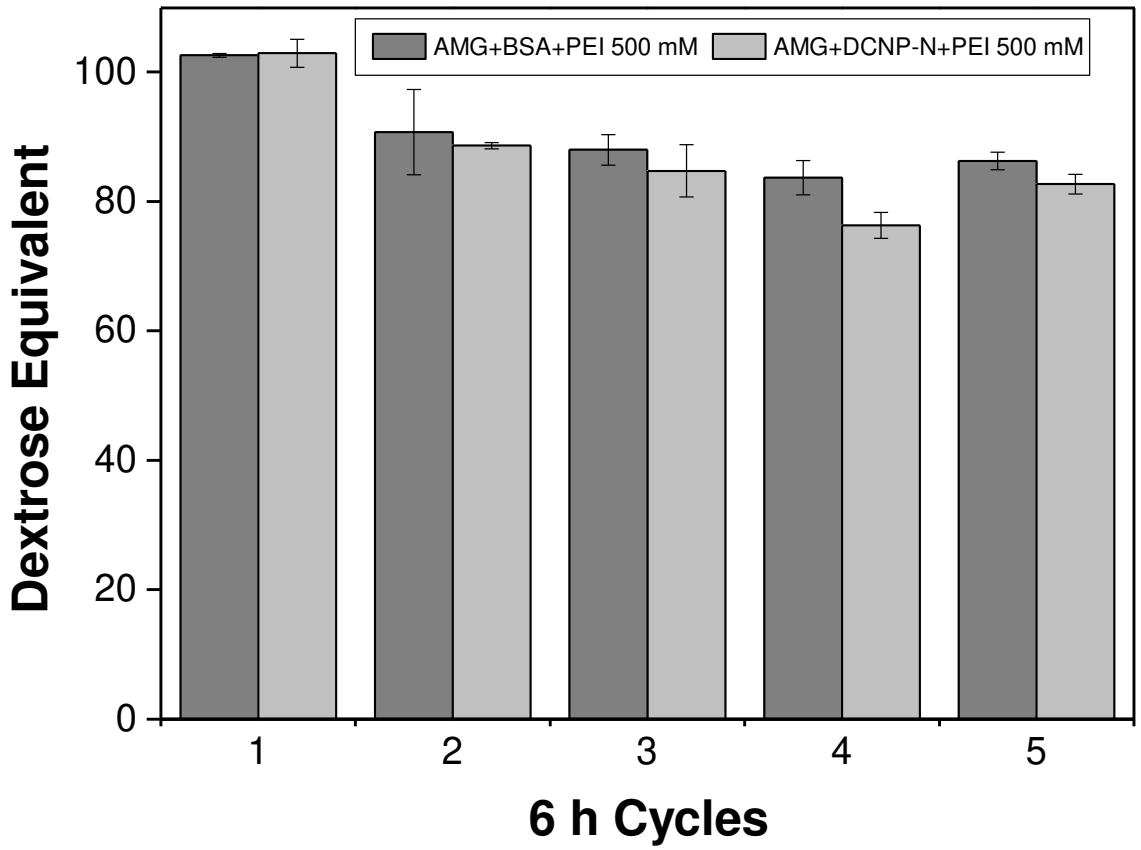
Amount of AMG: 2 mL of free enzyme/kg of starch (37,820 U/kg of starch) or equivalent units to the CLEAs. The reaction conversion (starch-to-glucose) was monitored by Dextrose Equivalent (DE) measuring reducing sugars by DNS method. Values are shown as the mean of duplicate experiments ± s.d.

It can be observed that the hydrolysis profiles for all biocatalysts (free and immobilized enzymes) were very closed, achieving a Dextrose Equivalent (DE) around 95 after a 6-h reaction, even at 45 °C using free AMG, which shows that the enzyme load is high enough to guarantee high hydrolysis rates. The increase in DE for larger reaction times is very low, not justifying longer hydrolysis because the productivity of the process drops a lot (in $g_{glucose}.L^{-1}.h^{-1}$): 6.6 for 6 h, 2.7 for 9 h, and 1.35 for 12 h. Maximum conversions of starch hydrolysis by soluble AMG ranging from 92 to 98% (TOMASIK; HORTON, 2012) had been previously reported, which shows the excellent performance of CLEAs of AMG prepared in this work.

3.3.8 Reuse assays

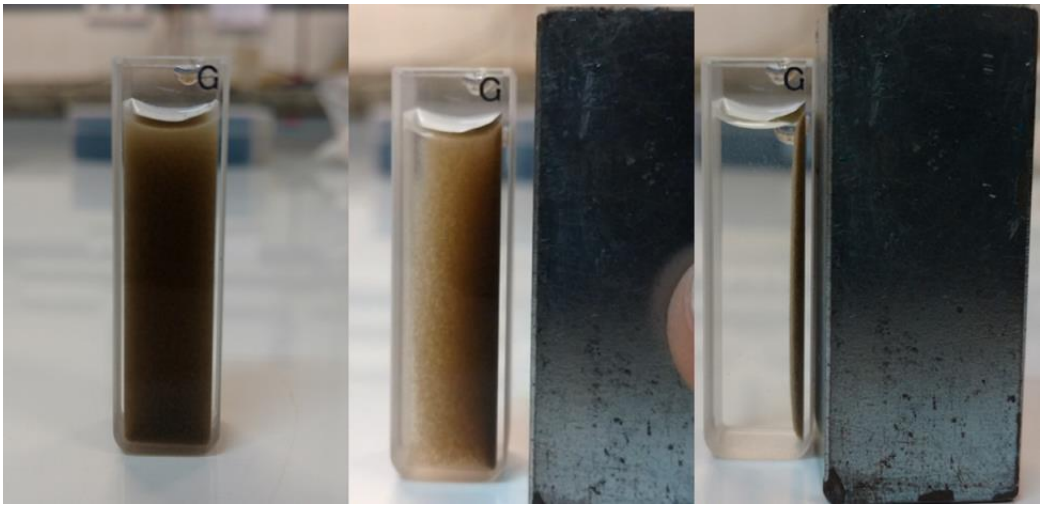
Because of the similar behavior of hydrolysis at 45 and 55 °C, achieving a DE around 95 after 6 h, the reuse assays were performed at 45 °C because of the high stability of AMG. Figure 3.16 shows that after five 6-h batches, DE is maintained around 85% using both biocatalysts (CLEAs of AMG prepared with BSA or MNP in presence of PEI). Handling and recovery of the CLEA were easy when MNP was used applying an external magnetic field (Figure 3.17).

Figure 3.16 — Hydrolysis of pre-hydrolyzed starch with α-amylase (35%, w/v) catalyzed by CLEAs of amyloglucosidase (AMG) prepared by enzyme treatment with polyethyleneimine (PEI) and by co-aggregation with the magnetic nanoparticles DCNP-N or bovine serum albumin (BSA) at 45 °C and pH 4.5 for 6 h reaction.



Values are shown as the mean of duplicate experiments ± s.d.

Figure 3.17 — Separation of the magnetic CLEAs of amyloglucosidase by the action of an external magnetic field.



3.4 CONCLUSIONS

This study showed that promising CLEAs of amyloglucosidase could be synthesized by co-aggregation with aminated magnetic nanoparticles (MNPs) or bovine serum albumin (BSA), using polyethyleneimine as an aid in the crosslinking step with glutaraldehyde. Both CLEAs showed around 40% of the offered activity, thermal stability approximately 4 times higher than the soluble enzyme, and small changes in the catalytic properties. Moreover, the starch saccharification at typical industrial conditions, i.e., 35% (w/v) pre-hydrolyzed starch with α-amylase, 55 °C and pH 4.5, catalyzed by the CLEAs (co-aggregated with MNPs or BSA) showed similar behavior to the soluble enzyme, yielding a Dextrose Equivalent around 95 after a 6-h reaction. In addition, both CLEAs could be reused in five 6-h cycles at 45 °C and maintain a DE above 85. These findings could be attractive to the amylolytic industry because highly concentrated starch solutions may be processed by immobilized AMG as well as the soluble enzyme (including 10 °C below the conventionally used temperature), having the additional advantage of being easily separated from the reaction medium and reused in the process when MNPs are used instead of the protein co-feeders commonly used in the CLEA methodology, such as BSA, whose cost is too high for the synthesis of CLEAs for large-scale applications.

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4 OPTIMIZATION OF SIMULTANEOUS SACCHARIFICATION AND ISOMERIZATION OF DEXTRIN TO HIGH FRUCTOSE SYRUP USING A MIXTURE OF IMMOBILIZED AMYLOGLUCOSIDASE AND GLUCOSE ISOMERASE BY DESIGN OF EXPERIMENTS

Abstract: High fructose syrup is a sweetener widely used as a substitute for sucrose by the food and beverage industry and is produced by enzymatic route. However, its current industrial production is limited to basically occurring in three sequential processes due to the use of three different enzymes (α-amylase, amyloglucosidase, and glucose isomerase) that operate under different pH and temperature conditions. Studies in the literature have evaluated the application of these enzymes in simultaneous processes of liquefaction and/or saccharification and isomerization, but with low conversion yields, or with low concentration substrates, or with the application of soluble enzymes. This makes it impossible to recover and reuse these enzymes extensively. In this study, a synergistic saccharification and isomerization process was proposed in a single step, using the commercial amyloglucosidase enzyme of *Aspergillus niger* immobilized by the CLEA technique, co-aggregated with magnetic nanoparticles and polyethyleneimine, and the commercial immobilized glucose isomerase enzyme produced by *Streptomyces murinus*. Using immobilized enzymes made it easier to work in a wider operating window, thus leading to the application of a factorial design with central a composite rotatable design that was able to define an optimal condition of pH and temperature of the process, as well as the best relation between the two enzymes. Simultaneous saccharification and isomerization from a 35% (w/v) dextrin solution reached a Dextrose Equivalent above 95%, with conversion yields around 48% of fructose at the end of 30 h of reaction. In addition, the catalysts could be reused for six consecutive cycles, maintaining conversion yields around 47% of fructose without loss of activity and with easy recovery of the biocatalysts. Furthermore, because they are of different natures (magnetic CLEA of amyloglucosidase and GI pellets), if one of these biocatalysts is inactivated, they can be easily separated and recharged individually.

Keywords: Simultaneous reaction. High fructose syrup. Amyloglucosidase. Glucose isomerase. Single-step saccharification and isomerization.

4.1 INTRODUCTION

High-Fructose Syrup (HFS) is a starch-based sweetener used as a sugar substitute (JENSEN et al., 2018; VUILLEUMIER, 1993; WHITE; HOBBS; FERNANDEZ, 2015). HFS is preferred by the food and beverage industries, because it presents a high solubility, does not undergo recrystallization, and has sweetness equal to or greater than sucrose (depending on the concentration of fructose) (PARKER; SALAS; NWOSU, 2010; WHITE; HOBBS; FERNANDEZ, 2015; WHITE, 2014). In the United States of America, the largest producer, and consumer, HFS is produced from corn starch, known as high fructose corn syrup (HFCS) (PARKER; SALAS; NWOSU, 2010; VUILLEUMIER, 1993; WALKER; DUMKE; GORAN, 2014).

The conventional industrial production of HFS basically involves three processes: (i) liquefaction, which hydrolyzes the starch to oligomers with hydrochloric acid and/or α-amylase (EC 3.2.1.1) (CRABB; MITCHINSON, 1997; NIGAM; SINGH, 1995); (ii) saccharification, which hydrolyzes the oligomers to glucose with amyloglucosidase (AMG) (EC 3.2.1.3, glucoamylase) (CRABB; MITCHINSON, 1997; PAZUR; ANDO, 1959); and (iii) isomerization, which isomerizes glucose to fructose by glucose isomerase (GI) (EC 5.3.1.5, xylose isomerase) (CRABB; MITCHINSON, 1997; MARSHALL; KOOI, 1957).

The saccharification and isomerization processes are carried out sequentially, both enzymatically under different conditions. Besides, the saccharification process currently uses soluble AMG, which is neither recovered nor reused (CRABB; MITCHINSON, 1997; HOBBS, 2009). This drawback does not occur with GI, which due to the high cost and large industrial application is already commercialized and applied in the immobilized form (DICOSIMO et al., 2013; JØRGENSEN et al., 1988; PARKER; SALAS; NWOSU, 2010).

To overcome these limitations, a single-step process, which hydrolyzes and isomerizes the pre-hydrolyzed starch in a process where the enzymes in the immobilized form can be reused several times has been suggested and reported (GE et al., 1999; HUI et al., 1992; MISHRA et al., 2002; SCHAFHAGSER; STOREY, 1992).

It is known that enzyme immobilization techniques are widely used in order to produce insoluble biocatalysts with operational stability, which may be recovered and reused continuously (GARCIA-GALAN et al., 2011; GUISAN, 2006; KENNEDY;

CABRAL, 1987; MATEO et al., 2007; RODRIGUES et al., 2013; SHELDON; VAN PELT, 2013). Several scientific papers report the immobilization of AMG using different techniques (AMARAL-FONSECA et al., 2018; BRYJAK, 2003; SHAH; SELLAPPAN; MADAMWAR, 2000; SILVA; ASQUIERI; FERNANDES, 2005; TARDIOLI et al., 2011b; WANG et al., 2013).

In addition, co-immobilization techniques using AMG and GI have also been reported. Katwa & Rao (1983) covalently coupled the enzymes α-amylase, AMG and GI in Sepharose-6MB activated with cyanogen bromide, applying the catalyst simultaneous hydrolysis, saccharification and isomerization on a reactor jacketed column, achieving conversion yields of 40% in fructose using soluble starch 5% (w/v) as substrate. Schafhagser & Storey (1992) immobilized the enzymes AMG, pullulanase, and GI by adsorption on a granular chicken bone (Biobone™) achieving conversion yields of 20% in fructose using glycogen 12% (w/v) as substrate. They also observed an increase of 5% in the conversion yields with the pH change from 6.0 to 7.2 after 1 h of reaction in order to initially favor hydrolysis followed by isomerization. GE et al. (1999) adsorbed GI on macroporous polystyrene trimethylamine followed by treatment with trimethylammonium iodate and subsequent adsorption of AMG, ending with glutaraldehyde cross-linking, achieving conversion yields of 42% in fructose after 25 h using dextrin-27 at 5% (w/v) as substrate.

These techniques can also create a microenvironment, where the product of the first enzyme is the substrate of the second enzyme, facilitating the diffusion of the substrate and favoring the production of the final product (GE et al., 1999; SCHAFHAGSER; STOREY, 1992). However, the co-immobilized enzymes do not present similar operational conditions (*e.g.*, pH and temperature) (KATWA; RAO, 1983; SCHAFHAGSER; STOREY, 1992). One of them may be inactivated first, leading to the loss of the whole biocatalyst. Thus, using a mixture of these enzymes immobilized separately could be more advantageous if one of them may be recovered using a simple separation method.

Previous work carried out in our research group has evaluated the immobilization of amyloglucosidase using the cross-linked enzyme aggregate (CLEA) technique. The biocatalyst co-aggregated with magnetic nanoparticles in the presence of polyethyleneimine (PEI), followed by crosslinking with glutaraldehyde, showed operational resistance and ease of recovery by an external magnetic field (AMARAL-FONSECA et al., 2018).

In this context, this work aimed to develop a process for simultaneous saccharification and isomerization using magnetic CLEAs of AMG and commercial immobilized GI. In order to find the best conditions of pH, temperature, and GI/AMG ratio to achieve maximum substrate hydrolysis and fructose production, a factorial design with central composite rotatable design (CCRD) was performed. The mathematical model was validated in the optimal conditions predicted for the simultaneous saccharification and isomerization of dextrin. These optimum conditions were also used in a process using a high concentration of dextrin to establish the time of maximum dextrin-to-glucose conversion and fructose production. In the best condition, assays of reusability were performed at the best operational conditions (pH, temperature, GI/AMG ratio and time reaction).

4.2 MATERIALS AND METHODS

4.2.1 Materials

AMG 300L™ from *Aspergillus niger* (EC 3.2.1.3, glucoamylase, amyloglucosidase) (≥300 AGU/mL) and Sweetzyme® IT Extra from *Streptomyces murinus* (EC 5.3.1.5, xylose isomerase, glucose isomerase) (≥400 IGIU/g) were obtained from Novozymes A/S (Bagsværd, Denmark), dextrin 10 and polyethylenimine (oligomer mixture, Mn~423) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and glutaraldehyde 25% (v/v) aqueous solution from Vetec (Duque de Caxias, RJ, Brazil). A mono colorimetric enzymatic reagent (GOD-POD) was purchased from Gold Analisa (Belo Horizonte, MG, Brazil). Aminated magnetic nanoparticles (marketed under the trademark DCNP-N) were purchased from Kopp Technologies (São Carlos, SP, Brazil). All other chemicals (analytical grade) were purchased from Synth (Diadema, SP, Brazil) and Vetec (Duque de Caxias, RJ, Brazil).

4.2.2 Preparation of magnetic AMG-CLEA

Magnetic AMG-CLEAs were prepared according to the methodology described by Amaral-Fonseca et al. (2018). An aqueous solution of PEI (at pH 7.0) was mixed with the enzyme solution (20 mg/mL in 50 mM phosphate buffer pH 7.0) at a protein/PEI mass ratio of 1:1. After 30 min stirring, the aminated magnetic nanoparticle (MNP) was added at a final protein/MNP mass ratio of 1:1. This solution was precipitated with ethanol at a volume ratio of 1:9 (enzyme/precipitant) for 30 min in an ice bath. Afterwards, glutaraldehyde 25% (*v*/*v*) was added to a final concentration of 500 mM and the homogenized suspension was incubated at 4 °C for 16 h.

4.2.3 Experimental design

The pH and temperature are the most significant effects on enzymatic efficiency. Aiming to use two different enzymes (AMG and GI) in a single simultaneous process, a factorial design was performed to evaluate the effect of pH (X_1), temperature (X_2) and the enzymatic loading ratio between GI and AMG (X_3). The response variables dextrose equivalent (DE) and glucose-to-fructose conversion were analyzed using the central composite rotatable design (CCRD). In total, 17 experimental runs were carried out in random order. Statistica software (StatSoft, version 7.0) was used to analyze the experimental data (DE and conversion) and construct the second-order polynomial mathematical model to establish the correlation between the variables and each response *y* (DE or conversion), according to Equation (4.1):

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad \text{Equation (4.1)}$$

Where *y* is the predicted response variable; β are the constant terms, representing regression coefficients of individual linear and quadratic effects and interaction effects between the variables; X_1, X_2, and X_3 are the coded independent variables.

The polynomial equation for DE and conversion were used to create the response surfaces to visualize the relationship between the process variables and the

responses studied.

4.2.4 Saccharification and isomerization

For experimental design assays, a dextrin 10 solution (1%, w/v) was prepared in 50 mM citrate buffer containing $MgSO_4.7H_2O$ (50 mM) and $CoCl_2.6H_2O$ (2.5 mM), the ion concentration applied was the same as GI activity measures. The pH used ranged from 3.98 to 6.51 and the temperature ranged from 41.6 to 58.4 °C. The enzyme loading of AMG offered was constant at 15 U, varying the enzyme loading offered of GI from 2.02 to 23.4 U. The reactions were carried out for 12 h in a 70 mL batch reactor (8.5 x 3.33 cm (length x diameter) containing 30 mL of reaction medium, mechanically stirred at 1500 rpm using a cylindrical impeller without blades (8.4 x 0.80 cm (length x diameter). Samples of the reaction medium were withdrawn at the end of the reaction to quantify sugars formed by HPLC and to measure released reducing sugars (RS) by the dinitrosalicylic acid method (DNS) (MILLER, 1959). The dextrose equivalent (DE) (KEARSLEY; DZIEDZIC, 1995) and glucose-to-fructose conversion were calculated by Equations (4.2) and (4.3).

$$Dextrose\ Equivalent = \frac{Amount\ of\ RS\ (expressed\ as\ glucose)}{Dextrin\ dry\ mass} \times 100 \qquad \text{Equation (4.2)}$$

$$Conversion\ (\%) = \frac{Fructose\ concentration}{Glucose\ conc.+Fructose\ conc.} \times 100 \qquad \text{Equation (4.3)}$$

The hydrolysis and isomerization profiles with the time under optimal conditions were evaluated with different enzyme loads (5, 20, 37.8, 50, 100 U_{AMG}/g dextrin and 7.8, 31.2, 59, 78, 156 U_{GI}/g dextrin) using dextrin 10 (35%, w/v) prepared in 50 mM citrate buffer (pH 5.7) containing $MgSO_4.7H_2O$ (250 mM) and $CoCl_2.6H_2O$ (2.5 mM). The reactions were performed at 50 °C and aliquots of the reaction medium were withdrawn at regular time intervals to measure DE and conversion as described above.

4.2.5 Operational stability

The reusability assays were performed in the same 70 mL batch reactor at 50 °C under 1500 rpm mechanical stirring using a cylindrical impeller without blades, using an enzyme load of 37.8 U_{AMG}/g dextrin and 59 U_{GI}/g dextrin. The substrate solution (20 mL) was Dextrin 10 (35%, w/v) prepared in a 50 mM citrate buffer pH 5.7 containing $MgSO_4.7H_2O$ (250 mM) and $CoCl_2.6H_2O$ (2.5 mM). After each cycle of 30 h reaction time, the biocatalysts were recovered by centrifugation (1500× g for 5 min at 4 °C) and applying an external magnetic field, were washed with distilled water and re-suspended in a fresh substrate solution. The yield of the dextrin hydrolysis, quantified as DE, and the glucose-to-fructose conversion were determined using the supernatant of the reaction medium.

4.2.6 Amyloglucosidase activity

Amyloglucosidase activity of magnetic AMG-CLEA was determined by measuring the glucose released at 55 °C from a dextrin 1% (w/v) solution prepared in a 50 mM sodium citrate buffer (pH 4.5) (AMARAL-FONSECA et al., 2018), using the glucose oxidase and peroxidase colorimetric enzymatic test (GOD-POD) (TRINDER, 1969). The reaction was carried out for 10 min under 900 rpm stirring and aliquots were withdrawn at 2.5 min intervals and quenched with HCl (1 M). One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol of glucose per minute under the conditions above.

4.2.7 Glucose isomerase activity

Enzymatic activity of glucose isomerase was determined by calculating the initial velocity of fructose formation catalyzed by a known amount of enzyme. The standard substrate was 2 M glucose solution in 50 mM Tris-maleate buffer (pH 7.0) containing $MgSO_4.7H_2O$ (50 mM) and $CoCl_2.6H_2O$ (2.5 mM) (GIORDANO; GIORDANO; COONEY, 2000; MILESSI-ESTEVES et al., 2019). The reaction was carried out at 60 °C for 30 min under 1500 rpm stirring. Samples were withdrawn at 10 min intervals, the reaction was quenched with 1 M HCl, and fructose was quantified by HPLC. One unit of enzyme activity (U) was defined as the amount of enzyme required

to release 1 µmol of fructose per minute under the assay conditions.

4.2.8 Analytical methods

The concentration of maltose, glucose and fructose was determined by high-performance liquid chromatography (HPLC) in a Waters Breeze™2 1525 chromatograph equipped with a refractive index detector (Waters 2414). The compounds were separated in an Aminex® HPX-87H column (300 x 7.8 mm, Bio-Rad) at 45 °C, using 5 mM H_2SO_4 solution as eluent at a flow rate of 0.6 mL/min. All samples were filtered using a 0.22 µm filter previous to its injection into the equipment.

4.3 RESULTS AND DISCUSSION

4.3.1 Experimental design

In order to define the best conditions for the simultaneous saccharification and isomerization process for the production of High Fructose Syrup, the pH and temperature conditions need to be determined in a condition where the enzymes used in this process presents its maximum operational activity, as shown in Table 4.1.

Table 4.1 —. Range of pH and temperature for maximum activity.

Enzyme	pH	Temperature	Reference
Amyloglucosidase (CLEA)	4.5 – 6.0	45 – 55°C	(AMARAL-FONSECA et al., 2018)
Glucose Isomerase (Sweetzyme® IT Extra)	6.5 – 8.0	55 – 60°C	(JØRGENSEN et al., 1988)

These enzymes, have different biochemical, structural, and functional properties, therefore they work in different pH and temperature ranges (TOMASIK; HORTON, 2012). Thus, in order to work synergistically and efficiently, it is also crucial to evaluate the relationship between them, since the supplementation of one of the biocatalysts can overcome the deficit generated by the operating conditions (XUE; WOODLEY, 2012).

For this purpose, a statistical experimental design (2^3 + 2(3) + 3 CP) was carried to evaluate pH, temperature and U_{GI}/U_{AMG} ratio (independent variables), as shown in Table 4.2.

Table 4.2 — Variables and codified levels of the experimental design of the simultaneous saccharification and isomerization of dextrin to fructose syrup catalyzed by immobilized amyloglucosidase (AMG) and glucose isomerase (GI).

Independent variables	**Levels**				
	-1.68	-1	0	+1	+1.68
pH	3.98	4.5	5.25	6.0	6.51
Temperature (°C)	41.6	45	50	55	58.4
U_{GI}/U_{AMG}	2.02/15	6.35/15	12.7/15	19.1/15	23.4/15

The ranges of pH (3.98 to 6.5) and temperature (41.6 to 58.4 °C) were defined to encompass the operational range of the two enzymes, as shown in Table 4.1. The AMG load was always constant at 15 U (measured at pH 4.5 and 55 °C), changing only the GI loads for the evaluated UGI/UAMG ratios. The influence of the variables pH, temperature and U_{GI}/U_{AMG} ratio were evaluated in the response variables, i.e., hydrolysis (dextrose equivalent) and isomerization (glucose-to-fructose conversion), in order to investigate the most suitable conditions for the production of HFS in a simultaneous saccharification and isomerization process. Table 4.3 shows the experimental and predicted results by the RCCD, calculated from the second-order polynomial equations, for DE and conversion in each condition of pH, temperature and U_{GI}/U_{AMG} ratio evaluated.

Multiple regression analysis of the experimental data using Statistica 7.0 software including all terms to express every variation of the model, regardless of its significance, generated the following mathematical models for DE and conversion:

$$\mathrm{DE} = -596.2495 + 88.1604X_1 - 8.4070X_1^2 + 19.1342X_2 - 0.1922X_2^2 + 58.7937X_3 - 6.2169X_3^2 - 0.0386X_1X_2 - 7.9780X_1X_3 - 0.1229X_2X_3; \qquad (R^2 = 0.8869)$$

$$Conversion\ (\%) = 410.9403 - 110.9392\,X_1 + 8.7529X_1^2 - 4.7133X_2 + 0.0273X_2^2 - 96.0640X_3 + 4.6656X_3^2 + 0.3653X_1X_2 + 15.1063X_1X_3 + 0.3384X_2X_3; \quad (R^2 = 0.9574)$$

The obtained polynomial models presented coefficients of determination (R^2) of

0.89 and 0.96 for DE and conversion, respectively, indicating a good fit of the models to the experimental data, according to the general rule that R^2 should be at least 0.80 (JOGLEKAR; MAY, 1987). The similarity between the observed and predicted results by the models corroborates the adequacy of the models in the prediction of the behavior of the simultaneous saccharification and isomerization process in the pH, temperature and GI/AMG ranges evaluated.

Table 4.3 — Experimental design for simultaneous saccharification and isomerization and experimental and predicted results for dextrose equivalent and conversion.

Run	Independent variable						Experimental		Predicted	
	X_1 (pH)		X_2 (Temp/°C)		X_3 (U_{GI}/U_{AMG})		Dextrose Equivalent	Conversion (%)	Dextrose Equivalent	Conversion (%)
1	-1	(4.5)	-1	(45)	-1	(6.35/15)	98.0	0.0	100.5	1.6
2	1	(6.0)	-1	(45)	-1	(6.35/15)	92.5	8.0	92.7	7.3
3	-1	(4.5)	1	(55)	-1	(6.35/15)	100.2	0.0	97.4	-0.3
4	1	(6.0)	1	(55)	-1	(6.35/15)	92.0	10.6	89.0	10.8
5	-1	(4.5)	-1	(45)	1	(19.1/15)	105.6	0.0	106.3	-2.6
6	1	(6.0)	-1	(45)	1	(19.1/15)	87.8	24.4	88.3	22.3
7	-1	(4.5)	1	(55)	1	(19.1/15)	104.7	0.0	102.1	-1.7
8	1	(6.0)	1	(55)	1	(19.1/15)	88.4	32.7	83.5	28.8
9	-1.68	(3.98)	0	(50)	0	(12.7/15)	103.0	0.0	103.2	0.7
10	+1.68	(6.51)	0	(50)	0	(12.7/15)	78.0	28.1	81.2	30.8
11	0	(5.25)	-1.68	(41.6)	0	(12.7/15)	98.8	0.7	95.4	1.8
12	0	(5.25)	+1.68	(58.4)	0	(12.7/15)	82.0	3.3	88.8	5.6
13	0	(5.25)	0	(50)	-1.68	(2.02/15)	101.5	0.0	102.3	-1.6
14	0	(5.25)	0	(50)	+1.68	(23.4/15)	100.0	4.9	102.6	9.9
15	0	(5.25)	0	(50)	0	(12.7/15)	108.3	2.0	105.6	1.8
16	0	(5.25)	0	(50)	0	(12.7/15)	103.7	2.2	105.6	1.8
17	0	(5.25)	0	(50)	0	(12.7/15)	105.4	1.8	105.6	1.8

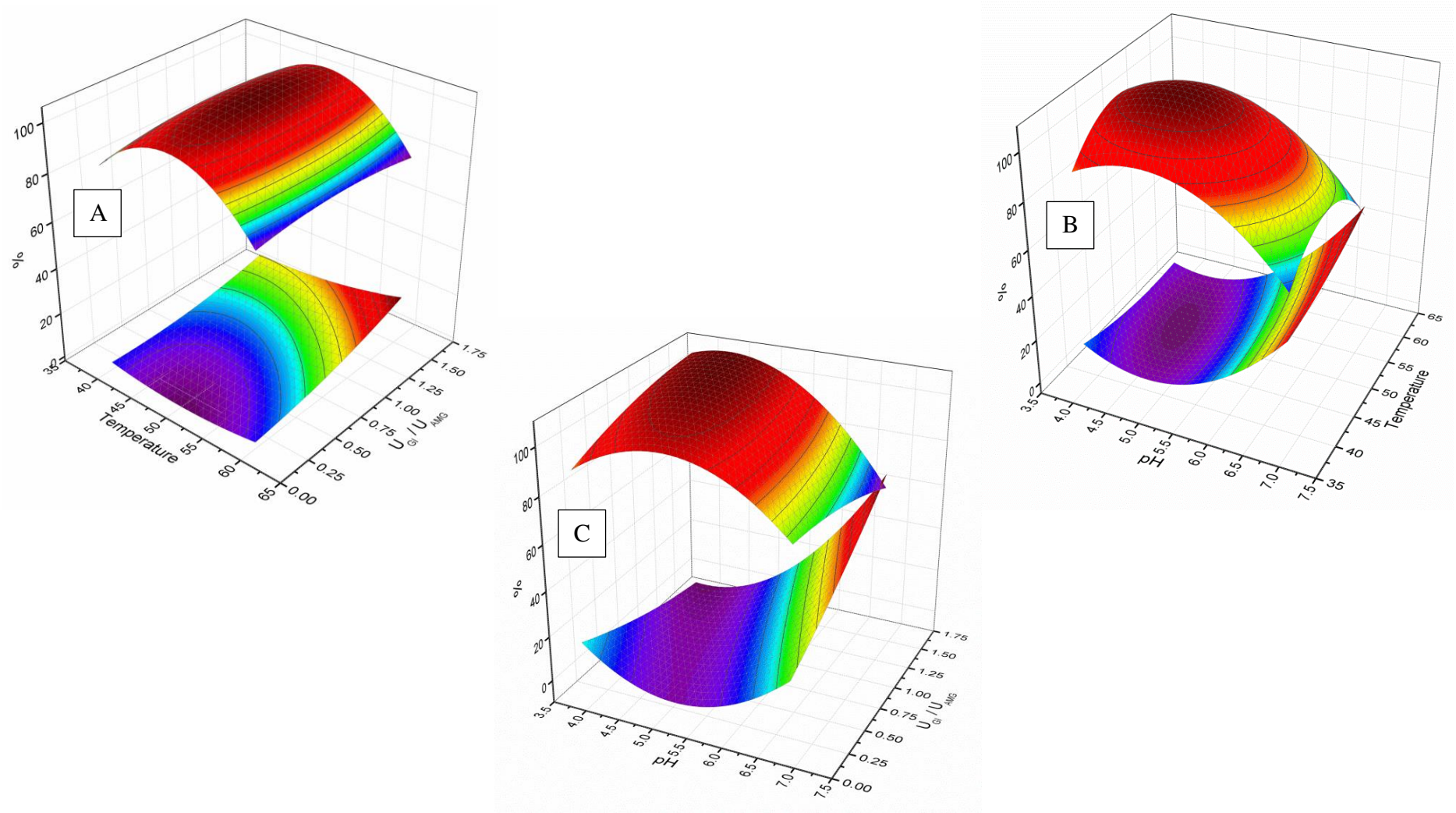
Analysis of variance (ANOVA) at a 95% confidence level (Table 4.S1, Supplementary Data) showed that the DE model was significantly influenced by the independent variables pH (X_1), temperature square (X_2^2), and pH square (X_1^2), whose effects were negative, indicating that the higher the value of these parameters, the lower the DE obtained. The lack of fit presented a P-value of 0.183, non-significant ($P > 0.05$), which shows that the model had a good fit. The ANOVA also indicated a regression coefficient R^2 of 0.8869, which represents that only 11.31% of the total variations are not explained by the model.

For the conversion, ANOVA showed (Table 4.S2, Supplementary Data) that the model was highly influenced by the significant variables, pH (X_1), pH square (X_1^2), the interaction of pH * U_{GI}/U_{AMG} ratio (X_1X_3), and U_{GI}/U_{AMG} ratio (X_3). Besides these, temperature (X_2) and the interaction of pH * temperature (X_1X_2) were also significant. All the significant terms had positive effects, which mean that higher conversion values are expected when the values of the parameters are higher. The proposed model presented a P-value for lack of fit of 0.06 (non-significant) and a regression coefficient R^2 of 0.9574 confirming the good fit of the model to the obtained data.

The response surfaces obtained by the model for DE and conversion (glucose-to-fructose) were plotted together as shown in Figure 4.1. In Figure 4.1 (A), at constant pH (5.25), it can be observed that the variable DE (upper surface) presents the highest values around the temperature of 50 °C. Far from the optimum region, at temperatures higher or lower than 50 °C, the DE values decrease due to the significant influence of the term temperature square (X_2^2). For the U_{GI}/U_{AMG} ratio, the variation of values did not have a significant influence. For the conversion (lower surface), the higher U_{GI}/U_{AMG} values significantly increased the conversion obtained, which is also improved at higher temperatures.

In Figure 4.1 (B), at a constant U_{GI}/U_{AMG} ratio (0.8467), it can be noted that the pH has a very significant and distinct effect on both response variables, DE (upper surface) and conversion (lower surface). For DE, the increase in pH causes a decrease in AMG activity, thus reducing the hydrolysis yield of dextrin. For the conversion, the pH increase had a positive effect, reaching higher conversions at pH close to 7.0. Regarding the effect of the temperature, DE showed a maximum value around 50 °C and the conversion was not significantly influenced.

Figure 4.1 — Response surfaces showing the effects of independent variables on DE (superior surface) and Conversion (inferior surface). (A) Temperature and U_{GI}/U_{AMG}, (B) pH and temperature, and (C) pH and U_{GI}/U_{AMG}. DE and conversion values (in percentage) are given in the z-axis on a scale from 0 to 100.



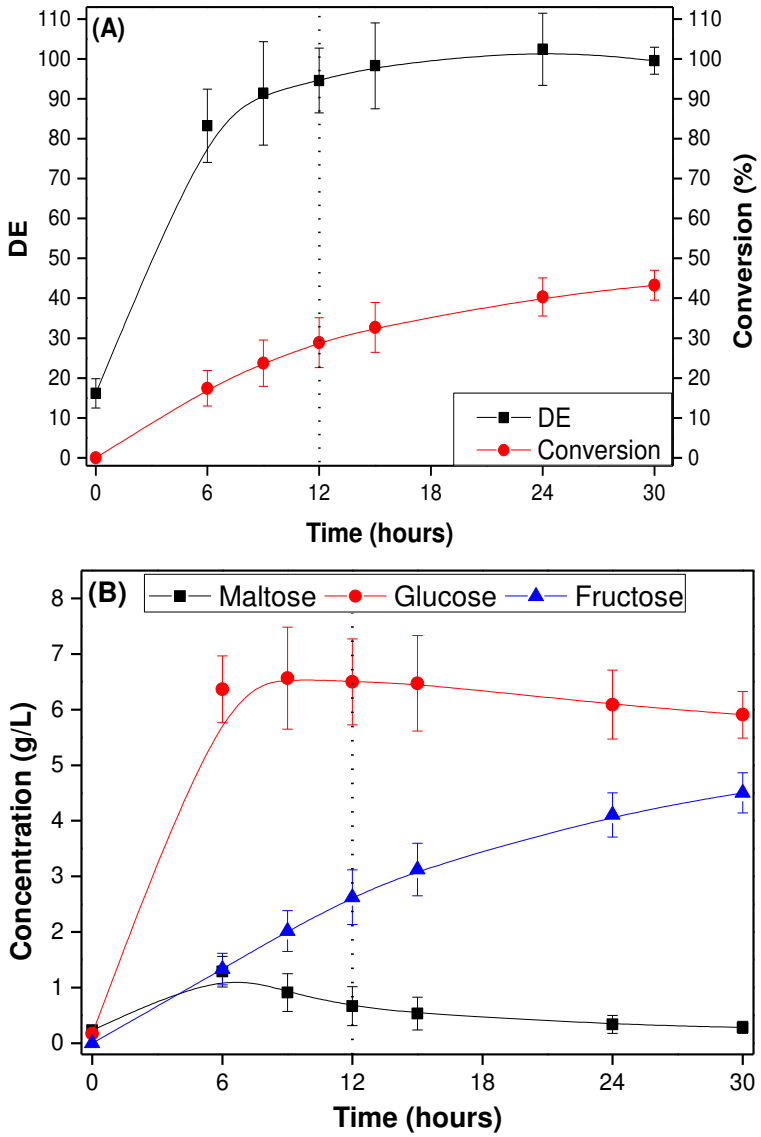
In Figure 4.1 (C), at a constant temperature (50 °C), the effect of pH on DE and conversion were similar to those discussed above, and the U_{GI}/U_{AMG} ratio positively influenced the conversion with a slight improvement in DE.

By analyzing the experimental data and comparing them with the response surfaces, it can be concluded that the best synergistic conditions for the simultaneous reaction of AMG and GI occurred at a temperature of 50 °C and a GI/AMG ratio of 1.56 (23.4 U_{GI}/15 U_{AMG}). However, the optimum pH condition is not so obvious to be defined. Therefore, the Derringer's desirability prediction tool was used to define all variables in order to maximize DE and conversion. Supported by this tool, the optimum operating conditions were set at pH 5.7, 50 °C and GI/AMG ratio of 1.56 (U_{GI}/U_{AMG}) (Figure 4.S1, Supplementary Data). Under these conditions, DE and conversion values of 94.2 and 22.2% are expected, respectively.

4.3.2 Validation of the mathematical model

Figure 4.2 shows the results of DE and conversion, as well as the glucose, fructose and maltose profiles obtained from the simultaneous saccharification and isomerization of the dextrin under the previously defined optimal conditions (50 °C, pH 5.7 and U_{GI}/U_{AMG} ratio of 1.56).

Figure 4.2 — Simultaneous saccharification and isomerization profiles at the optimum conditions obtained by the model generated by the central composite rotatable design.



As shown in Figure 4.2 (A), after 12 h of reaction, DE and conversion reached values of 94.6 ± 8.1 and 28.9 ± 6.3%, respectively, values close to those predicted by the model (94.2 and 22.2%). Furthermore, the glucose-to-fructose conversion reached equilibrium (42-46%, under optimum conditions of pH and temperature (BHOSALE; RAO; DESHPANDE, 1996; TOMASIK; HORTON, 2012), at the end of 30 h of reaction with 1% (w/v) dextrin solution Figure 4.2 (B) shows the maltose, glucose and fructose profiles, where it can be observed that the maximum concentration of glucose is reached in 9 h of reaction, however the reaction requires a longer time (30 h) to reach the conversion glucose-to-fructose of 43.3 ± 3.7%. The maltose released by dextrin

hydrolysis is accumulated at the beginning of the reaction (6 h), due to the lower activity and affinity of the AMG for short chain substrates (KUMAR; SATYANARAYANA, 2009). Afterwards, the maltose is hydrolyzed reducing its concentration almost to zero at the end of the reaction.

From the obtained data, it can be concluded that the proposed model, as well as the optimization, was validated. Furthermore, a longer reaction (about 30 h) is required for the glucose isomerase performance in these simultaneous processes.

4.3.3 Evaluation of the effect of $MgSO_4$

It is widely reported that the divalent cations Mg^{2+}, Co^{2+} and Mn^{2+} exert a positive effect to obtain the maximum activity of glucose isomerase (BHOSALE; RAO; DESHPANDE, 1996; HOBBS, 2009), where Mg^{2+} an essential ion that activates and stabilizes the enzyme (BHOSALE; RAO; DESHPANDE, 1996; JØRGENSEN et al., 1988). Milessi-Esteves et al. (2019) reported that supplementation of the medium of xylose isomerization catalyzed by soluble GI of *Streptomyces rubiginosus* with 250 mM $MgSO_4$ allowed an increase in xylose-to-xylulose isomerization rates of 45.7 and 38.1 times at pH 6.0 and 5.0, respectively. For this purpose, a study of the effect of the concentration of this ion on the activities and stabilities of the immobilized enzymes (GI and CLEA of AMG) was carried out individually.

Tables 4.4 and 4.5 show the activities of immobilized enzymes at different pH and temperature ranges supplemented with $MgSO_4$ (50 and 250 mM). Under the operational conditions previously established for the simultaneous saccharification and isomerization (50 °C, pH 5.7), the supplementation of $MgSO_4$ did not significantly influence the activities of GI and AMG. The enzymes work with approximately 40 and 56% of their maximum activities, respectively. However, the positive effect of the increase in the $MgSO_4$ concentration over the GI activity is evident at 60 °C and pH 5.7, in which this activity increase may be related to the activating and stabilizing effect of the ion Mg^{2+}.

Table 4.4 — Activities of glucose isomerase (Sweetzyme® IT Extra) at different conditions of temperature, pH and $MgSO_4$ concentrations.

pH and $MgSO_4$ concentration	**50°C**		**60°C**	
	Activity (U/g)	Relative activity (%)[a]	Activity (U/g)	Relative activity (%)
pH 7.5; $MgSO_4$ 250 mM	276.7 ± 18.8	47.9 ± 3.3	-	-
pH 7.0; $MgSO_4$ 50 mM	270.0 ± 17.2	46.7 ± 3.0	577.7 ± 24.2	100 ± 4.2
pH 5.7; $MgSO_4$ 50 mM	230.3 ± 9.7	39.9 ± 1.7	437.6 ± 12.8	75.7 ± 2.2
pH 5.7; $MgSO_4$ 250 mM	232.7 ± 17.9	40.3 ± 3.1	492.3 ± 30.4	85.2 ± 5.3

[a] Relative activity of 100% was taken as the activity measured at 60 °C and pH 7.0 in the presence of 50 mM $MgSO_4$.

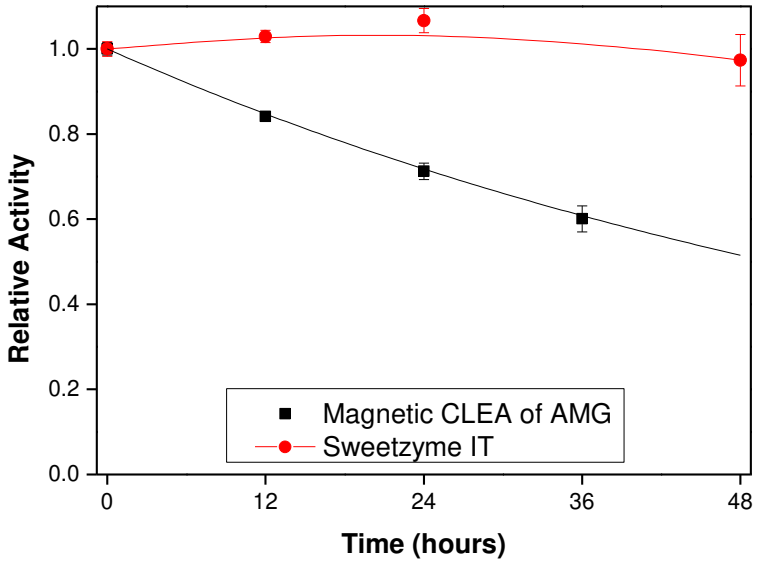
Table 4.5 — Activities of immobilized amyloglucosidase (magnetic CLEAs of AMG) at different conditions of temperature, pH and $MgSO_4$ concentrations.

Assay conditions	**Activity (U/mL)**	**Relative Activity (%)[a]**
pH 4.5; T= 55 °C	121.2 ± 8.0	100 ± 6.6
pH 5.7; T= 50 °C;	72.5 ± 3.8	59.8 ± 3.2
pH 5.7; T= 50 °C; $MgSO_4$ 50 mM	70.0 ± 1.2	57.7 ± 0.9
pH 5.7; T= 50 °C; $MgSO_4$ 250 mM	67.3 ± 2.0	55.8 ± 1.6

[a] Relative activity of 100% was taken as the activity measured at 55 °C and pH 4.5.

Figure 4.3 shows the thermal inactivation profiles at 50 °C, pH 5.7 at the presence of 250 mM $MgSO_4$, in which a high stability of GI can be observed even in the absence of substrate, where the initial activity is preserved after 48 h incubation. On the other hand, magnetic CLEA of AMG in the presence of 250 mM $MgSO_4$ exhibited lower stability (half-life around 48 h), but similar to the findings of Amaral-Fonseca et al. (2018) in the absence of $MgSO_4$.

Figure 4.3 — Thermal inactivation of immobilized glucose isomerase (Sweetzyme® IT Extra) and amyloglucosidase (magnetic CLEA of AMG) at 50 °C and pH 5.7 (in presence of 250 mM $MgSO_4$ and absence of substrate).



It is important to point out that the lower stability of immobilized AMG is not a problem from an operational point of view, because the magnetic properties of the CLEA of AMG allow its separation from the GI easily by an external magnetic field, therefore allowing the reuse of GI in the process.

The use of 250 mM $MgSO_4$, even at 50 °C, does not negatively influenced the AMG, and is probably advantageous for GI, since it is working out of ideal conditions for long periods of time. In addition, to apply the simultaneous saccharification and isomerization process directly after the liquefaction of the starch, Ca^{2+} ions (in the order of 50-70 ppm) in the hydrolyzed solution are expected. Thus, in order to avoid inhibitory interactions between Ca^{2+} ions and the GI structure, high concentrations of $MgSO_4$ may be applied to maintain the regular activity of GI, as reported by Jørgensen et al. (1988). Thus, the concentration of 250 mM of $MgSO_4$ was selected for further experiments and was able to avoid inhibitions even at 135 ppm of Ca^{2+}.

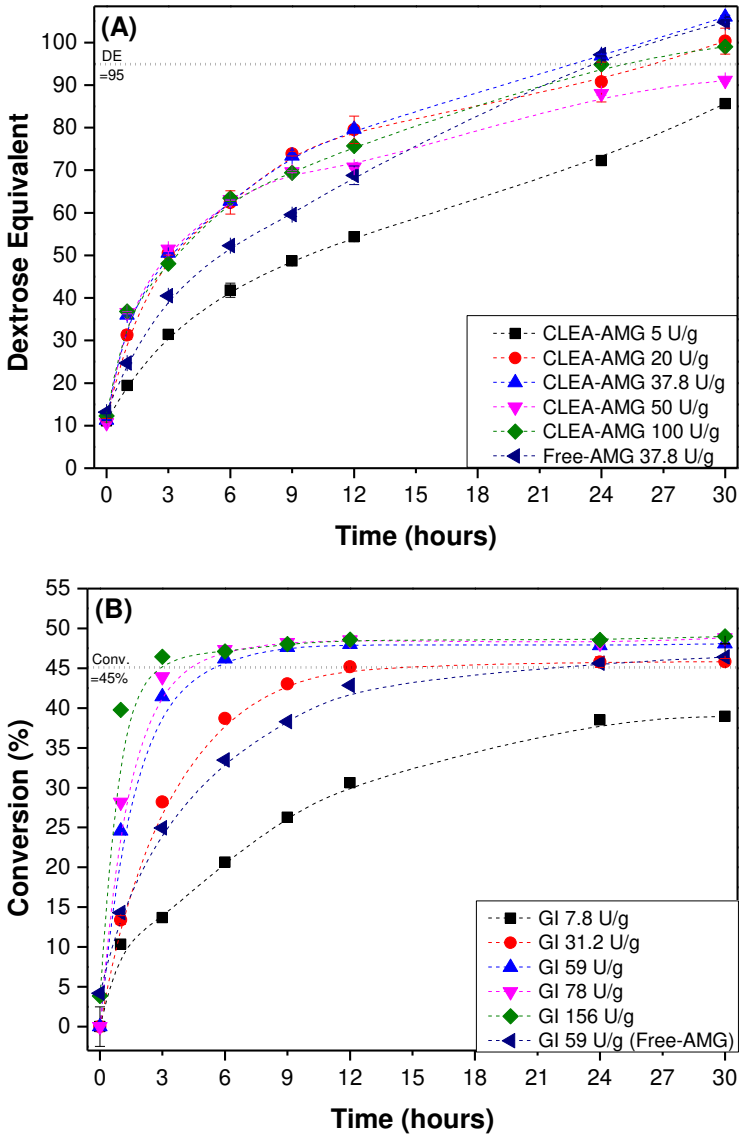
4.3.4 Effect of the enzyme loads in the saccharification and isomerization of dextrin at high concentrations

Industrially, the production of HFS is typically produced using high substrate concentration (35%, w/v) in the starch saccharification step (CRABB; MITCHINSON, 1997; KUMAR; SATYANARAYANA, 2009). Thus, in this work, it was evaluated the simultaneous saccharification and isomerization of 35% (w/v) dextrin was evaluated with different AMG and GI loads but the U_{GI}/U_{AMG} ratio constant was maintained (1.56, previously established).

Figure 4.4 (A) shows that the saccharification profiles are very close for the AMG loads ranging from 20 to 100 U/g dextrin (around 95% DE after 30 h reaction), where the optimal loads given by the statistical design (50 U/g dextrin) are included in this range. Moreover, it can be observed that the immobilized AMG at 37.8 U/g dextrin (corresponding to 2 mL of soluble AMG per kg of starch reported in the literature (CEREDA, 2001)) exhibits a better performance than the free AMG, at least in the initial stage of the reaction, showing that the saccharification step catalyzed by immobilized AMG is not limited by diffusional problems. The similarity of the profiles for all loads in the range evaluated could be an indicative of the pH influence on the reaction rate, because AMG works well at pH 4.5.

Figure 4.4 (B) shows the isomerization profiles for GI loads ranging from 7.8 to 156 U/g dextrin, but they maintain the GI/AMG ratio constant, which was previously established. The results corroborate with the statistical design results, i.e., higher GI load higher glucose-to-fructose conversion. However, except for 7.8 U/g, all GI loads allowed the system to achieve the equilibrium conversion (around 45%) after 30 h reaction.

Figure 4.4 — Effect of the enzyme load (U/g dextrin) on the saccharification and isomerization profiles at 50 °C and pH 5.7 (in the presence of 250 mM $MgSO_4$).

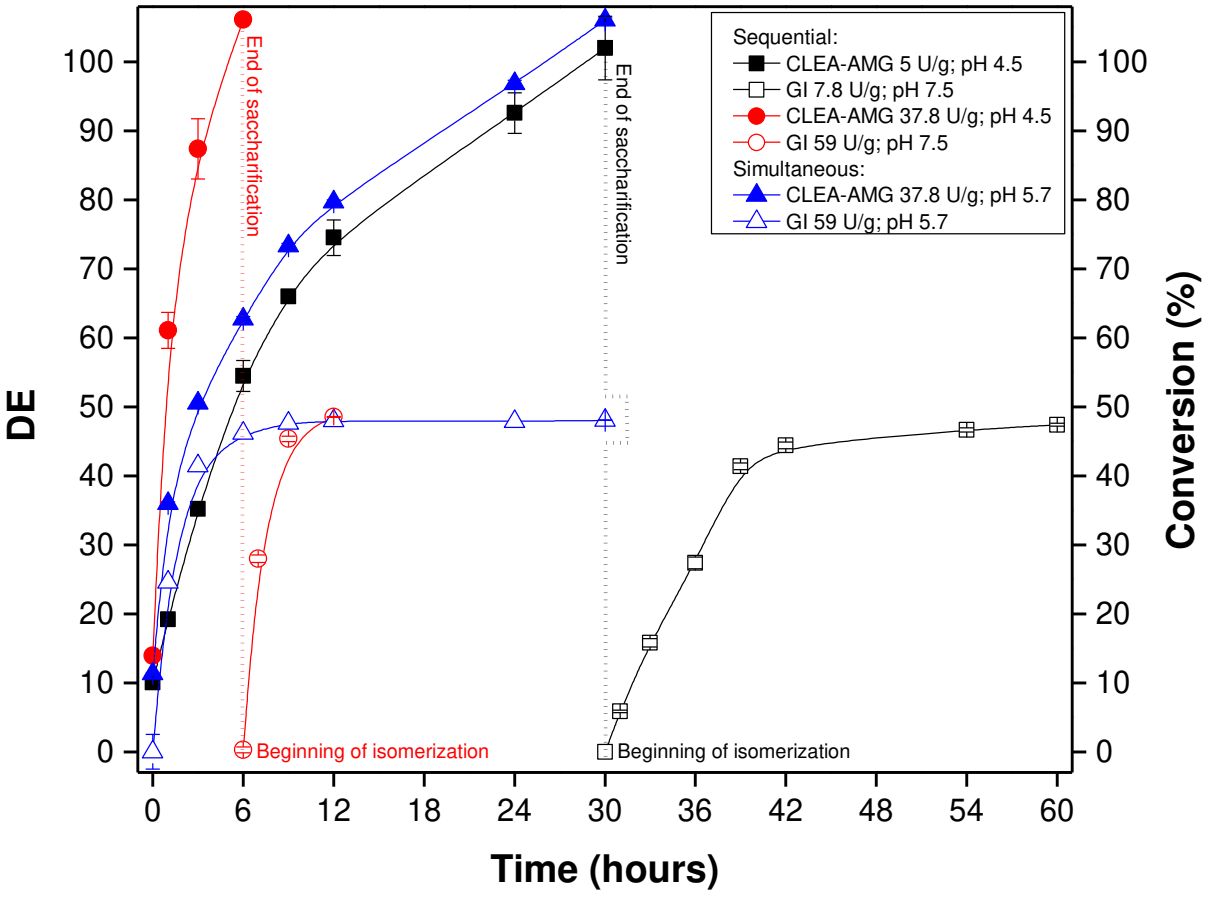


To verify the pH influence in the HFS production, sequential saccharification and isomerization at optimal pHs 4.5 and 7.5, respectively, were carried out at 50 °C, and compared to the simultaneous saccharification and isomerization at pH 5.7 and 50 °C, using a GI/AMG ratio of 1.56 (59 U_{GI}/37.5 U_{AMG}).

Figure 4.5 shows that at pH 4.5 using the same AMG load (37.8 U_{AMG}/g dextrin) the saccharification reaction achieved a DE of 95% after 6 h (similar to the findings of Amaral-Fonseca et al., 2018), while this same DE is only achieved after 30 h reaction in the simultaneous process. However, in the glucose-to-fructose isomerization (both

sequential at pH 7.5 and simultaneous at pH 5.7), the reaction equilibrium (48.5% conversion) was achieved after the same reaction time (approximately 6 h reaction). Thus, it is evident that AMG is much more influenced by the pH than GI.

Figure 4.5 — Profiles of sequential saccharification and isomerization with two enzyme loadings under optimum conditions of each enzyme and simultaneous saccharification and isomerization at the optimum conditions obtained by the model for both enzymes. Closed symbols represent the process of saccharification and open symbols the process of isomerization.



Indeed, a dextrin saccharification at pH 4.5 using an AMG load of 5 U_{AMG}/g dextrin showed a reaction profile very close to that at pH 5.7 in the simultaneous process. Thus, at pH 5.7 the dextrin saccharification reaction occurs as if the system had approximately 8 times less AMG. In this case, the overall process (simultaneous and sequential saccharification and isomerization) would require 30 h to achieve 90% DE and equilibrium glucose-to-fructose conversion, using high GI load (59 U_{GI}/g dextrin). If this load is reduced to 7.8 U_{GI}/g dextrin, the sequential isomerization is preceded more slowly, requiring 30 h only to achieve the reaction equilibrium. Thus,

the overall process would require a minimum time of 36 h to reach the end point (using 37.8 and 7.8 U/g of AMG and GI, respectively) or a maximum time of 60 h (using 5 and 7.8 U/g of AMG and GI, respectively).

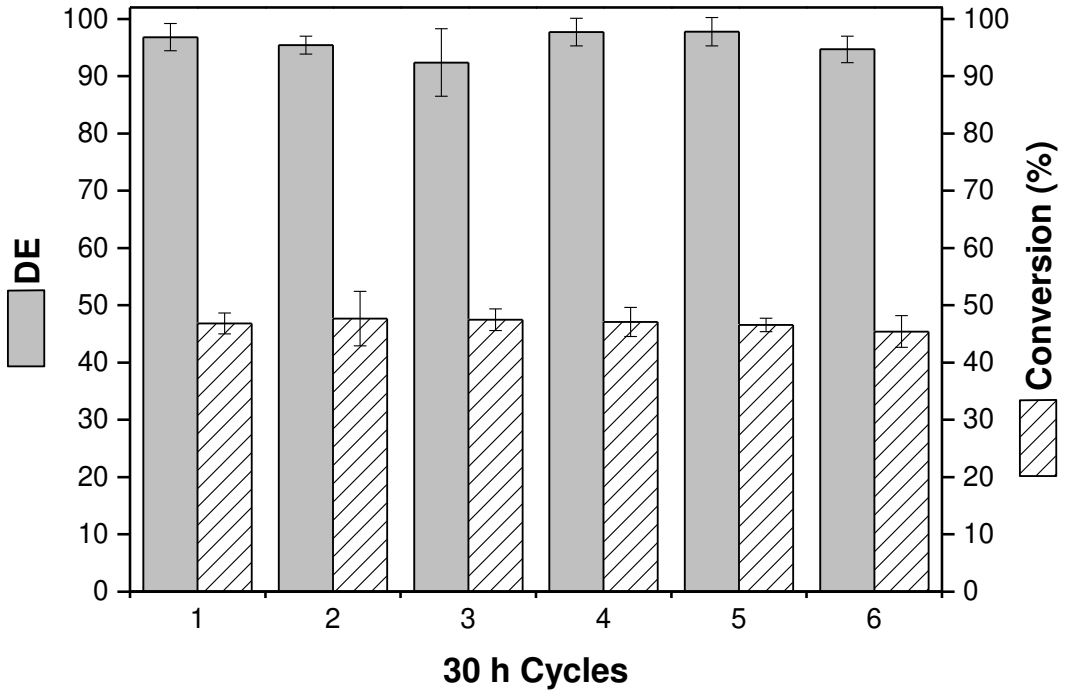
In conclusion, although the sequential process of HFS production can be carried out for 12 h, this procedure requires additional time to recover the AMG, adjustment of pH, and loads the reactor with GI. Besides, the process needs to be performed in two reactors, while the simultaneous process is globally one-pot at constant pH, which can represent an economy in the process, at least in terms of equipment.

4.3.5 Reuse assays

The reusability of the biocatalysts in the simultaneous saccharification and isomerization process was evaluated under optimal conditions previously established, that is, 50 °C, pH 5.7, presence of 250 mM MgSO4, 35% (w/v) dextrin, and enzymes' ratio of 59 U_{GI}/37.8 U_{AMG}.

Figure 4.6 shows that the dextrose equivalent (DE) and equilibrium conversion glucose-to-fructose could be maintained at maximal values (around 90% DE and 47% conversion) for six 30 h-batches. These findings show that the process evaluated in this work, using both AMG and GI immobilized could be advantageous from an industrial point of view. Of course, a technical-economic evaluation would have to be carried out to prove the real advantage of this simultaneous process, which will still be evaluated in our research group. An additional advantage of our process is the use of magnetic AMG CLEAs, because the enzymes can be easily separated by applying an external magnetic field, thus recovering the active enzyme to be reused in the process, avoiding the loss of all biocatalyst as in the case of AMG and GI co-immobilized.

Figure 4.6 — Reusability assay of simultaneous saccharification and isomerization of dextrin (35%, w/v) at 50 °C and pH 5.7 (in presence of 250 mM $MgSO_4$), catalyzed by magnetic CLEA of AMG (37.8 U/g dextrin) and commercial immobilized glucose isomerase (Sweetzyme® IT Extra, 59 U/g dextrin).



4.4 CONCLUSIONS

Central composite rotatable design methodology and surface response were very important to define the operational conditions (pH, temperature and glucose isomerase/amyloglucosidase ratio) to perform a simultaneous saccharification and isomerization (SSI) of dextrin aiming to produce fructose syrup under different conditions from those optimal ones for each enzyme. The mathematical model was accurate to predict dextrose equivalent (DE) and glucose-to-fructose conversion, which could be experimentally validated. Under optimal conditions (50 °C, pH 5.7 and glucose isomerase/amyloglucosidase ratio of 1.56), the SSI yielded a DE around 90% and a glucose-to-fructose conversion around 47% after a 30 h process, which could be maintained for six consecutive batches with recovery and reuse of the immobilized biocatalysts. Thus, the evaluated process could be attractive from an industrial point of view, at least as the simplicity of the process is carried out in a single step and in a single reactor, making it possible to easily separate the biocatalysts when one of them

is inactivated.

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SUPPLEMENTARY MATERIAL

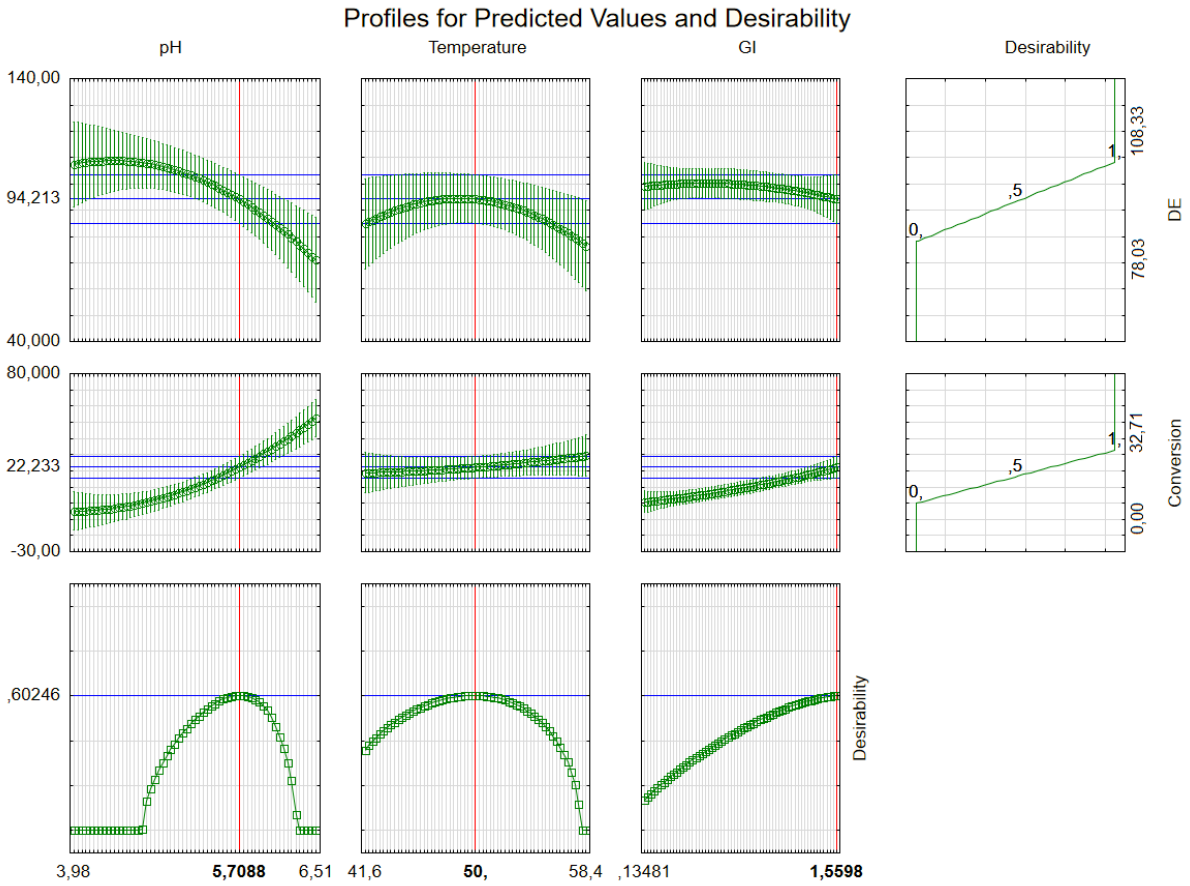
Table 4.S1 — Analysis of variance of a quadratic model and coefficient estimated for dextrose equivalent.

Source of variation	Sum of squares (SS)	Degree of freedom (df)	Mean squares (MS)	F-value	P-value
Model	1106.61	9	122.96	6.10	
X_1	596.36	1	596.36	108.87	0.0091*
X_2	52.54	1	52.54	9.59	0.0904
X_3	0.13	1	0.13	0.02	0.8912
X_1 X_2	0.17	1	0.17	0.03	0.8774
X_1 X_3	51.41	1	51.41	9.38	0.0921
X_2 X_3	0.54	1	0.54	0.10	0.7829
X_1^2	254.47	1	254.47	46.46	0.0209*
X_2^2	259.23	1	259.23	47.32	0.0205*
X_3^2	14.03	1	14.03	2.56	0.2507
Residual	141.13	7	20.16		
Lack of fit	130.17	5	26.03	4.75	0.1830
Pure error	10.96	2	5.48		
Total	1247.77	16			R^2=88.69%

Table 4.S2 — Analysis of variance of a quadratic model and coefficient estimated for conversion (glucose-to-fructose).

Source of variation	Sum of squares (SS)	Degree of freedom (df)	Mean squares (MS)	F-value	P-value
Model	1743.33	9	193.70	15.43	
X_1	1114.49	1	1114.49	26785.82	0.0055*
X_2	17.21	1	17.21	413.69	0.0440*
X_3	159.69	1	159.69	3838.05	0.0145*
X_1 X_2	15.01	1	15.01	360.82	0.0471*
X_1 X_3	184.30	1	184.30	4429.71	0.0135*
X_2 X_3	4.11	1	4.11	98.81	0.0895
X_1^2	229.38	1	229.38	6629.83	0.0121*
X_2^2	4.69	1	4.69	126.00	0.0839
X_3^2	6.96	1	6.96	189.90	0.0690
Residual	75.34	6	12.55		
Lack of fit	75.33	5	15.06	183.01	0.0561
Pure error	0.082	1	0.082		
Total	1820.91	15			R^2=95.74%

Figure 4.S1 — Profiles for predicted values and desirability



5 GENERAL CONCLUSIONS

This study evaluated the commercial amyloglucosidase enzyme immobilization from *Aspergillus niger* (AMG 300L™) using the cross-linked enzyme aggregate (CLEA) technique and glucose production from pre-hydrolyzed commercial starch. It also evaluated the application of AMG CLEA in conjunction with the commercial enzyme glucose isomerase from *Streptomyces murinus* (Sweetzyme® IT Extra) in high fructose syrup production in a simultaneous saccharification and isomerization process. The most important results are presented below.

Amyloglucosidase immobilization in the absence of amino feeders required high concentrations of glutaraldehyde, which corroborates with reports in the literature that low amounts of lysine groups are on the surface of amyloglucosidase. Using aminated magnetic nanoparticles (MNPs) helped to form CLEAs even at lower concentrations of crosslinking agents, reaching activities recovered around 20%.

Glutaraldehyde showed strong reactivity with the enzyme and was able to inactivate 70% of the total enzymatic activity when incubated with the free enzyme. Using starch or polyethyleneimine helped protect and stabilize the final catalyst, respectively, increasing the recovered activity, reaching 40%. However, the CLEA prepared with starch lost much activity after a hydrolysis cycle.

Moreover, under the same immobilization conditions, AMG was also co-aggregated with PEI and BSA, replacing the MNPs. Both CLEAs (co-aggregates with PEI and MNPs or BSA) showed very similar recovered activities and were four times more thermally stable than the soluble enzyme. These CLEAs were also evaluated on starch hydrolysis at 35% (w/v), achieving a conversion of starch to glucose over 95 %, measured as Dextrose Equivalent (DE).

Although both types of CLEAs can be reused for five cycles maintaining a DE around 90%, the magnetic CLEAs were more attractive due to their easy separation by an external magnetic field.

Regarding the synergistic application of magnetic CLEA of AMG and the commercial immobilized GI catalyst, after the initial survey of the pH and temperature performance ranges of both enzymes, the variation range of each variable in the

experimental design was defined. From the 17 assays performed, a wide variation of experimental results was observed, which by multiple regression analysis generated two mathematical models for DE and conversion of glucose to fructose. The mathematical models showed a good fit to the experimental data, indicating an optimum condition for the simultaneous reaction at 50 °C, pH 5.7 and U_{GI}/U_{AMG} ratio of 1.56, predicting a DE of 94.2% and conversion of 22.2%. The model was validated reaching a DE of 94.6% and conversion of 28.9%, demonstrating the good model fit.

To obtain higher concentrations of fructose, the reaction should occur for 30 h, resulting in DE around 95% and conversions of 43%. The same values were obtained when substrate concentration and enzyme loading were increased proportionally. It has also been shown that the longer reaction time (30 h) occurs mainly due to pH limitations.

Finally, it was observed that the biocatalysts under optimum conditions showed a DE around 90% and conversion of glucose to fructose around 47% after 30 h, which can be maintained for at least six consecutive cycles with recovery and reuse of the immobilized biocatalysts. Thus, the evaluated process proved to be attractive from an industrial point of view, at least as the simplicity of the process is carried out in a single step and in a single reactor, thus making it easy to separate the biocatalysts when one of them is inactivated.

In view of the results obtained, the following is suggested for future work:

a) applying biocatalysts to fixed bed reactors, as in industry, continuously operating with parallel reactors;
b) producing AMG CLEAs using the millifluidic process in syringe pumps, aiming to improve the final catalyst;
c) applying pre-hydrolyzed starch from several amylaceous sources to the simultaneous saccharification and isomerization process.