UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

BIOCATALYST ENGINEERING APPLIED TO THE IMPROVEMENT OF CROSS-LINKED ENZYME AGGREGATES AIMING AT THE MULTIENZYMATIC CONVERSION OF SUCROSE TO GLUCONIC ACID AND FRUCTOSE SYRUP

Agnes Cristina Oliveira Mafra

DOCTORAL THESIS

UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

ENGENHARIA DE BIOCATALISADORES APLICADA À SÍNTESE DE CLEAS PARA A APLICAÇÃO NA CONVERSÃO MULTIENZIMÁTICA DE SACAROSE À ÁCIDO GLUCÔNICO E XAROPE DE FRUTOSE

Agnes Cristina Oliveira Mafra

SÃO CARLOS/SP

2017

UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

BIOCATALYST ENGINEERING APPLIED TO THE IMPROVEMENT OF CROSS-LINKED ENZYME AGGREGATES AIMING AT THE MULTIENZYMATIC CONVERSION OF SUCROSE TO GLUCONIC ACID AND FRUCTOSE SYRUP

Agnes Cristina Oliveira Mafra

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

Supervisor: Prof. Dr. Paulo Waldir Tardioli

Co-supervisor: Prof. Dr. Marcelo Perencin de Arruda Ribeiro

SÃO CARLOS/SP

2017

MEMBROS DA BANCA EXAMINADORA DA DEFESA DE TESE DE AGNES CRISTINA OLIVEIRA MAFRA APRESENTADA AO PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA DA UNIVERSIDADE FEDERAL DE SÃO CARLOS, EM 17 DE FEVEREIRO DE 2017.

BANCA EXAMINADORA:

Paulo Waldir Tardioli
Orientador, UFSCar

Marcelo Perencin de Arruda Ribeiro
Coorientador, UFSCar

Olga Luisa Tavano
UNIFAL

Angélica Marquetotti Salcedo Vieira
UEM

Alberto Colli Badino Junior
UFSCar

To Tamires (in memoriam).

AKNOWLEDGEMENTS

To my advisors Professor Dr. Paulo Waldir Tardioli and Professor Dr. Marcelo Perencin da Silva, for his dedication, teachings and opportunity to my personal and professional growth.

To all the Chemical Engineering Department Professors, important people in my scientific and academic qualification. In particular, to Professor Dr. Ruy de Sousa Júnior who kindly took part in the Monitoring and Qualifying Examination Board; and therefore could give suggestions of great value to the paper progress. Professor Raquel de Lima Camargo Giordano who was part of the Qualifying Examination Board. Doctor William Kopp who was part of the Monitoring Examination Board, and that for many years has divided knowledge and laboratory bench with me, thank you! Professor Monica Lopes Aguiar from the Laboratory of Environmental Control (DEQ/UFSCar) by the microscopy images of CLEAs. Professor Alberto Colli Badino from Laboratory of Fermentation (DEQ/UFSCar) by pneumatic reactors and oxygen measuring equipment used in this study. I thank the Embrapa Instrumentation (São Carlos, SP, Brazil) for granting use of SEM equipment.

To the staff because of their prompt help and patience, in particular the technician Amadeus.

I thank the scientific initiation students Maisa Beltrame and Letícia Ulrich who collaborated in experimental practices of this thesis and so that I could progress as a Professor.

The study herein was supported by the following Brazilian Foundations: National Council for Scientific and Technological Development (CNPq), São Paulo

Research Foundation (FAPESP), and Coordination for the Improvement of Higher Education Personnel (CAPES).

SPECIAL AKNOWLEDGEMENTS

First of all, I am grateful to God for the unconditional care throughout my life and especially during the four years of doctoral programs.

I would like to thank the friends from the Enzymatic Technology Laboratory: Felipe Silva, Laiane Antunes, Thaís Milessi e Paula Novelli; and the friends from Fermentation Laboratory: Mateus, Diego, Nayana, Kaio, Carol, Jorge e Paula Carvalho, for friendship, conversation and support during the study. I thank my great high school friends Keila Zeni and Eduardo Mandu, for friendship, support and consideration!! To "café com bobagem" friends. Ricardo, Andreza, Guilhermina, Duda, Gabriel, Erich, Felipe, Mateus, Paula Rosa, Anderson.

I am grateful to the love of my life, William, for encouragement, emotional and financial support. For all our years of partnership, for having been strong at the defense final stretch cheerful and patiently!! I love you dear!

To all my relatives for love and encouragement which made me courageous to face all challenges until the conclusion of this paper. A special thanks to my parents, Jô e Aguinaldo, who with great love supported me emotionally and financially during all years of study.

To the class BIT 7613 and Dr. Dalton Pessoa for affection, love, support and strength so that I would be able to finish the doctorate. I would also like to thank my Ballet and tap dance class - Estudio K that made of my nights more exciting and relaxing!! Many Thanks to Professors Deise, Munique and Isabela. I am grateful to cell MSOD that with much love welcomed me and connects me with the bible teachings so lightly and easily.

“Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained.” —Marie Curie.

ABSTRACT

Sugarcane sugar (sucrose) is a raw material produced in abundance in Brazil; it is very attractive to produce high added-value products. Gluconic acid (GA) can be obtained by multienzymatic conversion of sucrose, using three enzymes. Firstly, invertase (INV), responsible for the inversion of sucrose into glucose and fructose, next glucose oxidase (GOD) for the glucose oxidation and the last enzyme is catalase (CAT) used for the decomposition of hydrogen peroxide (H_2O_2, by-product from glucose oxidation). In this study, the sucrose was inverted in a fed-batch process catalyzed by INV immobilized as cross-linked enzyme aggregate (CLEA). The GA was produced by glucose oxidation catalyzed by combinate CLEAs (Combi-CLEAs) of CAT from bovine liver and GOD from *Aspergillus niger* in a batch pneumatic reactor. CAT has a tetrameric structure which complicates its stabilization through conventional immobilization technique. CLEAs of CAT were prepared, evaluating the effect of precipitant and cross-linking agents, as well as bovine serum albumin (BSA) as feeder protein on the catalytic properties, thermal stability, and mass transport resistance of the derivatives. The most active derivatives were prepared using ammonium sulfate as precipitant agent, 50 mM glutaraldehyde as cross-linker, and BSA/CAT mass ratio of 3.0. These derivatives were almost completely active (yield of immobilization up to 100%) and highly stable at 40 ^{o}C and pH 7.0 (~ 80% of the initial activity was recovery after 200 h under these conditions). The co-precipitation of BSA together with CAT reduced the size of clusters suggesting a decrease of diffusive effects within the biocatalyst. Empirical kinetic model was fitted to the experimental data of initial rate vs. substrate concentration and used to make a comparative analysis of mass transfer into derivatives with and without BSA. Results suggested that the main effect that differentiates the free enzyme and the two derivatives analyzed was of diffusive nature. In fact, the effectiveness factor of the crosslinked aggregates of catalase with BSA increased approximately 4 times. The statistical experimental design and the analysis of the response surface methodology showed that the immobilization did not alter the conditions of maximum activity of the CAT, which were found to be 30 ^{o}C and pH ~ 7.0 for all biocatalysts. Secondly, INV was immobilized by CLEA methodology using soy protein as feeder molecules as an alternative to the commonly used expensive

BSA. The immobilized INV retained around 30% of the initial activity after enzyme leaching assay. The immobilized INV was recycled tenfold in 4 h-batches of hydrolysis of sucrose at 40 ^{o}C and pH 6.0, maintaining the reaction conversion above 75 %. The hydrolysis of sucrose catalyzed by immobilized INV showed to be economically feasible in an operational window built based on economic metrics for a fed-batch process with three intermittent sucrose feeds to restore the substrate concentration at 100 g.L^{-1} when the conversion reaches 95 %. This work represents an advance in the field, because using a carrier-free and recyclable biocatalyst the specific productivity (gram of products per gram of biocatalyst per hour) of inverted sugar syrup was as high as those previously reported for INV immobilized on solid carriers that may dilute its volumetric activity and increase the cost of the biocatalyst. Combi-CLEAs of CAT and GOD were prepared, evaluating the influence of precipitant and cross-linking agents, as well as BSA as feeder protein on enzyme immobilization yield and thermal stability of each enzyme. Combi-CLEAs were prepared using dimethoxyethane as precipitant, 25 mM glutaraldehyde and mass ratio BSA/enzymes of 5.45 (w/w) were selected, their activities and stabilities at 40 ^{o}C, pH 6 and 250 rpm for five hours were evaluated. The selected Combi-CLEAs were used in GA production in a pneumatic reactor with 26 g.L^{-1} glucose at 40 ^{o}C, pH 6 and 10 vvm. Results showed conversion of 100 % and kinetic profile very similar to the free enzymes process. The reusability of Combi-CLEAs was also studied in ten batch-cycles of 5 hours. Operational half-life was calculated from kinetic profiles and first order inactivation model and presented a value of 31.50 hours. Combi-CLEAs of GOD and CAT showed to be relevant robust biocatalyst for GA application and production of glucose.

Keywords: Gluconic acid; Inverted sugar; Invertase; Glucose-oxidase; Catalase; CLEA; BSA; Soy Protein; Biocatalyst engineering; Multiconversion.

RESUMO

O açúcar de cana-de-açúcar (sacarose) é uma matéria-prima produzida em abundância no Brasil, sendo muito atraente para produzir produtos com alto valor agregado. O ácido glucônico (AG) pode ser obtido por conversão multienzimática de sacarose, utilizando três enzimas. Na primeira etapa a enzima invertase (INV) faz inversão da sacarose em glicose e frutose, na segunda etapa a enzima glicose-oxidase (GOD) oxida a glicose formando AG e peróxido de hidrogênio (H_2O_2) e na última etapa a enzima catalase (CAT) decompõem H_2O_2. Neste estudo, a sacarose foi invertida num processo de batelada alimentada catalisado por agregados reticulados(CLEA) de INV. AG foi produzido por oxidação de glicose catalisada por CLEAs combinados (Combi-CLEAs) de CAT de fígado bovino e GOD de *Aspergillus niger* em reator pneumático operado em batelada. A CAT apresenta uma estrutura tetramérica que dificulta a sua estabilização através da técnica convencional de imobilização. Assim, prepararam-se CLEAs de CAT separadamente, avaliando a influência de agentes precipitantes e intercruzantes, bem como albumina de soro bovino (BSA) como proteína de alimentação sobre as propriedades catalíticas, estabilidade térmica e resistência ao transporte de massa dos derivados. Os derivados mais ativos foram preparados utilizando sulfato de amônia como agente precipitante, glutaraldeído 50 mM como agente de entrecruzamento e proporção de massa BSA/CAT de 3,0. Estes derivados foram quase totalmente ativos (rendimento de imobilização até 100 %) e altamente estáveis à 40 °C e pH 7,0 (~ 80% da atividade inicial foi recuperação após 200 h nestas condições). A co-precipitação de BSA em conjunto com CAT reduziu o tamanho dos clusters, sugerindo uma diminuição dos efeitos difusivos dentro do biocatalisador. Um modelo cinético empírico foi ajustado aos dados experimentais de velocidades iniciais *vs*. concentração de substrato e, foi utilizado para fazer uma análise comparativa da transferência de massa em derivados com e sem BSA. Os resultados sugeriram que o principal efeito que diferencia a enzima livre e os dois derivados analisados foi de natureza difusiva. De fato, o fator de efetividade dos agregados reticulados de CAT com BSA aumentou cerca de 4 vezes. O delineamento experimental e a análise da metodologia da superfície de resposta mostraram que a imobilização não alterou as condições de atividade máxima da CAT, que se verificou ser de 30 ^{o}C e pH ≥ 7,0

para todos os biocatalisadores. Na segunda parte do trabalho, a INV foi imobilizada pela metodologia CLEA utilizando proteína de soja como proteína espaçadora como uma alternativa à BSA, comumente usada. A INV imobilizada reteve cerca de 30 % da atividade inicial após ensaio de lixiviação enzimática. A INV imobilizada pôde ser reciclada em dez ensaios (bateladas de 4 h) de hidrólise de sacarose a 40 ^{o}C e pH 6,0, mantendo a conversão da reação acima de 75 %. A hidrólise de sacarose catalisada por INV imobilizada mostrou ser economicamente viável numa janela operacional construída com base em métricas econômicas para um processo de batelada alimentada com três alimentações de sacarose na concentração de substrato a 100 g.L^{-1} quando a conversão atingia 95 % . Este trabalho representa um avanço nesta área, pois utilizando um biocatalisador isento de suporte e reciclável; a produtividade específica (grama de produto por grama de biocatalisador por hora) de xarope de açúcar invertido foi a mais elevada já reportada pela literatura. Prepararam-se Combi-CLEAs de CAT e GOD, avaliando a influência de agentes precipitantes e intercruzantes, bem como BSA como proteína espaçadora sobre o rendimento de imobilização enzimática e estabilidade térmica de cada enzima. Selecionaram-se os Combi-CLEA preparados utilizando dimetoxietano como precipitante, glutaraldeído 25 mM e razão de massa BSA / enzimas de 5,45 (m/m), avaliando as suas atividades e estabilidades a 40 ^{o}C, pH 6 e 250 rpm durante cinco horas. Os Combi-CLEAs selecionados foram utilizados na produção de AG num reator pneumático a partir de 26 g.L^{-1} de glucose a 40 ^{o}C, pH 6 e 10 vvm. Os resultados mostraram conversão de 100% e perfil cinético muito semelhante ao processo de enzimas livres. A reutilização de Combi-CLEAs foi também estudada em 10 ciclos reacionais de 5 horas. A meia-vida operacional foi calculada a partir dos perfis cinéticos e do modelo de inativação de primeira ordem e apresentou valor de 31,50 horas. Este trabalho pode concluir que os Combi-CLEAs de GOD e CAT são biocatalisadores relevantes e robustos para aplicação na produção de GA a partir de glicose.

Palavras-chave: Ácido glucônico; Açúcar invertido; Invertase; Glicose-oxidase; Catalase; CLEA; BSA; Proteína de Soja; Engenharia de biocatalisador; Multiconversão.

LIST OF FIGURES

LIST OF TABLES

CONTENTS

1. Introduction

1.1 Gluconic acid

In 1870 Hlasiwetz and Habermann discovered Gluconic Acid (GA) during glucose oxidation using chlorine as a biocatalyst. Later, Boutroux *et al*. (1880) reported that bacteria present in the acetic acid were capable to produce GA. In 1922, Molliard observed that fungus *Aspergillus niger* was able to produce GA, through glucose oxidase enzyme. Then other bacterial species such as *Pseudomonas, Gluconobacter, Acetobacter, Zymomonas* and fungi such as *Penicillium* were researched for GA production (HEINRICH & REHM, 1982; MILSON & MEERS, 1985; KIM & KIM, 1992; SILVEIRA *et al*., 1999; MUKHOPADHYAY *et al.*, 2005; AHMED *et al*., 2015; SAINZ *et al*., 2016; ZHANG *et al*., 2016).

Gluconic acid is an organic acid resulting from the oxidation of glucose. It has low corrosive capacity and good complexation with metal ions, which can be applied in the food, pharmaceutical, and textile industries (RAMACHANDRAN *et al.*, 2006; SINGH & KUMAR, 2007; PURANE *et al.*, 2011).

The glucose oxidation using enzymes as biocatalysts is a very viable method, allowing up to 100% conversion of glucose to GA. The literature has reported four enzymes capable of producing GA: glucose oxidase (β-D-glucose: oxygen-reductase 1, GOD, EC 1.1.3.4), glucose dehydrogenase (GDH, EC 1.1.1.47), hexose oxidase (HOX, EC 1.1.3.5) and glucose-fructose oxidoreductase (GFOR, EC 1.1.99.28). However, the literature has reported only two enzymes for GA production at industrial scales by fermentation processes: glucose oxidase from *Aspergillus niger* and glucose-fructose oxidoreductase from *Zymomonas mobilis.* Only the first

enzyme has been isolated and immobilized for *ex-vivo* GA production (MITCHEL & DUKE, 1970; GODJEVARGOVA, DAYAL & MARINOV, 2004; WONG, WONH & CHEN, 2008; BANKAR *et al*., 2009).

Despite the advantages of the microbiological conversion process, the biotransformation presents two major problems; the first is the decrease in productivity resulting from the inhibition caused by GA produced. In addition, there is also the difficulty to separate the product from the fermentation broth, since the transformation process produces a large amount of waste (LIU & CUI, 2007). Consequently, the use of GA and its derivatives is limited by its high cost (SINGH & KUMAR, 2007). According to Ramachandran *et al.* (2006), the average demand for GA is about 50,000-60,000 tones and the cost is estimated at 1.20-8.50 dollars per kilogram of GA.

1.2 Fructose syrup

Fructose, or fruit sugar, is a simple ketonic monosaccharide found in many plants, where it is often bonded to glucose to form the disaccharide sucrose (HANOVER, 1992).

Fructose syrup is produced in many parts of the world (WHITE, 2014). It can be produced by the complete hydrolysis of corn starch or sucrose catalyzed by acids or enzymes (WHITE, 2014), resulting in high-fructose corn syrup and isoglucose syrup or glucose-fructose syrup, respectively. Fructose syrup has many advantages over sucrose, including longer shelf life, ease of transportation, ease of blending a liquid vs. a powder, and lower cost in areas where corn is plentiful (HANOVER & WHITE, 1993).

High-fructose corn syrup (HFCS) is a mixture of glucose and fructose as monosaccharides; it is used in the soft drink and food industries as a direct replacement for sucrose due to its similar sweetness. First developed from corn in the early 1970s, HFCS's popularity has quickly grown and it is now the second-most consumed sweetener. HFCS is mainly obtainable in two forms. HFCS 55 contains 55% fructose and 45% glucose. It is commonly used in soft drinks, and is very similar in sweetness to table sugar. HFCS 42 contains 42% fructose and 58% glucose (WHITE, 2014). It is commonly used in canned fruits, ice cream, desserts and other sweetened processed foods. HFCS is included on the FDA's "Generally Recognized as Safe" status for use in food (VUILLEUMIER, 1993).

Glucose-Fructose Syrup (GFS) is a liquid sweetener used in the manufacturing of foods and beverages. GFS is composed of different simple sugars, mainly glucose and fructose, with varying compositions (HANOVER & WHITE, 1993; HULL, 2010). The fructose content can range from 5% to 50%. If the fructose makes up more than 50% of the syrup, the name on the ingredient listing should read Fructose-Glucose Syrup (FGS). Fructose and glucose exist in their free form in GFS, whereas in sucrose they are linked together (VUILLEUMIER, 1993; WHITE, 2014).

1.3 Immobilized multienzyme system

The Brazilian Sugarcane Industry Association (UNICA) reports that Brazil accounts for one third of the world sugar production. This is due to the country's favorable climate, to the advancement of research in the technological, industrial and agricultural areas, as well as the opportunity for more competitive market prices. These reasons justify the interest for the production of products with higher added value. In view of these advantages, Mafra *et al.* (2015) proposed a

multi-enzymatic system (invertase, glucose oxidase and catalase) for the production of GA and fructose syrup from sucrose in an airlift bioreactor, obtaining conversion and yields of 100%.

According to Xue and Woodley (2012), multi-enzymatic processes are considered an innovative method for the production of complex compounds of industrial interest. The major advantage of a multi-enzymatic process is that the mixture of enzymes intensifies the process, catalyzing several reactions, without the production of by-products, and thus the final product purification step can be eliminated. Consequently, the application of the multi-enzymatic process decreases the operation costs and increases the productivity and efficiency of the process.

However, using soluble enzymes in processes is largely limited by the low operational stability, difficulty in separating biocatalysts for reuse and, in some cases, the high cost. Aiming to improve the catalytic potential of enzymes and prevent some of the aforementioned limitations, there are studies on how to make them insoluble to the reactional medium (CAO, 2006; DUTTA, 2008; MOSIER & LADISCH, 2009). A promising immobilization technique for the detention of two or more enzymes is the aggregation and reticulation, known as CLEAs (Cross-linked Enzyme Aggregates) (SHELDON, 2007).

1.4 Scope of the work and specific research goals

This doctoral project aimed at developing the process of enzyme immobilization and stabilization of the invertase, glucose oxidase and catalase, using the aggregation and reticulation technique, for the production and application of

gluconic acid and fructose syrup from sucrose. The specific objectives of this study were:

❖ Study of the enzymatic immobilization method using the CLEAs technique with feeder protein for the enzymes: invertase, glucose oxidase and catalase.

❖ Characterization of the derivatives developed regarding the process stability, substrate concentration, temperature and pH of maximum catalytic activity and reuse.

❖ Kinetic characterization of the sucrose inversion process and of gluconic acid production.

❖ Production of gluconic acid using Combi-CLEAs from glucose-oxidase and catalase enzymes.

1.5 Thesis outline

This text is organized into chapters, in which each chapter is an article, as described below:

Chapter 2, entitled **"Literature Review"**, addresses the enzymatic immobilization techniques, highlighting the aggregation and reticulation technique or CLEAs.

Chapter 3, entitled **"Diffusion effects of bovine serum albumin on cross-linked aggregates of catalase"**, reports the immobilization of catalase from bovine liver by aggregation and reticulation technique, which investigated the influence of the cross-linker, precipitant reagent and feeder protein in catalase

CLEAs. The influence of the substrate concentration on the initial reaction speed was been investigated, enabling the fitting of kinetic models to the experimental data. The influence of the inert protein on the diffusivity of catalase CLEAs was studied based on the kinetic model.

Chapter 4, entitled **"Enzymatic production of inverted sugar using SOY-CLEAs of invertase"**, reports the immobilization of invertase from *Saccharomyces cerevisiae* by CLEAs technique using soy proteins as feeder protein. In this study, the effect of the soy proteins and the concentration of the cross-linker agent on the overall yield of invertase CLEAs was investigated, which evaluated the overall performance and stability to leaching. The biocatalysts produced were characterized kinetically regarding pH, temperature and substrate concentration. The invertase CLEAs were used in the invert sugar production under conditions of 40 ^{o}C and pH 6.0 using 50 g.L^{-1} of sucrose as substrate; the biocatalyst reuse was evaluated under the same conditions. These studies have allowed assessing the viable economic prospect of the production process of invert sugar by using 100 g.L^{-1} of sucrose.

Chapter 5, entitled **“Combi-CLEAs of glucose-oxidase and catalase for multiconversion of glucose to gluconic acid in a pneumatic reactor”** proposes the preparation of Combi-CLEAs of glucose oxidase from *Aspergillus niger* and catalase from bovine liver and its application in the gluconic acid production. The Combi-CLEAs were prepared by coprecipitation and cross-linking of glucose-oxidase, catalase and bovine serum albumin (BSA). The effects of precipitant agents (organic and inorganic salts), cross-linker (glutaraldehyde) and feeder protein (BSA) concentrations on the activities of each enzyme were investigated. Additionally, the stability to lixiviation of all derivatives was studied at 40^{o}C and pH 6.0. Combi-CLEAs

were used in GA production in a pneumatic reactor, besides their reusability in ten batch-cycles of 5h.

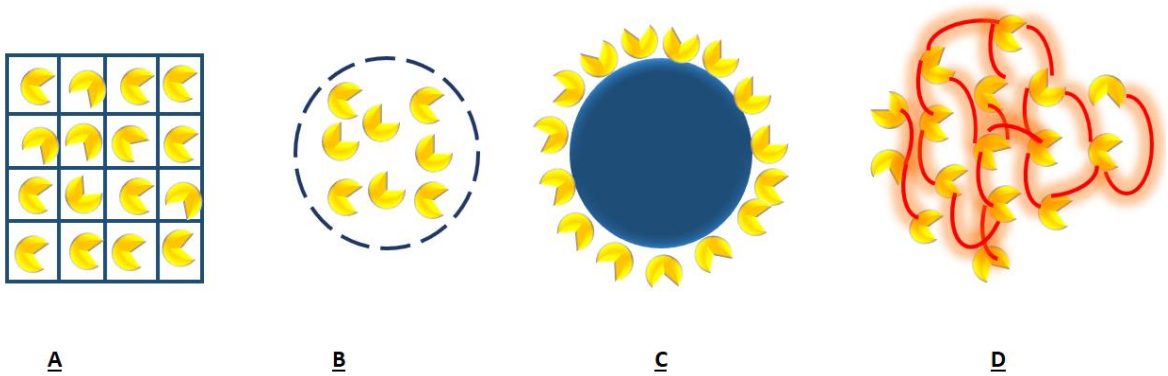
The last part of the text, **"Concluding remarks and future perspectives"**, is a summary of the main results obtained in this work and some suggestions for further studies.

2. Immobilization of enzymes as cross-linked enzyme aggregates: review

2.1 Introduction

Enzymes are proteins with catalytic functions, which play an important role in biological reactions and industrial processes. Enzymes are used in chemical conversions, biosensors and processes of bioremediation (O'FAGAIN, 2003). The great interest in the use of enzymes for industry is due to several factors, as for instance the wide range of substrates and reactions that enzymes catalyze, without generating by-products (BRADY and JORDAAN, 2009). However, the use of enzymes in some processes is limited by low operational stability and by the difficulty in separating the biocatalyst for its reuse in other processes (DUTTA, 2008). The enzymatic immobilization can overcome these problems and make the enzymes attractive from the industry`s point of view (CAO, 2006; MOSIER AND LADISCH, 2009). There are four main methods to immobilize enzymes, i.e., entrapment within porous matrices, encapsulation, covalent binding, and cross-linking, as shown in Figure 2.1 (CAO, 2006).

Figure 2.1 – Methods of enzymatic immobilization: (A) entrapment within porous matrices, (B) encapsulation, (C) covalent binding and (D) cross-linking.



The covalent binding method occurs by covalent bonds (between reactive groups from the support and biocatalyst), ionic interactions or physical adsorption. The enzymes are easily attached to various organic or inorganic materials, such as acrylic resins, starch, cellulose, chitosan, silica, etc. (ROTHENBERG, 2008; ILLANES, 2008). The main positive point of immobilization by adsorption is the ease and simplicity of the procedure, with little change in the structural conformation of the enzyme; however, the enzyme desorption can occur by changes in ionic strength and temperature (CAO, 2006).

The immobilization by means of entrapment within matrices is usually performed with the formation of the porous matrix surrounding the biocatalyst, and this matrix may be, for example, alginate, polyacrylamide gels, cellulose triacetate, agar, gelatin and carrageenan (CAO, 2006; ILLANES, 2008). The matrix pore size can be adjusted with the concentration of the polymer used. However, there is a possibility of low molecular weight enzyme leakage from the matrix and the pore size could hinder the mass transfer (ILLANES, 2008).

The immobilization technique through permeable membranes or encapsulation comprises the use of preformed membranes (hollow fiber type reactors) or the formation of membranes around the biocatalyst (CAO, 2006). This is an inexpensive and simple method and a large quantity of enzymes can be immobilized by encapsulation. The capsule is made up of semi permeable membrane such as nitro cellulose or nylon. However, the pore size could hinder a large substrate molecule to move cross the membrane (ILLANES, 2008).

In the cross-linking methodology, there is no need to use immobilization support, because this method involves the direct cross-linking of enzymes (dissolved,

dried or crystallized) or cross-linking after aggregation and precipitation of the enzymes naturally or artificially induced (CAO, 2006).

The best method to immobilize an enzyme must be determined based on the experiments, because the success of the immobilization process depends on the reaction nature, the biocatalyst stability and the activity after the immobilization process. This review aims to show the advances in the immobilization technique through self-aggregation and crosslinking with an emphasis on applications of these biocatalysts in industrial processes.

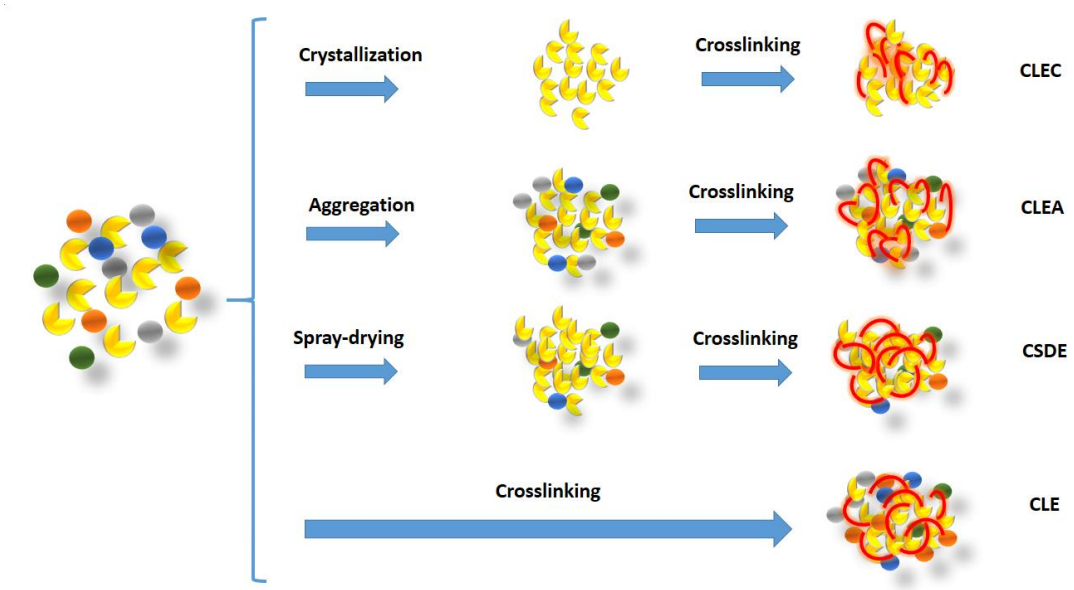
2.2 Carrier-free immobilized enzymes

In the 1960s, Doscher and Richards (1963) studied the crosslinking of enzymes dissolved through the reaction of their amino groups with a bifunctional crosslinking agent, such as glutaraldehyde, to produce cross-linked enzymes (CLE). However, CLE had several drawbacks, such as poor reproducibility, low mechanical stability, low activity recovery, low volumetric activity, and handling difficulties. In a subsequent study, Quiocho and Richards (1964) investigated the crosslinking of a crystallized enzyme in order to obtain cross-linked enzyme crystals (CLEC). However, to crystallize an enzyme required high purity, which greatly increases the process cost. Amotz (1987) proposed the crosslinking of dry enzymes by the spray-drying (CSDE) process. However the drying technique inactivated part of the enzymes, producing a low activity derivative. Then Cao, van Rantwijk and Sheldon (2000) developed a new method to immobilize enzymes, i.e., cross-linked enzyme aggregates (CLEAs) (SCHOEVAART *et al*., 2004; SHELDON, 2007). This approach is extremely simple and does not require great enzymatic solution purity and can be applied to enzymes that have different characteristics. With the technique

enhancement, many researchers have successfully proposed the use of feeder proteins in the CLEAs preparation and also the crosslinking of more than one enzyme in a CLEA, the so-called Combi-CLEAs (discussed below).

Figure 2.2 summarizes the four possible crosslinking techniques of enzymes discussed earlier.

Figure 2.2 – Enzyme's crosslinking in the absence of support.

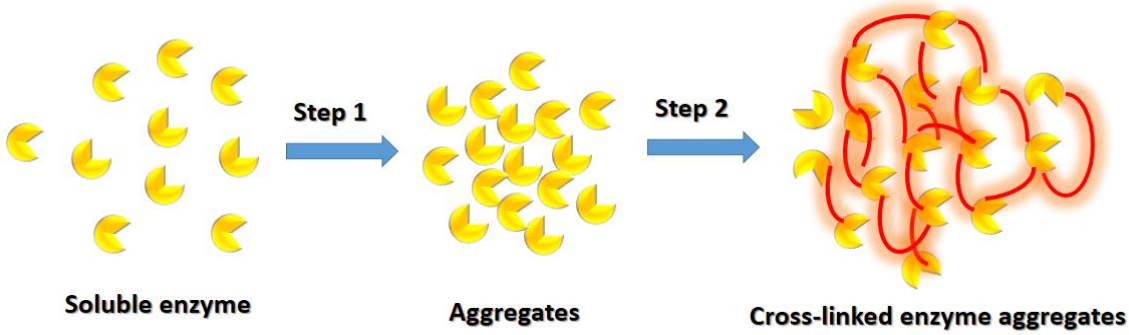


2.3 CLEAs

In the enzyme immobilization by aggregation and crosslinking, proteins are precipitated as aggregates, without denaturing or disruption of the tertiary structure. The aggregation is carried out by the addition of salts, solvent organic miscible in water or non-ionic polymers in the enzyme aqueous solution. To keep the aggregates chemically bound requires a crosslinking step (CAO, VAN RANTWIJK & SHELDON, 2000). Glutaraldehyde is often used as a crosslinking agent due to its low cost and high reactivity in the formation of Schiff's bases (-C=N-, reaction between the terminal aldehyde from glutaraldehyde and ε-lysine amino acid from the protein). The concentration of the crosslinking agent is always based on optimizing the

parameter, because in excess it can cause enzyme inactivation and at a low concentration it may promote a low crosslinking, allowing the enzyme molecules to leach (SCHOEVAART *et al*., 2004; SHELDON, 2007). Figure 2.3 shows a preparation representation of the cross-linked enzyme aggregates.

Figure 2.3 – Representation of cross-linked enzyme aggregates preparation. In the first step the soluble enzyme is aggregated by the action of a precipitating agent. In the second step the crosslinking of the enzymatic aggregates occurs by the action of a crosslinking agent.



Several studies report the optimization of various parameters that affect the properties of CLEAs, such as temperature, pH, enzymatic concentration, feeder protein (spacer agent), agitation, precipitating and crosslinking agents. Recent research has shown promising results with the aggregation and crosslinking technique using different precipitating and crosslinking agents, as highlighted in Table 2.1 (WILSON *et al*., 2004; TALEKAR *et al*., 2012; KIM *et al*., 2013). These derivatives showed high activity retention and stability in organic solvents (*e.g*., tert-butyl alcohol, acetone, dimethoxyethane). It should be noted, as also highlighted by Sheldon (2011), that the vast majority of CLEAs reported in the literature are enzymes belonging to the hydrolase family, particularly because these enzymes have more industrial applications and their immobilization enables the reuse and the cost reduction with the biocatalyst.

Table 2.1 – Application of immobilization by CLEAs in various enzymes

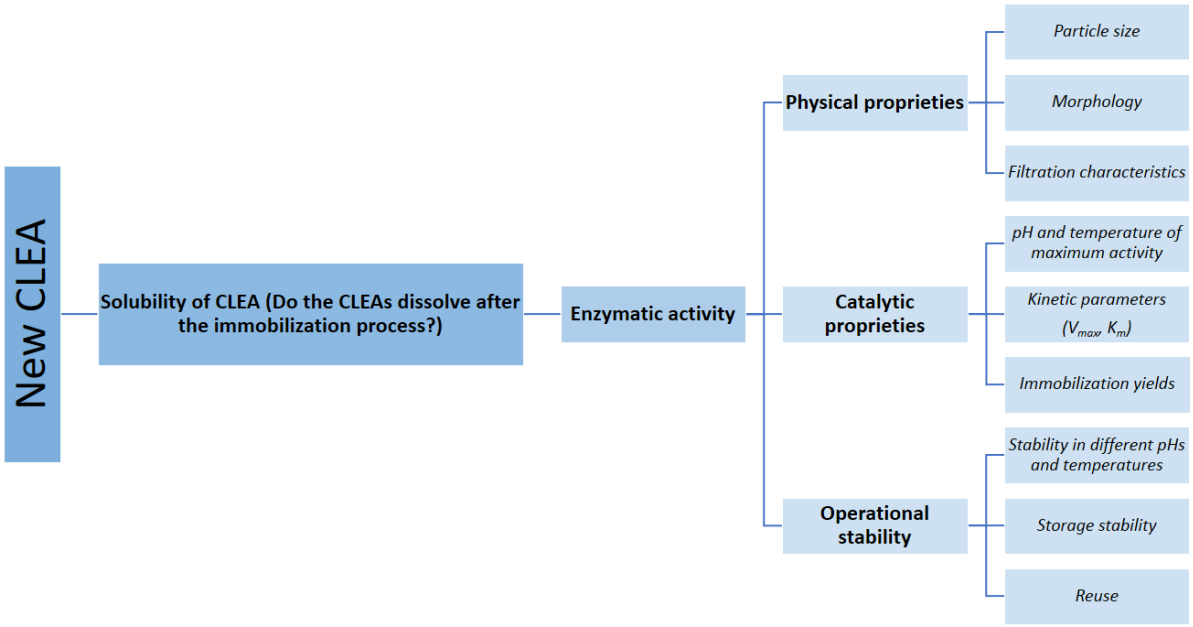
Enzyme (Class)	Source	Precipitant agent	Glutaraldehyde concentration	Feeder protein	Application	Reference
Alfa-Amylase (Hydrolase)	*Bacillus amyloliquefaciens*	Ammonium sulphate (70% V/V)	2% V/V	N.A.	Hydrolysis of starch into glucose	Talekar *et al*., 2012
Catalase (Oxidoreductase)	Bovine liver	Ammonium sulphate (70% V/V)	140 mM	1 mg enzyme/1mg BSA	Decomposition of H_2O_2	Tukel *et al* , 2013
Catalase (Oxidoreductase)	Bovine liver	Ethylene glycol diethyl ether	5% V/V	N.A.	Decomposition of H_2O_2	Wilson *et al* ., 2004
Formato Dehydrogenase (Oxirredutase)	*Candida boidinii*	Ammonium sulphate (Saturated)	6 mM	2 mg enzyme/1mg BSA	Regeneration of NADH during the reduction process of chiral compounds	Kim *et al* ., 2013
Invertase (Hydrolase)	Saccharomyces cerevisiae	Ammonium sulphate (Saturated)	20 mM	0.4% (w/v) starch	Sucrose hydrolysis in glucose and fructose	Talekar *et al* ., 2012
Lipase (Hydrolase)	Thermomyces lanuginosus	N.A.	2% V/V	10 mg enzyme / 20ml of egg white	Production of alkyl esters	Karimpil & D'Souza, 2011
Lipase (Hydrolase)	*Candida antarctica*	Ammonium sulphate (Saturated)	1.67 mg glutaraldehyde /1mg precipitated protein)	480 mg precipitated enzyme /1mg BSA	Production of alkyl esters	Torres *et al* ., 2014
Lpnal (Liase)	*Lactobacillus plantarum WFCS1 Recombinante*	Ammonium sulphate (90% V/V)	1% V/V	5 mg enzyme / 1mg BSA	Production of sialic acid	García-García *et al*., 2011
Protease (Hydrolase)	*Bacillus subtilis*	Ammonium sulphate (70% V/V)	4% V/V	N.A.	Hydrolysis of peptide bonds	Sangeetha, Emilia Abraham, 2008

Note: NA = Not applicable

2.4 CLEAs performance evaluation

Talekar *et al.* (2013) proposed a protocol for the development of stable CLEAs for large-scale applications; this protocol defines the main characteristics that should be observed in CLEAs, as shown in Figure 2.4.

Figure 2.4 – Protocol for the characterization of new CLEAs.



Font: Based on Talekar *et al*. (2013)

The particle size and morphology are important characteristics in CLEAs, since they may influence the intraparticle mass transport (especially when the substrate is macromolecular) and the biocatalyst filtering for reuse in the process (SCHOEVAART *et al.*, 2004; SHELDON, 2007; TALEKAR *et al*., 2013).

The study of enzymatic kinetics contributes to the immobilization performance evaluation and the formulation of kinetic models contributes to the choosing the procedure to be adopted. The enzyme immobilization through CLEAs technique can change the three-dimensional structure of the enzyme or may form microenvironments inside the biocatalyst. In both cases, the enzymatic activity is

affected and the parameters that allow demonstrating these effects are the constant of Michaelis-Menten (K_m) and the maximum reaction rate (V_{max}). The CLEAs enzymatic activity can also be affected by changes in pH and temperature. The immobilization usually results in a change of pH and temperature of maximum catalytic activity in comparison with the free enzyme. The pH change alters the charge distribution at the active site and the formation of the enzyme-substrate complex can occur in a favorable manner or not (SCHOEVAART *et al*., 2004; SHELDON, 2007; TALEKAR *et al*., 2013).

The formation of covalent bonds between the essential amino acid residues of the enzyme and the glutaraldehyde at the crosslinking step can change the flexibility of the immobilized enzyme and thus change the point of maximum catalytic activity and stability regarding the temperature. The half-life of an immobilized enzyme is a parameter that rules the economic feasibility of the process. However, some interference may occur in the measurement stability procedure. For example, the initial CLEA activity with mass transfer limitations may seem to have less activity than it really has and throughout the thermal stability experiment a false positive stability is observed, i.e., the CLEA loses activity, however the inactivated enzymes are "replaced" by hidden ones due to diffusional problems. To avoid these errors, the use of productivity in U/kg of product or the reverse is recommended. The reuse, i.e., the number of times that the enzyme can be recycled, is also a factor that contributes to choosing a process and to the economic feasibility assessment (SCHOEVAART *et al*., 2004; SHELDON, 2007; TALEKAR *et al*., 2013).

2.5 Feeder protein

One of the main advantages of CLEAs compared to enzymes immobilized on insoluble substrates is their high volumetric activity. However, high volumetric activity may cause diffusion limitations. The use of a feeder protein to dilute the enzyme in the CLEA matrix may decrease the mass transfer limitation; however, this also represents an additional cost in the preparation of the biocatalyst.

Talekar *et al.* (2012) proposed the immobilization of *S. cerevisiae* invertase by CLEAs in the presence of starch as a porogen, which is eluted from the biocatalyst by hydrolysis with soluble alpha-amylase and successive washes. The use of starch was proposed because invertase CLEAs without porogenic agent presented severe diffusional limitation to mass transfer. The biocatalysts prepared with the porogenic agent presented 100% recovered activity and with no diffusion limitations to the internal mass transfer.

The addition of inert protein contributes to the increase of amino group available for crosslinking. Some studies have proposed CLEAs using bovine serum albumin (BSA), chicken egg or egg protein as an inert protein (Cruz *et al*., 2012; Shah *et al*., 2006; Talekar *et al*., 2013). BSA is a high commercial value protein. In order to obtain cheaper options, the use of soy protein, which is a byproduct of the extraction of soybean oil, has been proposed by our research group. This option is 250 times cheaper than BSA.

2.6 Cascade process and Combi-CLEAs

The production of pharmaceutical and fine chemicals by typical industrial processes includes many steps, in which the product of each step is

produced and purified separately, and these procedures require more energy, raw materials, labor and processing time. Over the last decades, much research has been directed to the development of new processes that integrate steps, the so-called cascade processes. Catalytic cascade-based processes have numerous advantages, such as, lower number of unit operations, lower reactor volume, increased volumetric productivity, shorter cycle times, and reduced waste generation. In addition, the cascade processes shift the chemical equilibrium toward product formation (XUE & WOODLEY, 2012).

The combination of more than one enzyme can imitate multi-enzymatic complex processes that only happen in cells. Despite the great advantages of enzymatic cascade processes, enzyme combinations are sometimes incompatible. The temperatures and pHs of maximum catalytic activity and the stabilities may be very different, making the use of multi-enzymatic biocatalyst very complex. Some studies report that the co-precipitation and crosslinking of enzyme mixtures, known as Combi-CLEAs, contribute to overcome some of the problems identified (CAO, VAN RANTWIJK & SHELDON, 2000; SHELDON, 2011)

2.7 Final considerations

There is no simple and universal protocol for the immobilization of an enzyme. The choice of the immobilization method requires prior knowledge of the soluble enzyme properties, such as protein structure, pH, and temperature of maximum catalytic activity and stability prior to the immobilization conditions. Based on this prior study, the chances of success in choosing the immobilization methodology are more likely. Moreover, the biocatalyst cost is a very important variable, which can make its industrial use attractive or not. In this context, the

immobilization technique by aggregation/crosslinking (CLEAs) that does not comprise the use of solid support and does not require the use of pure enzymatic preparations has been presented as an alternative to commercially available conventional immobilized biocatalysts. Furthermore, CLEAs enable preparing simpler multi-enzymatic biocatalysts, also allowing better stability when multimeric enzymes are used.

2.8 References

AHMED, A. *et al.* Production of gluconic acid by using some irradiated microorganisms. Journal of Radiation Research and Applied Sciences, v. 8, n. 3, p. 374-380, 2015.

AMOTZ, S**. Method for production of an immobilized enzyme preparation by means of a crosslinking agent**. US 4665028 A. Novo Industri A/S, 1987.

BANKAR, S. *et al.* Glucose oxidase - An overview. **Biotechnology Advances**, v. 27, n. 4, p. 489-501, 2009.

BOUTROUX, L. Chimie physiologique, Sur une fermentation nouvelle de glucose (Physiological chemistry. About a new glucose fermentation). **Comptes rendus de l'Académie des Sciences**.,v. 91, p. 236–238, 1880.

BRADY, D.; JORDAAN, J. Advances in enzyme immobilisation. **Biotechnology Letters**, v. 31, n. 11, p. 1639-1650, 2009.

CAO, L. **Carrier-bound Immobilized Enzymes.** Wiley, 2006.

CAO, L. Q.; VAN RANTWIJK, F.; SHELDON, R. A. Cross-linked enzyme aggregates: A simple and effective method for the immobilization of penicillin acylase. **Organic Letters**, v. 2, n. 10, p. 1361-1364, 2000.

DOSCHER, M. S.; RICHARDS, F. M. The Activity of an Enzyme in the Crystalline State: Ribonuclease S. **The Journal of Biological Chemistry,** v. 238, p. 2399 - 2406, 1963.

DUTTA, R. **Fundamentals of Biochemical Engineering**. Springer Berlin Heidelberg, p.306, 2008.

FIEDUREK, J. Production of gluconic acid by immobilized in pumice stones mycelium of *Aspergillus niger* using unconventional oxygenation of culture. **Biotechnology Letter**, v.23 (21), p.1789-1792, 2001.

GARCÍA-GARCÍA, M. I*. et al.* New stabilized FastPrep-CLEAs for sialic acid synthesis. **Bioresource Technology**, v. 102, p. 6186–6191, 2011.

HANOVER, L. M. Crystalline fructose: production, properties, and applications. In: Schenck, F. W.; Hebeda, R. E. editors. **Starch hydrolysis products: worldwide technology, production, and application**. New York, NY: VCH; . p. 201–31,1992.

HANOVER, L. M.; WHITE, J. S. Manufacturing, composition, and applications of fructose. **The American Society for Clinical Nutrition**, v. 58, p. 724-732, 1993.

HEINRICH, M.; REHM, H. J. Formation of gluconic acid at low pH-values by free and immobilized Aspergillus niger cells during citric acid fermentation. **European journal of applied microbiology and biotechnology**, v. 15, n. 2, p. 88-92, 1982.

HULL P. **Glucose syrups: technology and applications**. Chichester, UK: Wiley-Blackwell Pub.; 2010.

ILLANES, A. **Enzyme Biocatalysis - Principles and Applications**. Springer, 2008.

KARIMPIL, J. J.; MELO, J. S.; D'SOUZA, S. F. Hen egg white as a feeder protein for lipase immobilization. **Journal of Molecular Catalysis B: Enzymatic***,* v.71, p. 113–118, 2011.

KIM, D.; KIM, H. Continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an oxidoreductase of *Zymomonas mobilis* and inulinase. **Biotechnology and Bioengineering**, v. 39, n. 3, p. 336-342, 1992.

KIM, M. H. *et al*. Immobilization of formate dehydrogenase from Candida boidinii through cross-linked enzyme aggregates. **Journal of Molecular Catalysis B: Enzymatic**, v. 97, p. 209 - 214, 2013.

LIU, J.; CUI, Z. Optimization of operating conditions for glucose oxidation in an enzymatic membrane bioreactor. **Journal of Membrane Science**, v. 302, p. 180-187. 2007.

MAFRA, A. C. O. *et al.* Gluconic acid production from sucrose in an airlift reactor using a multi-enzyme system. **Bioprocess and Biosystems Engineering**, v. 38, n. 4, p. 671-680, 2015.

MILSON, P. E.; MEERS, J. L. Gluconic acid, itaconic acid. In: Blanch HW, Drew S, Wang DIC (eds) **Comprehensive biotechnology**. Pergamon, Oxford, v.3. p. 681–700, 1985.

MITCHELL, R. E.; DUKE, F. R., Kinetics and equilibrium consents of the gluconic acid-gluconolactone system. **Annals of the New York Academy of Sciences**, v.172, n.7, p.131-138, 1970.

MOSIER, N. S.; LADISCH, M. R. **Modern Biotechnology: Connecting Innovations in Microbiology and Biochemistry to Engineering Fundamentals**. Wiley, P.464, 2009.

MUKHOPADHYAY, A. R. *et al.* Production of gluconic acid from whey by free and immobilized *Aspergillus niger*. **The International Dairy Journal**, v.15, p. 299 – 303, 2005.

O'FAGAIN, C. Enzyme stabilization - recent experimental progress. **Enzyme and Microbial Technology**, v. 33, n. 2-3, p. 137-149, 2003.

PURANE, N. K. *et al.* To study the various parameters for bioconversion of glucose to gluconic acid by *Penicillium chrysogenum* in submerged culture. **International Journal of Biotechnology**, v. 4, n. 6, p 589-593, 2011.

QUIOCHO, F. A.; RICHARDS, F. M. Intermolecular cross linking of a protein in the crystalline state: carboxypeptidase-A*. **Proceedings of the National Academy of Sciences of the United States of America**, v. 52, n. 3, p. 833-9, 1964.

RAMACHANDRAN, S. *et al.* Gluconic Acid: properties, applications and microbial production. **Food Technology Biotechnology**, v. 44, n.2, p. 185-195, 2006.

ROTHENBERG, G. **Catalysis: Concepts and Green Applications**. Wiley-VCH. Wiley-VCH, 2008.

SAINZ, F *et al.* Comparison of d-gluconic acid production in selected strains of acetic acid bacteria. International Journal of Food Microbiology, v. 222, p. 40-47, 2016.

SANGEETHA, K.; ABRAHAM, T. E. Preparation and characterization of cross-linked enzyme aggregates (CLEA) of Subtilisin for controlled release applications. **International Journal of Biological Macromolecules**,v. 43, p.314–319, 2008.

SCHOEVAART, R. *et al.* Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). **Biotechnology and Bioengineering**, v. 87, n. 6, p. 754-762, 2004.

SHELDON, R. A. Cross-Linked Enzyme Aggregates as Industrial Biocatalysts. **Organic Process Research & Development**, n. 15, p. 213 - 223, 2011.

SHELDON, R. A. Enzyme Immobilization: The Quest for Optimum Performance. **Advanced Synthesis & Catalysis**, v. 349, n. 8-9, p. 1289-1307, 2007.

SILVEIRA, M. M. *et al.* Bioconversion of glucose and fructose to sorbitol and gluconic acid by untreated cells of *Zymomonas mobilis*. **Journal of Biotechnology**, v. 75, n. Issues 2–3, p. 99–103, 1999.

SINGH, O. V.; KAPUR, N.; SINGH R. P. Evaluation of agrofood by-products for gluconic acid production by *Aspergillus niger* . **World Journal of Microbiology and Biotechnology,** v.21, p.519-524, 2005.

SINGH, O. V.; KUMAR, R. Biotechnological production of gluconic acid: future implications. **Applied Microbiology and Biotechnology**, v. 75, n. 4, p. 713-722, 2007.

TALEKAR, S. *et al*. Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). **The Royal Society of Chemistry**, v. 3, p. 12485 - 12511, 2013.

TALEKAR, S. *et al.* Porous cross linked enzyme aggregates (p-CLEAs) of *Saccharomyces cerevisiae invertase*. **The Royal Society of Chemistry** v. 2, p. 1575 - 1579, 2012a.

TALEKAR, S. *et al*. Preparation and characterization of cross linked enzyme aggregates (CLEAs) of *Bacillus amyloliquefaciens* alpha amylase. **Journal of Biochemical Technology**, v. 4, n. 4, p. 349 - 353, 2012b.

TORRES, M. P. G.; FORESTI, M. L.; FERREIRA, M. L. CLEAs of *Candida antarctica* lipase B (CALB) with a bovine serum albumin (BSA) cofeeder core: Study of their catalytic activity. **Biochemical Engineering Journal** , v. 90, p.36–43, 2014.

TUKEL, S. S. *et al.* Preparation of crosslinked enzyme aggregates (CLEA) of catalase and its characterization. **Journal of Molecular Catalysis B-Enzymatic**, v. 97, p. 252-257, 2013.

VUILLEUMIER, S. Worldwide production of high-fructose syrup and crystalline fructose, **The American Society for Clinical Nutrition** , v. 58, p. 733-736, 1993.

WHITE, J. S. Sucrose, HFCS, and Fructose: History, Manufacture, Composition, Applications, and Production. In: Rippe J.M. editor, **Fructose, High Fructose Corn Syrup**, Sucrose and Health, Nutrition and Health, 2014.

WILSON, L. *et al.* Cross-linked aggregates of multimeric enzymes: a simple and efficient methodology to stabilize their quaternary structure. **Biomacromolecules**, v. 5, n. 3, p. 814-7, 2004.

WONG, C.; WONG, K.; CHEN, X. Glucose oxidase: natural occurrence, function, properties and industrial applications. **Applied Microbiology and Biotechnology**, v. 78, n. 6, p. 927-938, 2008.

XUE, R.; WOODLEY, J. Process technology for multi-enzymatic reaction systems. **Bioresource Technology**, v. 115, p. 183-195, 2012.

ZHANG, H.; ZHANG, J.; BAO, J. High titer gluconic acid fermentation by Aspergillus niger from dry dilute acid pretreated corn stover without detoxification. **Bioresource Technology**, v. 203, p. 211-219, 2016.

3. Diffusion effects of bovine serum albumin on cross-linked aggregates of catalase

Preface

It was proposed to start the study of this thesis by immobilization of the enzyme catalase from bovine liver through the technique of aggregation and crosslinking, in view of the great difficulty reported in the literature for the stabilization of its four protein subunits. Once defined the technique and conditions of the enzyme immobilization that presents the greatest complexity (catalase) we can apply more easily the CLEAs technique for the other two enzymes involved in the multi-enzymatic conversion of sucrose (invertase and glucose oxidase).

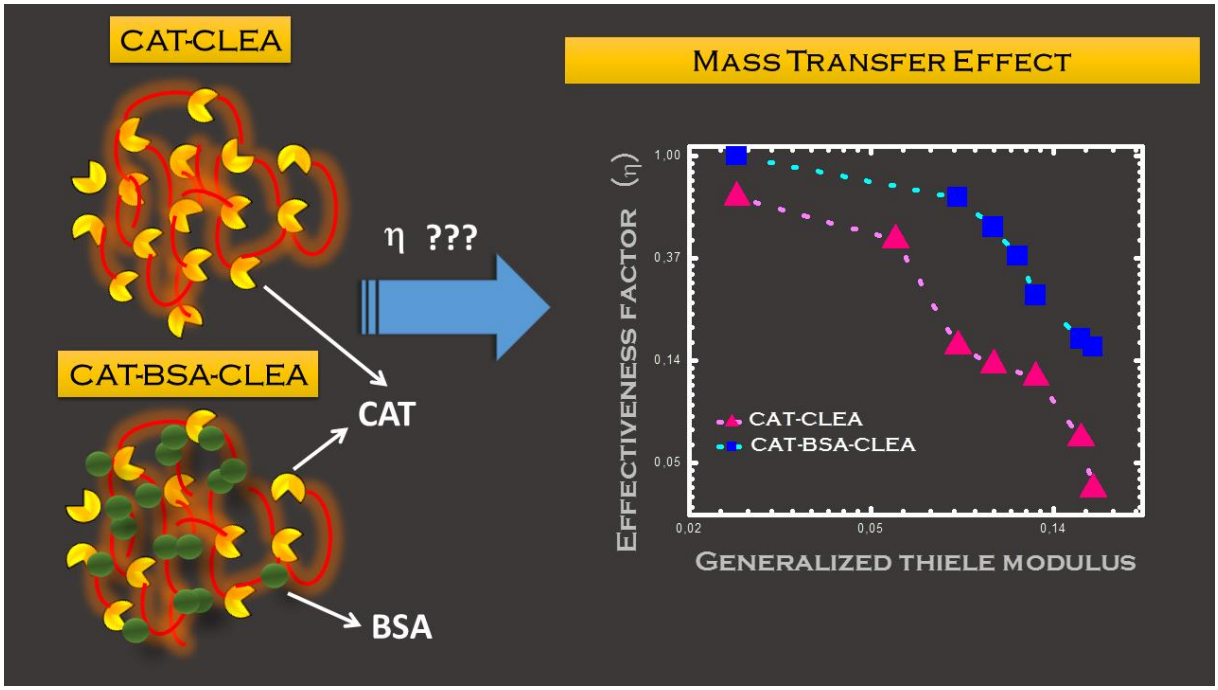
Regarding catalase immobilized as cross-linked aggregates, studies on kinetics and intraparticle mass transfer have not been reported in the literature. The stabilization of multimeric enzymes, such as the catalase, is a hard task in biocatalysis, due to the inactivation of the enzyme by dissociation of its subunits and consequent contamination of the reaction product.

The immobilization by precipitation/aggregation, followed by crosslinking is an alternative to avoid the subunits dissociation, however, the activity of the catalyst can decrease due to diffusional problems. In this study, it was evaluated the immobilization of the catalase from bovine liver using bovine serum albumin (BSA) as feeder protein; it was also evaluated its influence in the intraparticle mass transfer. The biocatalyst prepared using BSA showed high recovered activity and was stable under the conditions of the decomposition process of hydrogen peroxide into oxygen and water (40^{o}C and pH 7). This study could show that the

addition of BSA in CLEAs of catalase reduces the sizes of clusters formed after centrifugation, also decreasing the intraparticle diffusive effects. Empirical models were fitted to the experimental data of enzymatic activity with the aim to compare the mass transfer models in CLEAs with and without BSA. The results suggested that the difference of the models for free and immobilized catalase was of diffusive nature.

Finally, we believe that this work represents an advance to the knowledge of immobilization of enzymes by the CLEAs technique, having been published in November 2016, at the indexed journal: Journal of Molecular Catalysis B: Enzymatic (Print), volume 133, pages 107-116.

Graphical abstract



Highlights files

- Catalase was free-carrier immobilized using CLEA technique.
- Cross-linked catalase showed to be very stable at 40 ^{o}C and pH 7.0.
- Empirical kinetic model was used to analyze mass transfer within the particles.
- Cross-linked catalase with BSA reduced diffusional delays within the particles

Abstract

Stabilization of multimeric enzymes is one of the major challenges in biocatalysis since dissociation of subunits can inactivate the enzyme. Particularly, catalase that breaks down hydrogen peroxide in water and molecular oxygen is an enzyme difficult to stabilize by conventional immobilization techniques, because it is a tetrameric structure containing Fe-protoporphyrin IX in its active site. Cross-linking of enzyme aggregates is a methodology that can overcome this bottleneck, but diffusional delay of mass transport within the particles is a recurrent drawback. In this work, cross-linked aggregates of catalase from bovine liver were prepared, evaluating the influence of precipitant and cross-linking agents, as well as bovine serum albumin (BSA) as feeder protein on the catalytic properties, thermal stability, and mass transport resistance of the derivatives. The most active derivatives were prepared using ammonium sulfate as precipitant agent, 50 mM glutaraldehyde as cross-linker, and mass ratio BSA/catalase of 3.0. These derivatives in the absence of diffusive effects showed recovered activity of 98 ± 1.7% and high stability at 40 ◦C and pH 7.0 (~80% of the initial activity was recovery after 200 h under these conditions). The co-precipitation of BSA together with catalase reduced the size of clusters suggesting a decrease of diffusive effects within the biocatalyst. Empirical kinetic model was fitted to the experimental data of initial rate vs. substrate concentration and used to make a comparative analysis of mass transfer into derivatives with and without BSA. Results suggested that the main effect that differentiates the free enzyme and the two derivatives analyzed was of diffusive nature. In fact, the effectiveness factor of the cross-linked aggregates of catalase with BSA increased around 4 times. Statistical design of experiments and the analysis of the response surface methodology showed

that the immobilization did not alter the conditions of maximum activity of the catalase, which were found to be 30 ◦C and pH ∼7.0 for all biocatalysts.

Keywords: Catalase; CLEA; BSA; Kinetic model; Diffusional delay.

3.1 Introduction

Catalase (CAT; EC 1.11.16) is a homotetramer containing Fe-protoporphyrin IX in its active site (FITA & ROSSMANN, 1985), which is normally obtained from bovine liver or from microbial sources. CAT from bovine liver has a molecular mass of 250 kDa and each subunit has a molecular weight above 65 kDa (KISELEV *et al*., 1967).

CAT catalyzes the hydrogen peroxide (H_2O_2) decomposition through Bi–Bi Ping-Pong mechanism (SCANDALIOS, 2005; SWITALA & LOEWEN, 2002; ADÁNYI *et al*., 2007). The reaction of CAT with H_2O_2 follows two steps. In the first step of the reaction, a molecule of H_2O_2 oxidizes the ion Fe^{3+} in the prosthetic group, with the condensation of one molecule of water. In the second step, a second molecule of H_2O_2 reduces the prosthetic group, which was oxidized in the first step ($O–Fe^{4+}$), generating Fe^{3+} and releasing H_2O and O_2 (SCANDALIOS, 2005; SWITALA & LOEWEN, 2002; ADÁNYI *et al*., 2007).

Commercially, CAT is used to remove H_2O_2 from milk before cheese processes (POLAINA, 2007). It can also be found in disinfectants and food containers to prevent oxidation (POLAINA, 2007), keeping the food fresh for longer periods of time, and as an oxygenator for skin rejuvenation (CUTLER, 2005).

Enzymes play an important role in industrial chemical reactions. However, low operational stability and high costs may limit their usage in some processes. In the case of multimeric enzymes, as CAT, dissociation of the subunits often leads to enzyme inactivation and product contamination. Thus, stabilization of the quaternary structure of the enzyme is necessary (WILSON *et al*., 2004; FERNANDEZ-LAFUENTE, 2009).

An alternative to improve the performance of enzymes in industrial processes is the enzyme immobilization (GUISÁN, 2006). Adsorption, encapsulation, entrapment and covalent binding on insoluble carriers (KONDO *et al*., 1993; OZYILMAZ *et al*., 2007; ALPTEKIN *et al*., 2008,2009,2010) have been used to immobilize CAT. However, having four subunits to stabilize, CAT immobilization using these methods has shown troublesome (WILSON *et al*., 2004). On the other hand, CLEAs methodology showed to be efficient to stabilize its quaternary structure (WILSON *et al*., 2004).

CLEAs methodology (SHELDON, 2007; TALEKAR *et al*., 2012; CABANA *et al*., 2007) was developed by Sheldon *et al.* (2005) and consists in precipitating the enzyme from an aqueous solution then linking the enzymes together. First, a precipitant agent (e.g., salts, water-miscible organic solvents or non-ionic polymers) is added to an aqueous solution containing proteins. The proteins are precipitated as physical aggregates, and to keep the aggregates insoluble once the precipitant is removed, cross-linking is performed between the adjacent proteins in the CLEAs supramolecular structure (CAO *et al*., 2000). Glutaraldehyde is often chosen as cross-linking agent since it is available commercially at lower prices.

One of the main advantages of the CLEAs, compared to immobilization on insoluble carriers, is its high volumetric activity. Unfortunately, high volumetric activity can also cause severe mass transfer limitations (SHELDON, 2007; TALEKAR *et al*., 2012; CABANA *et al*., 2007; ZHEN *et al*., 2013; TALEKAR *et al*., 2013). Talekar et al. (2012) proposed a porous CLEA where starch was co-aggregated with the enzyme. The starch was subsequently removed by α-amylase action, resulting in large pores in the CLEA structure. This technique however (using an additional enzyme), may significantly increase the costs of the biocatalyst preparation. Bovine

serum albumin (BSA) has also been used to dilute the enzyme in the CLEA, reducing its volumetric activity. Tukel *et al*. (2013) and Shah *et al.* (2006) showed that the addition of BSA could increase the recovery activity of CLEAs, which may be an evidence of a reduction on diffusional resistance. On the other hand, Cabana *et al.* (2007) obtained low activity for CLEAs of laccase (EC 1.10.3.2) when BSA was added. The authors attributed the activity reduction to diffusional problems. The role that BSA plays in CLEAs may be of several natures. BSA may dilute the enzyme (reducing diffusional resistance) or make the biocatalyst denser (increasing diffusional effects). Free amino groups of the BSA compete with the amino groups of the enzyme reducing enzyme cross-linking. This competition may avoid excessive cross-linking of the enzyme reducing enzyme denaturation. On the other hand, it may prevent the necessary cross-linking of the molecules of enzymes.

The aim of this work was to evaluate and optimize the preparation of CLEAs of catalase from bovine liver (henceforth CAT-CLEAs). First, the effects of different precipitant agents (organic and inorganic salts) and cross-linking agent concentration (Glutaraldehyde) were evaluated on the recovered activity of CAT-CLEAs. Then, the amount of a feeder protein (BSA) was also evaluated. Soluble CAT and the best CAT-CLEAs without and with BSA (henceforth CATBSA-CLEAs) were characterized in terms of pH and temperature by using response surface methodology. The thermostability of the enzyme at 40 ^{o}C was measured. Empirical models were proposed to describe the kinetic of free and immobilized CAT until complete inactivation in the presence of high concentration of substrate. Finally, generalized Thiele modulus was used to verify if diffusional limitations could explain the behavior of the biocatalysts.

3.2 Material and methods

3.2.1 Material

Bovine liver catalase (CAT; EC:1.11.1.6) (30 U/mg protein) and bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO). For CAT-CLEAs and CAT-BSA-CLEAs syntheses and for the evaluation of their properties, dimethoxyethane (DME) (Fluka), tert-butyl alcohol (TBA) (Vetec, Brazil), acetone (A) (Vetec, Brazil), polyethylene glycol 600 (PEG 600) (Synth, Brazil), ammonium sulfate salt (AS) (Vetec, Brazil), glutaraldehyde 25% (w/w) aqueous solution (Vetec, Brazil) and hydrogen peroxide (H_2O_2) (Sinth) were used.

3.2.2 Methods

3.2.2.1 CAT activity assay

Activities of soluble CAT and CAT-CLEAs were determined spectrophotometrically at 240 nm, following the decomposition of a H_2O_2 solution (0.35 mM H_2O_2 in 0.05 M phosphate buffer, pH 7.5) at 25 ^oC. Briefly, 10 µL of a solution of CAT or suspension of CAT-CLEAs were added to 25 mL of substrate in a stirred and thermostatically controlled reactor. Samples of 1 mL at intervals of 1 min were withdrawn (free of solids) up to 3 min of reaction for reading the absorbance at 240 nm. The initial rate of the peroxide decomposition was calculated from the slope of the curve absorbance versus time, where absorbance was converted to H_2O_2 concentration from a standard curve. One CAT unit was defined as the amount of enzyme that catalyzes the decomposition of 1 µmol of H_2O_2 per minute.

The low substrate concentration used in this work to evaluate CAT activity was chosen to maintain a low reaction rate. High reaction rates would lead to an accumulation of bubbles inside the biocatalyst, such as reported by Ozilmaz *et al.*

(2007). Presence of bubbles inside the biocatalyst could impair the analysis of the diffusive effects inside the particles because of the complexity of the phenomenon. However, the reaction rate is very sensitive to substrate concentration, when its value is low. Therefore, during the activity measurements, extreme care was taken to assure that the rate remained constant, at initial velocity. This was accomplished by assuring a linear increase of absorbance in time and low consumption (~5%) of H_2O_2.

3.2.2.2 Protein concentration

Lowry method was used to measure protein concentration in the enzyme preparations (LOWRY, 1951). The protein content was spectrophotometrically quantified at 750 nm using BSA as standard protein.

3.2.2.3 Preparation of CAT-CLEAs and CAT-BSA-CLEAs

40 mg or 20 mg of CAT powder was dissolved in 1 mL of sodium phosphate buffer (100 mM, pH 7.0). Protein aggregation was induced by mixing 1 mL of the enzymatic solution and 1 mL of precipitant (saturate solution of ammonium sulfate, tert-butyl alcohol, polyethylene glycol, dimethoxyethane or acetone). After 1 min of mixing, glutaraldehyde was slowly added to the final concentration of 0, 25, 50, 100 or 200 mM.

For the CAT-BSA-CLEAs preparation, BSA (20, 40, and 60 mg) was added as a feeder protein into the enzyme solution. Then, the same protocol was used in precipitation and cross-linking. However, in this case, only saturated ammonium sulfate was used as precipitant and glutaraldehyde was added only to the final concentration of 50 mM. All the reactions were performed at 4 ^{o}C.

After 3 h of cross-linking at 4 ^{o}C, the biocatalyst solution was centrifuged at 10,000 × g for 10 min at 4 ^{o}C. CLEAs were recovered as pellets and washed twice with 100 mM sodium phosphate buffer (pH 7.0). After preparation, the enzyme was stored in the same buffer (2 mL) at 4 ◦C at concentrations of 20 mg.mL^{-1} and 10 mg.mL^{-1}.

The overall yield of immobilization (*Y*) for CAT-CLEAs and CAT-BSA-CLEAs was calculated as follows:

$$Y(\%) = \frac{A_{final}\left(\frac{U}{mg_{protein}}\right)}{A_{initial}\left(\frac{U}{mg_{protein}}\right)} \times 100\ \% \quad (3.1)$$

Where A_{final} is the total activity of CLEAs per mass of CAT used in the preparation, and $A_{initial}$ is the total activity of the crude (soluble) CAT for CLEAs production per mass of CAT. In other words, *Y* is the activity of the biocatalyst divided by the activity of the same amount of soluble enzyme used in the biocatalyst preparation.

3.2.2.4 Thermo stability study

Stability of soluble and immobilized CAT were evaluated at 40 ^{o}C and pH 7.0 (100 mM sodium phosphate buffer) for 200 h. In all assays, the initial activity was taken as 100%. The retained activity was calculated as follow:

$$Retained\ activity(\%) = \frac{A_{final\ after\ stability}}{A_{initial}} \times 100\ \% \quad (3.2)$$

Where A_{final} is the total activity of CLEAs per mass of CAT used in its preparation after the stability test, and $A_{initial}$ is the total activity of the crude CAT for CLEAs production per mass of CAT. It is worth to notice that the retained activity is defined here as the enzymatic activity of the biocatalyst after the stability assay

divided by the activity of the same amount of free enzyme used in the biocatalyst preparation.

The residual activity is defined here as follow:

$$Residual\ activity(\%) = \frac{A_{final\ after\ stability}}{A_{before\ stability}} \times 100\ \% \quad (3.3)$$

where $A_{\text{before stability}}$ is the total activity of CLEAs before the stability assay.

3.2.2.5 Kinetic analysis

Kinetic parameters of soluble CAT and derivatives were estimated by measuring initial reaction rates using different substrate (H_2O_2) concentrations in the range of 0.35-140 mM in sodium phosphate buffer (0.1 M, pH 7) at 25 ^{o}C. Except for the concentration of substrate used, the assays were performed as described in section 3.2.2. In order to describe the influence of the substrate in the CAT activity two models were tested. The first was the Hill model (HILL, 1910) (Eq. 3.4). The second is an empirical model based on the Hill model and a model proposed by Wu *et al.* (1988) (Eq. 3.5).

$$r = \frac{k_{cat} \cdot [S]^n}{K_m^n + [S]^n} \quad (3.4)$$

$$r = \frac{k_{cat} \cdot [S]^n}{[S]^n + K_m^n + \left(\frac{[S]}{K_i}\right)^w} \quad (3.5)$$

where, r is the specific reaction rate (μmol.min^{-1}.mg$_{CAT}^{-1}$), k_{cat} is the *turnover* number (μmol.min^{-1}.mg$_{CAT}^{-1}$), K_m is the Michaelis-Menten constant (mM); [*S*] is the substrate concentration (mM), w is an inactivation degree, and n is a cooperativity parameter.

k_{cat} ,K_m, K_i, *n* and *w* values of soluble CAT, CAT-CLEAs and CAT-BSA-CLEAs were calculated from nonlinear regression fitting the models (Eq. 3.4-3.5) to the experimental data of *initial reaction rates* versus H_2O_2 *concentrations*.

3.2.2.6 Influence of pH and temperature

A factorial design followed by response surface analysis was used to evaluate the effect of temperature (from 15.2 to 44.1 ^{o}C) and pH (from 4.2 to 9.8) in the soluble CAT, CAT-CLEAs and CAT-BSACLEAs activities (response variable). The activities were measured as described in Section 3.2.2. The Rotatable Central Composite Design (RCCD) used is shown in Table 3.1. The twelve runs were carried out in a random order. Statistica software (Statsoft, version 7.0) was used to analyze the experimental data, generate the ANOVA (analysis of variance) data, and build response surfaces.

A second-order polynomial model of the form of (Eq. (3.6)) was used to fit the data:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (3.6)$$

Where Y is the predicted response variable; X_1 and X_2 are the coded independent variables representing the temperature and the pH, respectively; and, β are empirical parameters.

3.2.2.7 SEM

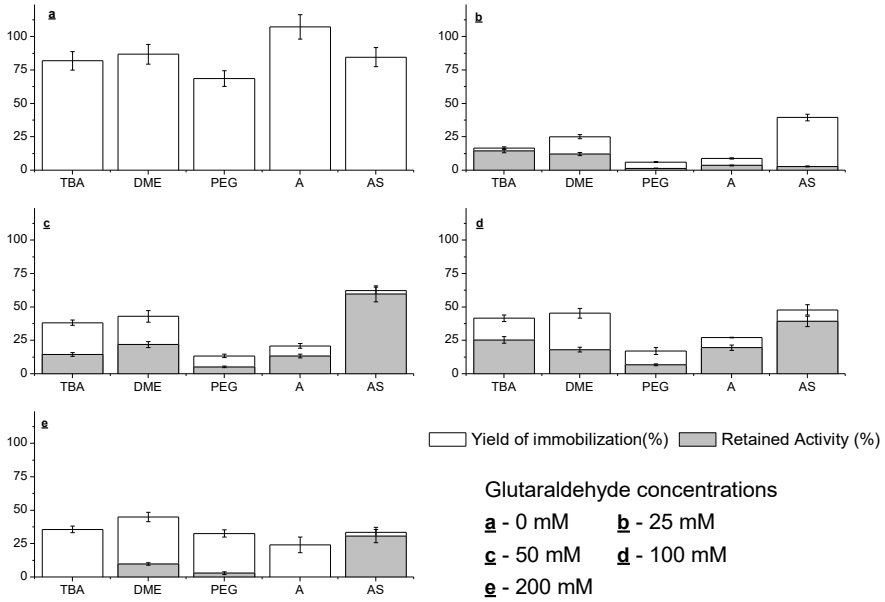
The surface morphology of CAT-CLEAs and CAT-BSA-CLEAs were obtained by scanning electron microscopy (SEM). Micrographs were taken on a JSM 6510/JEOL (5KV) SEM instrument.

3.3 Results and discussion

3.3.1 Preparation CAT-CLEAs

Aiming to precipitate all protein in the reaction medium with maximum recovered activity, several protein precipitants were tested: tert-butyl alcohol (TBA), polyethylene glycol (PEG), dimethoxyethane (DME), acetone (A), and ammonium sulfate (AS). The effect of the glutaraldehyde concentration (0, 25, 50, 100 and 200 mM) in the properties of CAT-CLEAs was also investigated. Preparations with 40 mg of CAT were used in these assays.

Figure 3.1 – (White column) Overall yield of immobilization of CAT-CLEAs prepared with different precipitant agents: ammonium sulfate solution (AS), tert-butyl alcohol (TBA), polyethylene glycol (PEG), dimethoxymethane (DME), acetone (A), and glutaraldehyde concentrations at 0 (a), 25 mM (b), 50 mM (c), 100 mM (d), and 200 mM (e). (Black column) activity retained by CAT-CLEAs after 200 h at 40 ^{o}C, pH 7 (100 mM sodium phosphate buffer).



After precipitation and centrifugation, supernatant of the enzyme solution was removed and the precipitate was resuspended in buffer solution. Then protein content was analyzed. The protein content in the re-suspended solution was greater than 95% of the initial mass for all precipitant agents (data not shown). Similar findings were observed when AS was used as precipitant agent to immobilize by CLEA methodology the following enzymes: invertase (TALEKAR *et al*., 2012), alpha amylase (TALEKAR *et al*., 2012), laccase (TOUAHAR *et al*., 2014), versatile peroxidase (TOUAHAR *et al*., 2014), glucose oxidase (TOUAHAR *et al*., 2014), and formate dehydrogenase (KIM *et al*., 2013). The activity of re-suspended enzyme solution was measured and it is depicted in Fig. 3.1a. Notice that the decrease in the activity of the re-suspended solution in some cases cannot be attributed only to the loss of enzymes in the supernatant. There is also an enzyme inactivation due to the precipitant agent. For instance, the lowest activity obtained when PEG was used as precipitant agent can be attributed to the chelating ability of PEG that could attack the iron in the protoporphyrin rings of CAT (SOKMEN *et al*., 2008).

Fig. 3.1(b–e) shows the overall yield of immobilization for CAT-CLEAs prepared using five precipitant agents and five glutaraldehyde concentrations. Fig. 3.1 also shows the retained activities after 200 h of incubation at 40 ^{o}C and pH7.0. Notice that the retained activity is the activity after stability assay divided by the initial activity of the same amount of free enzyme used in the biocatalyst preparation.

The CAT-CLEAs prepared with AS as precipitant agent and 50 mM-glutaraldehyde as cross-linking agent yielded the most active (around 62 %) and the highest stable derivative (approximately 100 % of residual activity, i.e. the activity retained after 200 h at 40 ^{o}C, pH 7.0, was still around 60 %). The best derivative prepared in this work based on overall yield of immobilization and stability, showed to

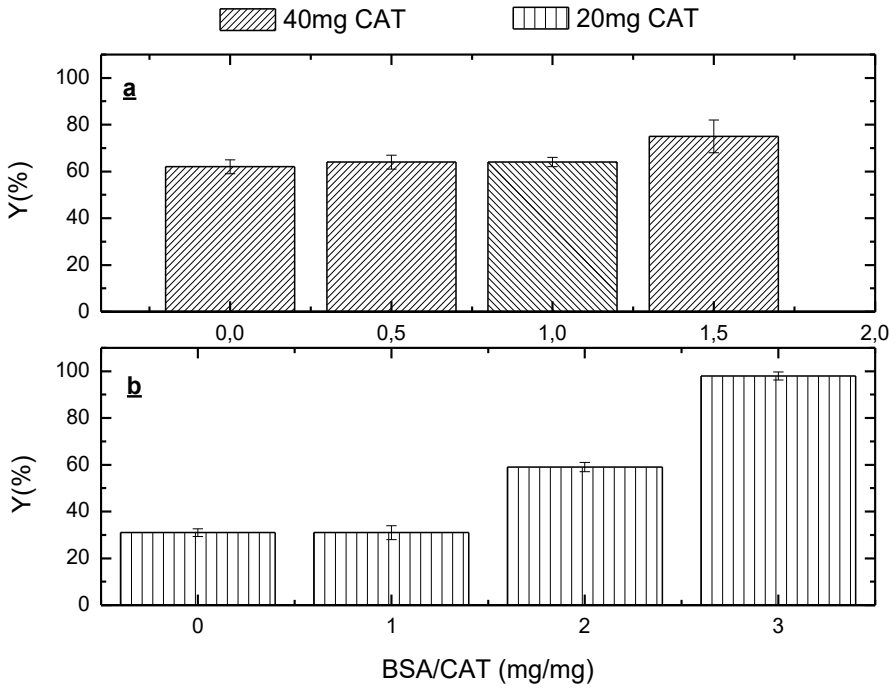
be more active than those reported by Wilson *et al*. (2004). The authors immobilized CAT from bovine liver and from *Micrococcus lysodeikticus* by CLEAs methodology using diethyleneglycol-dimethyl ether as precipitant agent and glutaraldehyde (5 % v/v) as cross-linking agent. Those derivatives retained 45 and 40 % of the offered activity, respectively.

3.3.2 Effect of BSA as a feeder protein

To study the effect of BSA as a feeder protein in CAT-CLEAs, only AS was used as precipitant agent. In addition, two masses of enzyme were used (20 and 40 mg) in 2 mL of enzyme solution. In both preparations glutaraldehyde concentration was the same, 50 mM. All CLEAs reported in this section showed high thermal stability at 40 ^{o}C, with a residual activity of around 80 % after 200 h (data not shown).

The difference between CAT-CLEAs using 20 and 40 mg of CAT without addition of BSA is shown in Fig. 3.2(a and b), when BSA/CAT = 0. It was observed that the overall yield of immobilization (Y) for the CLEAs using 40 mg of CAT was twice greater than the derivative using 20 mg of CAT. This behavior suggests that the increase of the cross-linking agent per mass of CAT may lead to an excessive cross-linking, which may yield: (a) an increase of diffusive resistance and, in extreme case, an impediment of the substrate in the access to the active site of a fraction of the enzymes; or (b) an inactivation of the enzyme by conformational alteration.

Figure 3.2 – Immobilization of CAT applying **(a)** 40 mg (CAT) and **(b)** 20 mg (CAT) of enzymatic protein and using 0, 20, 40 and 60 mg of BSA as a feeder protein**.** Residual activity (40 ^{o}C, pH 7, 200 h) was ~ 80% for all biocatalysts.



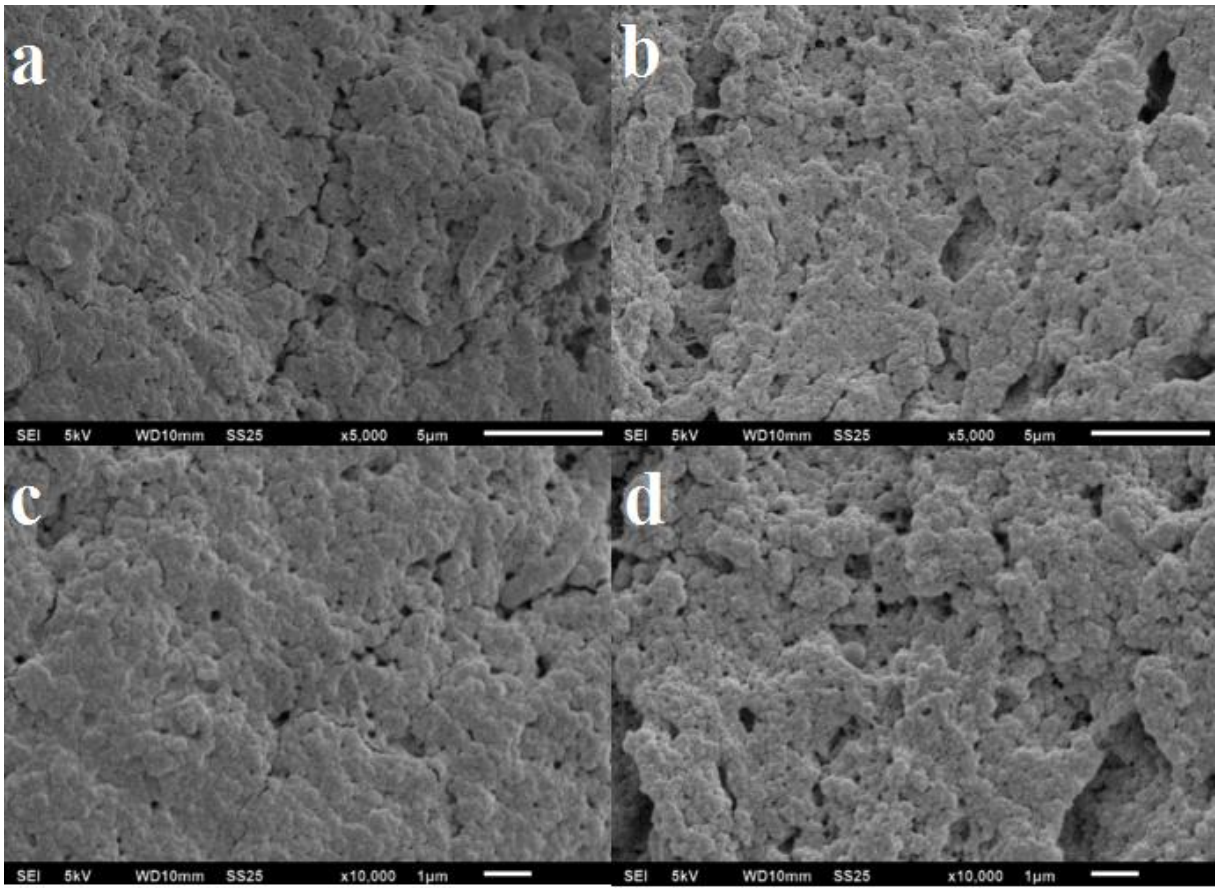
The effect of BSA as a feeder protein was investigated in the immobilization of CAT by CLEAs technique, using 40 and 20 mg of enzymatic protein and 20, 40 and 60 mg of BSA, (Fig. 3.2a and b). In both cases (using 20 and 40 mg of CAT) the concentration of feeder protein did not exert significant influence in the recovered activity of the CAT-BSA-CLEAs using mass ratio BSA/CAT ≤ 1. However, the results obtained using 20 mg of CAT (Fig. 3.2b) show that when the mass ratio BSA/CAT is greater than one, the recovery activity expressively increases. In this case (using 20 mg of CAT), Y reached values above 95 % when using BSA/CAT = 3.

The increasing in the recovery activity of CLEAs co-aggregated with BSA and other polyionic polymers have been reported (SHAH *et al*., 2006; WILSON *et al*., 2004). According to the authors, this strategy is favorable both when using low

protein concentration or using enzyme with low amine content. In these cases, standard cross-linking might not be expectedly effective, resulting in CLEAs with low mechanical stability or even releasing enzyme into the reaction media. Tukel *et al.* (2013) also reported an improvement in the CAT-CLEAs when BSA was added as a feeder protein. The authors assumed that the improvement was due to an increase of mass transport resistance. Cabana *et al.* (2007), synthesizing laccase-CLEAs, had an opposite behavior when using BSA as feeder protein, i.e., the addition of BSA reduced yield of immobilization. According to the authors, the mass transfer limitation could be increased when BSA is co-aggregated with CLEAs, since the internal structure of the aggregates is disturbed, resulting in narrower channels that limit the diffusion of the substrate within the biocatalyst. This hypothesis was based on visual evidence from the SEMs of laccase CLEAs with and without BSA and the kinetic parameters obtained.

SEMs from biocatalysts synthesized in this work are presented in Fig. 3.3. Small aggregates (type 2 as proposed by Schoevaart *et al*. (2004)) with approximately 0.1 µm in diameter can be visualized. SEMs show a small difference between CLEAs with and without BSA. However, CAT-BSA-CLEAs (Fig. 3.3a and c) seems to have smaller pores than CAT-CLEAs (Fig. 3.3b and d). Caves with openness greater than 1 µm, easily seen in CAT-CLEAs, are not present in CATBSA-CLEAs surface. Based only on SEMs, it can be expected that CAT-BSA-CLEAs have greater mass transfer limitation. However, CLEAs can form large clusters (Fig. 3.1S, Supplementary data), which may also impact on mass transport, especially with fast reactions (SCHOEVAART *et al*., 2004).

Figure 3.3 – SEM images of CAT-BSA-CLEAs (a, c) and CAT-CLEAs (b, d).



The Feret's statistical geometric diameters of the CLEAs were calculated from their microscopy images using the ImageJ software. The histograms of particle size distribution using step size of 10 µm are showed in Fig. 3.1S (Supplementary data). The particle diameter for CAT-CLEAs ranged from 20 to 60 µm, while for CAT-BSA-CLEAs it was less than 10 µm. CAT-BSA-CLEAs could form less clusters than CAT-CLEAs because at pH 7.0 BSA is negatively charged (the isoelectric point of BSA is around 5) and the repulsion between CLEA particles with BSA are greater than those without BSA. In fact, CLEAs prepared at pH 5.0 form clusters greater than at pH 7.0, as confirmed by microscopy images (Fig. 3.2S, Supplementary data). The pictures clearly show that the clusters of CAT-BSA-CLEAs are much smaller than CAT-CLEAs, indicating that BSA reduces the size of the cluster decreasing mass transport limitation.

3.3.3 CAT empiric kinetic

The kinetic mechanism of CAT is very complex. It is consensus among authors (SWITALA AND LOEWEN, 2002; SCANDALIOS, 20005; ADÁNYI *et al*., 2007) that CAT kinetic follow Bi–Bi Ping-Pong mechanism. However, substrate inhibition and inactivation may be also present. Lardinois *et al.* (1996) proposed a kinetic mechanism with two phases (α and β—without and with inhibition) into the H_2O_2 breakdown. The derived reaction rate expression was very complex but in each of these two distinct phases, the resulting expressions can be simplified to Michaelis-Menten models. However, when concentration of oxygen peroxide (substrate) further increases, CAT is severally inactivated (FITA & ROSSMANN, 1985; LARDINOIS *et al.*,1996).

Figure 3.4 – Effect of H_2O_2 concentration on the activities at 25 ^oC and pH 7.5 of soluble CAT: (a) the Hill model fitted to experimental data in the range without inhibition (k_{cat} = 21,537±799 μmol H_2O_2.min^{-1}.mg$^{-1}_{CAT}$, K_m = 32.08±1.24 mM and n = 2.47±0.19). **(b)** The Mix Hill and Wu models fitted to experimental data in the range with and without inhibition (k_{cat} = 19,971±928 μmol H_2O_2.min^{-1}.mg$^{-1}_{CAT}$, K_i = 38.09±5.94 mM, K_m = 29.89±1.54 mM, n = 2.67±0.36 and w = 9.95±1.26).

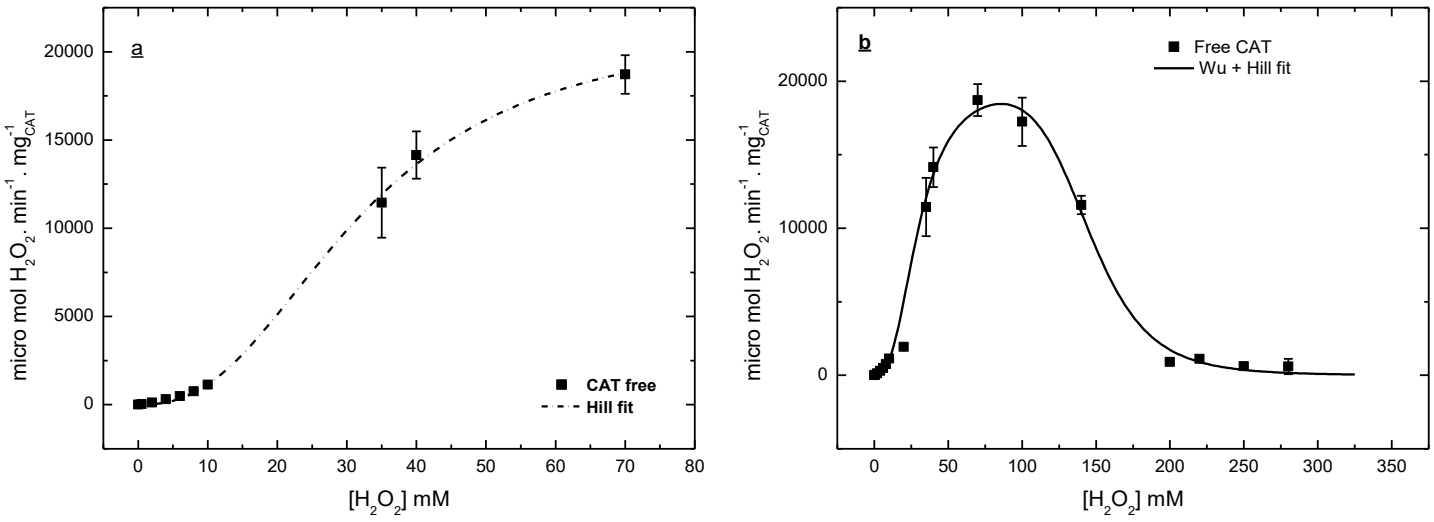
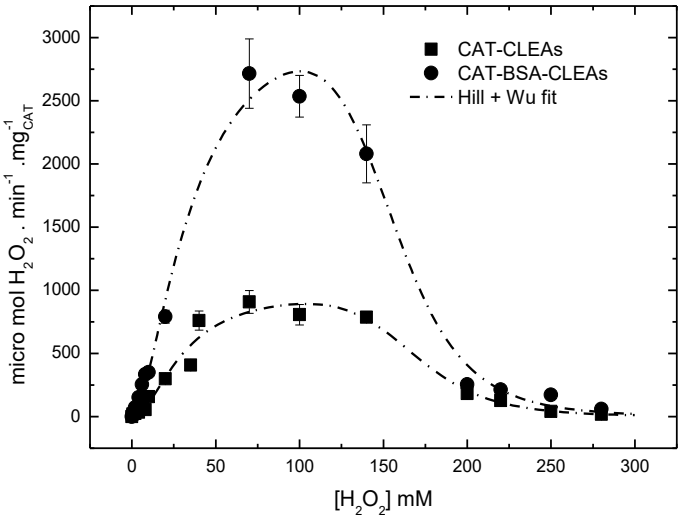


Fig. 3.4 shows experimental initial velocity of soluble CAT (25 ^{o}C and pH 7.5) in the range without (Fig. 3.4a) and with (Fig. 3.4b) H_2O_2 inactivation. Clearly, a Michaelis-Menten model cannot describe the behavior obtained in Fig. 3.4a. The sigmoid behavior showed here does not appear in Lardinois *et al*. (1996) data because the minimum concentration used by those authors was 10 mM. Therefore, in order to describe free CAT kinetic, the Hill model (a sigmoid model) was fitted against experimental data (Fig. 3.4a).

Figure 3.5 – Effect of H_2O_2 concentration on the activities at 25 ^{o}C and pH 7.5 of immobilized CAT. Mix Hill and Wu models fitted to experimental data in the range with inhibition of CAT-CLEAs (k_{cat} = 1,000±150 μmol H_2O_2.min^{-1}.mg$^{-1}_{CAT}$, K_i = 61.1±16.1 mM, K_m = 30.5±6.8 mM, n = 1.9±0.5 and w = 9.6±2.6) and CAT-BSA-CLEAs (k_{cat} = 3,305±387 μmol H_2O_2.min^{-1}.mg$^{-1}_{CAT}$, K_i = 60.9±8.0 mM, K_m = 35.12±6.7 mM, n = 1.68±0.25 and w = 9.25±1.30).



Initial velocity was obtained at same conditions for CAT-CLEAs and CAT-BSA-CLEAs (Fig. 3.5). Experimental data show (Fig. 3.4b) that soluble CAT, as well as immobilized CAT (Fig. 3.5), are severally inactivated at high concentration of oxygen peroxide (substrate), as previously reported by Fita *et al.* (1985). A similar

behavior was found by Wu *et al.* (1988). The authors developed an equation to describe the effect of chemical oxygen demand in a treatment of leachate. The kinetic expression proposed by these authors was used as an empirical model to describe the initial velocity for the whole range of substrate used (Figs. 3.4b, 3.5).

The results also show a maximum reaction rate at substrate concentration of around 100 mM. The range of maximum reaction rate is the same for the soluble CAT and for the immobilized CAT. However, the reaction rate value (per mass of CAT used in CLEAs preparation) is much lower for the immobilized enzymes.

3.3.4 Diffusional effects in CAT-CLEAs and CAT-BSA-CLEAs

Visual evidence based on Fig. 3.3 and Fig. 3.1S suggests that BSA decreases the mass transfer resistance by inhibiting large cluster formation as visually observed in CAT-CLEAs. If the only effect of BSA on CLEAs is of diffusive nature, kinetic evidence should support this claim. External limitation was supposed negligible under the stirring velocity adopted because 100% of activity was measured for CAT-BSA-CLEAs using low substrate concentration (Fig. 3.2b, BSA/CAT ratio of 3). A theoretical analysis based on the mathematical models obtained was used to at least verify whether the hypothesis is consistent with the data.

Generalized Thiele modulus, ϕ, (Eq. (3.7)) (FROMENT *et al*., 2010) can be used to investigate diffusive effects inside biocatalysts:

$$\phi = \frac{V}{S} \cdot \frac{r([S]_0) \cdot \rho}{\sqrt{2}} \cdot \left[\int_{[S]_{eq}}^{[S]_0} Deff([S]) \cdot r([S]_0) \cdot \rho \cdot d[S] \right]^{-\frac{1}{2}} \quad (3.7)$$

where *[V/S]* is the volume/superficial area ratio, *r* is the reaction rate of H_2O_2 decomposition by free CAT, *[S]* is the substrate concentration, D_{eff} is the

effectiveness diffusivity coefficient, ρ is the biocatalyst enzyme density and $[S]_0$ is the bulk concentration. $[S]_{eq}$ is assumed as zero.

The biocatalyst enzyme density, ρ, is defined by Eq. (3.8):

$$\rho = \frac{E_0 \cdot V_r}{V_d} \tag{3.8}$$

where E_0 is the enzyme loading ($g_{enzyme}/L_{reactor}$), V_r is the reactor volume ($L_{reactor}$) and V_d is the dried enzyme volume (L_{enzyme}).

Here, ϕ was evaluated for CAT-BSA-CLEAs and CAT-CLEAs in a range where substrate inhibition is negligible ([*S*] < 20 mM) assuming Hill model for the free enzyme. The Hill model fitted to the experimental data of free CAT could be approximate to an n^{th} order kinetic since $S^n << K_m^n$, and it may be described by Eq. 3.9:

$$r \cdot \rho = \left[\frac{k_{cat} \cdot S^n}{K_m^{\ n}} \right] \cdot \left[\frac{E_0 \cdot V_r}{V_d} \right] \tag{3.9}$$

Using Eq. 3.9 and defining $K = k_{cat}/ K_m^n$, ϕ simplifies to (FROMENT *et al.*, 2010):

$$\phi = \overbrace{\left[\sqrt{\frac{(n+1) \cdot K}{2}} \right]}^{A} \cdot \overbrace{\left[\frac{V}{S} \cdot \sqrt{\frac{\rho}{Deff}} \right]}^{B} \cdot \sqrt{S^{n-1}} \tag{3.10}$$

The term in the first bracket (A) relates to the kinetic of the free enzyme, the second (B) depends on the biocatalyst (geometry, enzyme density, pore size and shape).

Inner particle diffusive effects in a catalyst is often analyzed using the effectiveness factor (η) which relates the observed reaction rate (r^{obs}) with the reaction rate if there were no diffusion limitations, $r([S]_0)$ (Eq.3.11).

$$\eta = \frac{r^{obs}}{r([S]_0)} \tag{3.11}$$

In the region where inner diffusion controls the reaction rate (FROMENT *et al*., 2010):

$$\eta \approx \frac{\alpha}{\phi} \tag{3.12}$$

Where α is a number that depends on the geometry of the particle. Thus, in this region $ln(\eta)$ depends linearly on $ln(\phi)$.

$$\ln \eta = \ln \alpha - \ln \phi \tag{3.13}$$

Replacing ϕ given by Eq. 3.10, Eq. 3.13 becomes

$$\ln(\eta) \approx \ln \frac{\alpha}{A \cdot B} - \ln \sqrt{[S]^{n-1}} \tag{3.14}$$

Therefore, if the only effect that differentiate free CAT, CAT-CLEAs and CAT-BSA-CLEAs is of diffusive nature, η could be evaluated by Eq. (3.14) and the plot of ln(η) versus $ln(S^{(n-1)/2})$ should have slope of minus one in the range where the diffusion is the limiting mechanism. This plot can be seen in Fig. 3.6a, in which the data points close to η = 1 (Fig. 3.3S, Supplementary data) was not used to assess diffusion effects, since the observed specific activity was close to that of free enzyme. For $ln(S^{(n-1)/2})$ greater than one, both curve have similar slopes that approaches the unit. The behavior of the curve is a strong indicative that the effect is of the diffusive nature, both for CAT-CLEAs and CAT-BSA-CLEAs. In order to evaluate η in Fig. 3.6, the model of free CAT reaction rate, instead of experimental data, was used to reduce scattering of data.

Notice that the difference between the intercepts gives a relation between diffusive effects:

$$\text{intercept}_{CAT\text{-}CLEA} - \text{intercept}_{CAT\text{-}BSA\text{-}CLEA} = \ln\left(\frac{B_{CAT\text{-}BSA\text{-}CLEA}}{B_{CAT\text{-}CLEA}}\right) = \ln\left(\frac{h_{CAT\text{-}CLEA}}{h_{CAT\text{-}BSA\text{-}CLEA}}\right) \quad (3.15)$$

These results show a typical reactive-diffusive behavior that can be illustrated by plotting ln(η) by ln(ϕ/constant). Here, the constant used was $AB_{CAT\text{-}CLEA}$ obtained. Notice that, from Eq. 3.14 we can write Eq. 3.15 as:

$$\ln\eta = \ln\alpha - \ln\left[\phi\frac{AB_{CAT-CLEA}}{AB_{CAT-CLEA}}\right] = \ln\left[\frac{\alpha}{AB_{CAT-CLEA}}\right] - \ln\left[\frac{\phi}{AB_{CAT-CLEA}}\right] \quad (3.16)$$

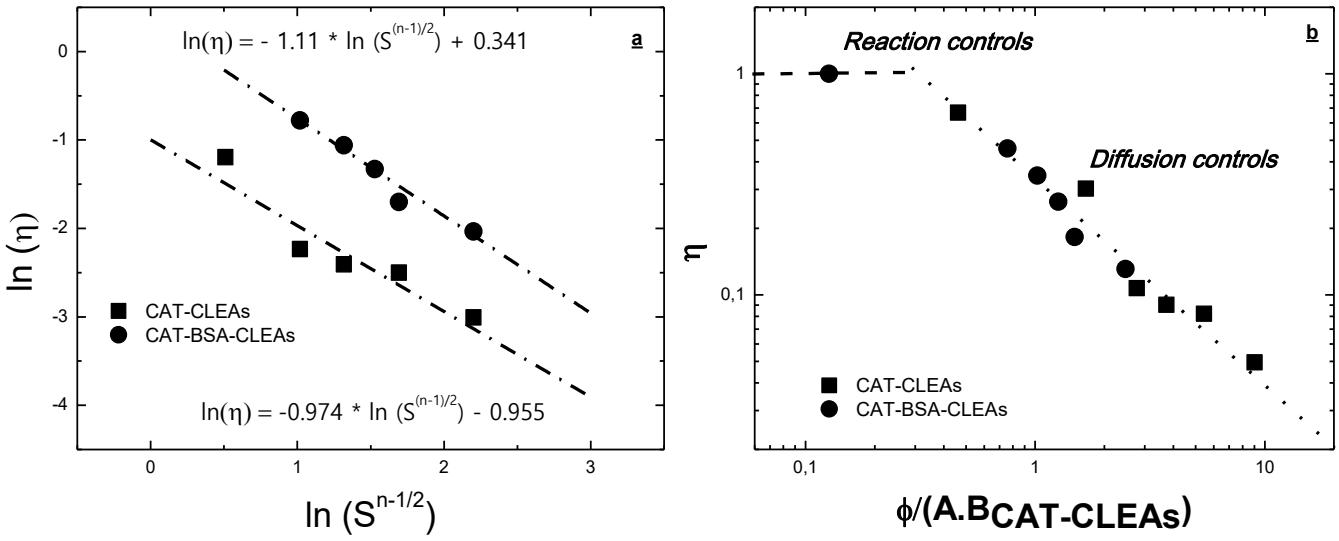
where, for CAT-CLEAs:

$$\frac{\phi_{CAT-CLEA}}{AB_{CAT-CLEA}} = \sqrt{S^{n-1}} \quad (3.17)$$

and for CAT-BSA-CLEAs:

$$\frac{\phi_{CAT-BSA-CLEA}}{AB_{CAT-CLEA}} = \frac{B_{CAT-BSA-CLEA}}{B_{CAT-CLEA}}\cdot\sqrt{S^{n-1}} \;\therefore\; \frac{\phi_{CAT-BSA-CLEA}}{AB_{CAT-CLEA}} = e^{-1,296}\cdot\sqrt{S^{n-1}} \quad (3.18)$$

Figure 3.6 – Effectiveness factors (η) for immobilized CAT (CAT-CLEAs and CAT-BSA-CLEAs) with *n* order intrinsic kinetic (*n* = 2.47) versus (a) $ln(\sqrt{S^{(n-1)}})$, and; (b) $\phi/AB_{CAT\text{-}CLEAs}$.



Plots of the logarithm of effectiveness factor (η) against the logarithm of

Thiele modulus divided by the constant $AB_{CAT\text{-}CLEA}$ ($\phi/AB_{CAT\text{-}CLEA}$) for CAT-BSA-CLEAs (Eq. 3.18) and CAT-CLEAs (Eq. 3.17) are depicted in Fig. 3.6b. The resulting graphic is similar to that expected if the difference among free CAT, CAT-CLEAs and CAT-BSA-CLEAs behavior were due to diffusive effects only. Therefore, the results not only represent kinetic evidence of the diffusive effect of adding BSA to CAT-CLEAs, but also indicate that the reduced recovery activity obtained for CAT-CLEAs is mainly due to effects of diffusive nature. From results we can estimate the effectiveness factor ratio $\eta_{CAT\text{-}BSA\text{-}CLEAs}$ / $\eta_{CAT\text{-}CLEAs}$ as 3.7 when diffusion controls the reaction rate.

3.3.5 pH and temperature effects on enzymatic activity

Temperature and pH effects on enzymatic activity were evaluated using the statistical design of experiments and the analysis of the response surface methodology. Three biocatalysts were chosen for this study: soluble CAT; CAT-CLEAs with 40 mg/mL of protein (CAT-CLEAs); and CAT-BSA-CLEAs with 20 mg/mL of enzymatic protein and 60 mg/mL of BSA (CAT-BSA-CLEAs). The effect of temperature was evaluated in the range of 44.1-15.2 ºC. The pH effect was evaluated in the range of 9.8-4.2.

Table 3.1 shows the results of the RCCD for soluble and immobilized CAT. The highest activity values were achieved in the central point assays. Eq. (3.6) was fitted to the experimental data of activities for soluble and immobilized CAT by multiple regressions using the Statistica 7.0 software, resulting in the Eq. 3.19-3.21, respectively.

$$A_{freeCAT} = 30.895 + 1.756 \cdot X_2 - 0.108 \cdot X_1 - 7.245 \cdot X_2^2 - 4.747 \cdot X_1^2 - 2.555 \cdot X_2 \cdot X_1 \quad (3.19)$$

$$A_{CAT-CLEAS} = 18.806 + 0.376 \cdot X_1 + 1.280 \cdot X_2 - 3.286 \cdot X_1^2 - 2.328 \cdot X_2^2 + 0.685 \cdot X_1 \cdot X_2 \quad (3.20)$$

$$A_{CAT-BSA-CLEAS} = 30.223 + 3.069 \cdot X_2 - 0.160 \cdot X_1 - 7.147 \cdot X_2^2 - 2.189 \cdot X_1^2 - 0.315 \cdot X_2 \cdot X_1 \quad (3.21)$$

At a significance level of 10% ($p<0.1$), the interaction between temperature and pH ($X_1.X_2$) was not statistically significant, as well as, the linear influence of temperature (X_1). Eliminating the non-significant parameters from the model (Eq. 3.6), the following equations were obtained:

$$A_{freeCAT} = 30.895 - 9.495 \cdot X_1^2 - 14.490 \cdot X_2^2 \quad (3.22)$$

$$A_{CAT-CLEAS} = 18.806 + 1.280 \cdot X_2 - 3.286 \cdot X_1^2 - 2.328 \cdot X_2^2 \quad (3.23)$$

$$A_{CAT-BSA-CLEAS} = 30.221 + 6.135 \cdot X_2 - 4.380 \cdot X_1^2 - 14.290 \cdot X_2^2 \quad (3.24)$$

The coefficients of determination (R-values) of 0.91, 0.95 and 1.00 indicate that the model was well fitted to the experimental data of activities for free and immobilized CAT. Variance analysis shows that the calculated F values were at least 4 times higher than the tabulated F values for a significance level of 10%. These results indicated a good agreement between the experimental data and those predicted by the models, allowing the construction of the response surfaces, which are illustrated in the Fig. 3.7.

From Eq. 3.22-3.24, the higher enzymatic activity was found to be close to the central point condition, at 30 ^{o}C for all derivatives and pH 7, 7.3 and pH 7.4 for soluble CAT, CAT-CLEAs and CAT-BSA-CLEAs, respectively. Therefore, immobilization procedure adopted did not alter the catalytic properties of the enzyme.

Figure 3.7 – Surface response showing the pH and temperature effect on (a) Free CAT, (b) CAT-CLEAs and (c) CAT-BSA-CLEAs activities.

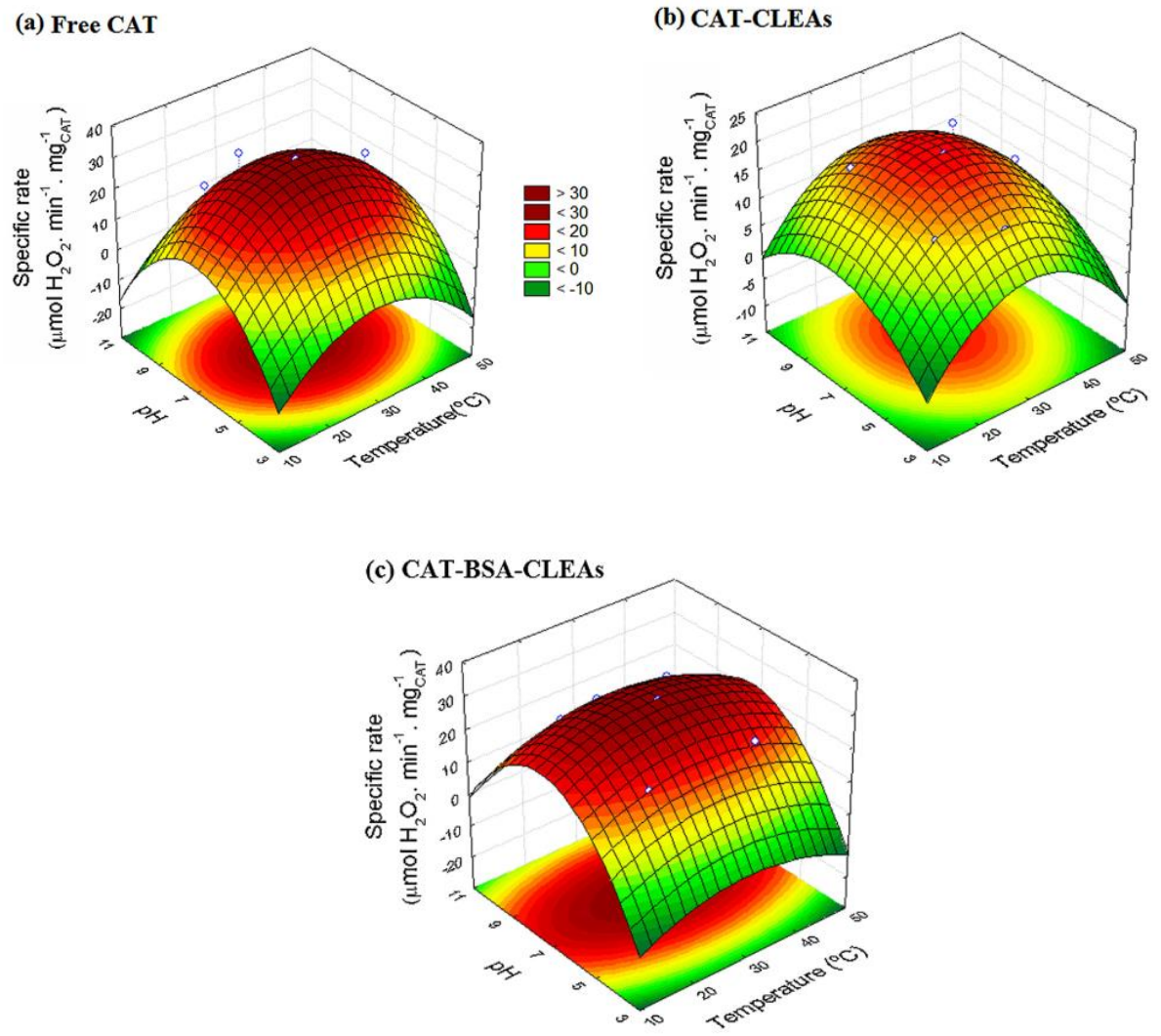


Table 3.1 – Experimental conditions and results of the statistical experimental design for free CAT, CAT-CLEAs, and CAT-BSA-CLEAs activities.

Exp.	X1 (T/°C)	X_2 (pH)	Free CAT (μmol H_2O_2.min^{-1}.mg^{-1}_{CAT})	CAT-CLEAs (μmol H_2O_2.min^{-1}.mg^{-1}_{CAT})	CAT-BSA-CLEAs (μmol H_2O_2.min^{-1}.mg^{-1}_{CAT})
1	-1 (20)	-1 (5)	17.65	12.96	19.68
2	-1 (20)	1 (9)	22.07	16.07	24.29
3	1 (40)	-1 (5)	19.05	10.77	20.66
4	1 (40)	1 (9)	13.24	16.61	24.01
5	-1.41 (15.2)	0 (7)	19.98	12.78	25.27
6	1.41 (44.1)	0 (7)	24.62	13.69	23.87
7	0 (30)	-1.41 (4.2)	11.85	9.68	8.79
8	0 (30)	1.41 (9.8)	22.76	12.96	20.52
9	0 (30)	0 (7)	30.66	18.62	30.57
10	0 (30)	0 (7)	30.43	18.26	30.43
11	0 (30)	0 (7)	32.06	18.99	31.41
12	0 (30)	0 (7)	30.43	19.35	28.48

3.4 Conclusions

Cross-linked catalase co-precipitate with bovine serum albumin (CAT-BSA-CLEAs) showed smaller clusters than without BSA (CAT-CLEA) suggesting that the use of BSA decreases the mass transfer resistance. Effectiveness factor (η) for CAT-BSA-CLEAs was 3.7 higher than for CAT-CLEAs. Kinetic evidence confirmed that the main difference among the activity of free catalase and its derivatives is of diffusive nature.

The best conditions to prepare CLEAs of catalase were found to be ammonium sulfate as precipitant agent, 50 mM glutaraldehyde as crosslinking agent, and mass ratio BSA/CAT of 3. Under these conditions, the CAT-BSA-CLEAs recovered almost 100% of the offered activity and showed high stability after 200 h at 40 ^{o}C and pH 7.0.

3.5 Acknowledgements

This work was supported by the National Council for Scientific and Technological Development (CNPq) [grant numbers 141647/2013-2 and 402850/2013-0), and the Federal Foundation CAPES. The authors thank the Embrapa Instrumentation (São Carlos, Brazil) for granting use of SEM equipment and Prof. Monica Lopes Aguiar from the Laboratory of Environmental Control (DEQ/UFSCar) by the microscopy images of CLEAs.

3.6 Reference

ADÁNYI, N. *et al.* Hydrogen Peroxide Producing and Decomposing Enzymes: their Use in Biosensors and other Applications. **Industrial Enzymes**, p. 441-459, 2007.

ALPTEKIN, O.; TUKEL, S. S.; YILDIRIM, D. Immobilization and characterization of bovine liver catalase on eggshell. **Journal of the Serbian Chemical Society**, v. 73, n. 6, p. 609-618, 2008.

ALPTEKIN, O. *et al.* Characterization and properties of catalase immobilized onto controlled pore glass and its application in batch and plug-flow type reactors. **Journal of Molecular Catalysis B-Enzymatic**, v. 58, n. 1-4, p. 124-131, 2009

ALPTEKIN, O. *et al.* Immobilization of catalase onto Eupergit C and its characterization. **Journal of Molecular Catalysis B-Enzymatic**, v. 64, n. 3-4, p. 177-183, 2010.

CABANA, H.; JONES, J. P.; AGATHOS, S. N. Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. **Journal of Biotechnology**, v. 132, n. 1, p. 23–31, 2007.

CAO, L. Q.; VAN RANTWIJK, F.; SHELDON, R. A. Cross-linked enzyme aggregates: A simple and effective method for the immobilization of penicillin acylase. **Organic Letters**, v. 2, n. 10, p. 1361-1364, 2000.

CUTLER, R. G. Oxidative Stress and Aging: Catalase Is a Longevity Determinant Enzyme. **Rejuvenation Research**, v. 8, n. 3, p. 138 - 140, 2005.

FERNANDEZ-LAFUENTE, R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. **Enzyme and Microbial Technology**, v. 45, n. 6–7, p. 405-418, 2009.

FITA, I.; ROSSMANN, M. G. The active center of catalase. **Journal of Molecular Biology**, v. 185, n. 1, p. 21–37, 1985

FROMENT, G. F.; BISCHOFF, K. B.; WILDE, J. **Chemical Reactor Analysis and Design**. Wiley, 2010.

GUISÁN, J. M. **Immobilization of Enzymes and Cells**, 2006.

HILL, A. V. The possible effects of the aggregation of the molecules of hæmoglobin on its dissociation curves. **Physiological society**, v. 40, 1910.

ITOH, T. *et al.* Catalase encapsulated in mesoporous silica and its performance. **Biochemical Engineering Journal**, v. 44, n. 2-3, p. 167-173, 2009.

KIM, M. H. *et al.* Immobilization of formate dehydrogenase from Candida boidinii through cross-linked enzyme aggregates. **Journal of Molecular Catalysis B-Enzymatic**, v. 97, n. 15, p. 209 - 214, 2013.

KISELEV, N. A., SHPITZBERG, C. L. , VAINSHTEIN, B. K. Crystallization of catalase in the form of tubes with monomolecular walls. **Journal of Molecular Biology**, v. 25, n. 3, p. 433–439, 1967.

KONDO, A. *et al.* Kinetic and circular-dichroism studies of enzymes adsorbed on ultrafine silica particles. **Applied Microbiology and Biotechnology**, v. 39, n. 6, p. 726-731,1993.

LARDINOIS, O. M.; MESTDAGH, M. M.; ROUXHET, P. G. Reversible inhibition and irreversible inactivation of catalase in presence of hydrogen peroxide. **Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology**, v. 1295, n. 2, p. 222–238, 1988.

LOWRY, O. H. *et al.* Protein measurement with the folin phenol reagent. **Journal of Biological Chemistry**, v. 193, n. 1, p. 265-275, 1951.

OZYILMAZ, G.; TUKEL, S. S.; ALPTEKIN, O. Kinetic properties and storage stability of catalase immobilized on to florisil. **Indian Journal of Biochemistry & Biophysics**, v. 44, n. 1, p. 38-43, 2007.

POLAINA, J.; MACCABE, A. P. **Industrial Enzymes - Structure, Function and Applications**. Springer, 2007. p.642

SCANDALIOS, J. G. **Oxidative stress and the molecular biology of antioxidant defenses**. Plainview, N.Y.: Cold Spring Harbor Laboratory Press, 1997. xi, 890 p.

SCHOEVAART, R. *et al.* Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). **Biotechnology and Bioengineering**, v. 87, n. 6, p. 754-762, 2004.

SHAH, S.; SHARMA, A.; GUPTA, M. N. Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. **Analytical Biochemistry**, v. 351, n. 2, p. 207–213, 2006.

SHELDON, R. A. Green solvents for sustainable organic synthesis: state of the art. **Green Chemistry**, v. 7, n. 5, p. 267 - 278, 2005.

SHELDON, R. A. Enzyme Immobilization: The Quest for Optimum Performance. **Advanced Synthesis & Catalysis**, v. 349, n. 8-9, p. 1289-1307, 2007.

SWITALA, J.; LOEWEN, P. Diversity of properties among catalases. Archives of **Biochemistry and Biophysics**, v. 401, n. 2, p. 145-154, 2002.

SÖKMEN, N. *et al.* Chelating Agent Effect on the Release of Gentamicin from PEG-DA Hydrogels. **Hacettepe journal of biology and chemistry**. 36: 347-352 p. 2008.

TALEKAR, S. *et al.* Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). **The Royal Society of Chemistry**, v. 3, p. 12485 - 12511, 2013.

TALEKAR, S. *et al.* Porous cross linked enzyme aggregates (p-CLEAs) of *Saccharomyces cerevisiae invertase*. **The Royal Society of Chemistry** v. 2, p. 1575 - 1579, 2012a.

TALEKAR, S. *et al.* Preparation and characterization of cross linked enzyme aggregates (CLEAs) of Bacillus amyloliquefaciens alpha amylase. **Journal of Biochemical Technology**, v. 4, n. 4, p. 349 - 353, 2012b.

TOUAHAR, I. E. *et al.* Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals. **Applied Microbiology and Biotechnology** , v. 481, p. 90–99, 2014.

TUKEL, S. S. *et al.* Preparation of crosslinked enzyme aggregates (CLEA) of catalase and its characterization. **Journal of Molecular Catalysis B-Enzymatic**, v. 97, p. 252-257, 2013.

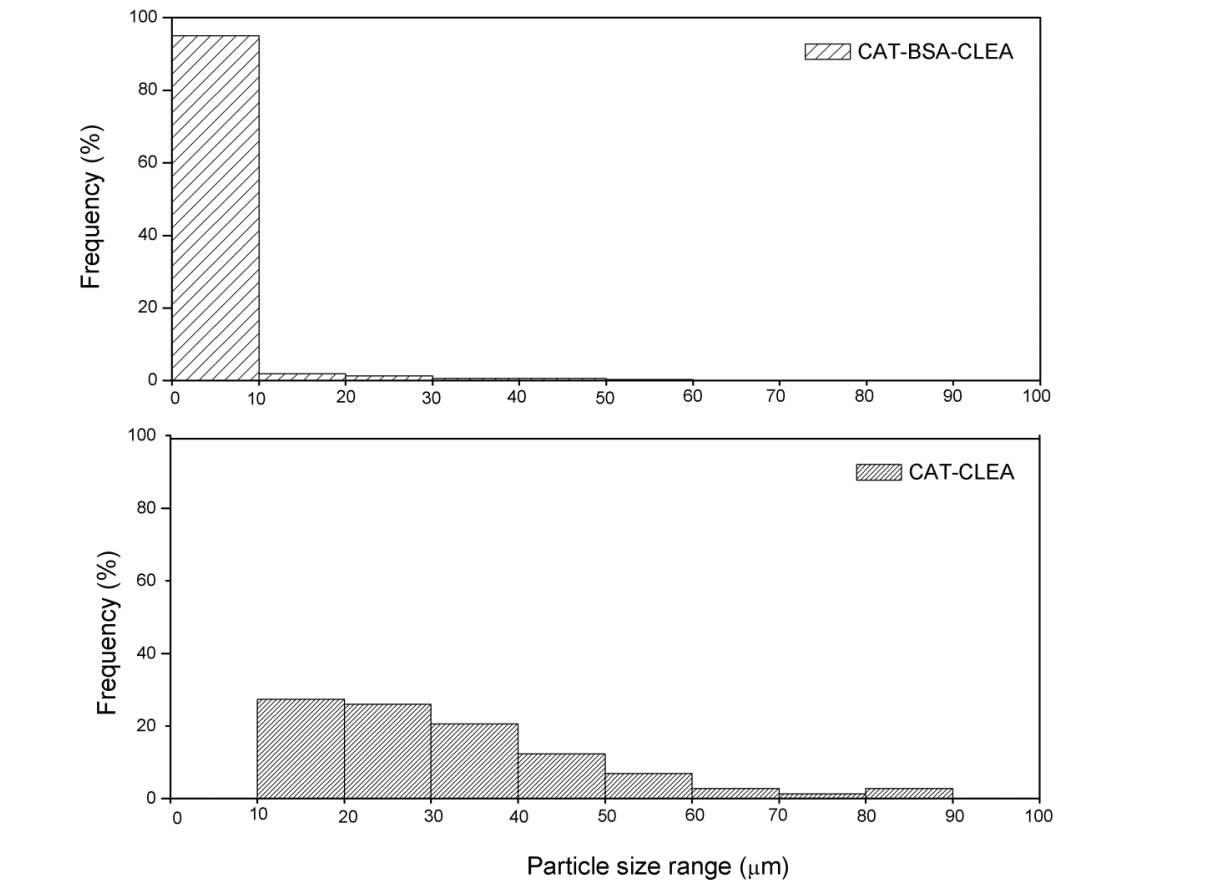
WILSON, L. *et al.* Cross-linked aggregates of multimeric enzymes: a simple and efficient methodology to stabilize their quaternary structure. **Biomacromolecules**, v. 5, n. 3, p. 814-7, 2004.

WILSON, L. *et al*. Co-Aggregation of Penicillin G Acylase and Polyionic Polymers: An Easy Methodology To Prepare Enzyme Biocatalysts Stable in Organic Media. **Biomacromolecules**, v. 5, n. 3, p. 852 - 857, 2004.

WU, Y. C. *et al*. Treatment of leachate from a solid waste landfill site using a two-stage anaerobic filter. **Biotechnology Bioengineering**, v. 31, n. 3, p. 257-66, 1988.

ZHEN, Q. *et al.* Preparation of β-mannanase CLEAs using macromolecular cross-linkers. **Catalysis Science & Technology**, v. 3, p. 1937 - 1941, 2013.

3.7 Supplementary data



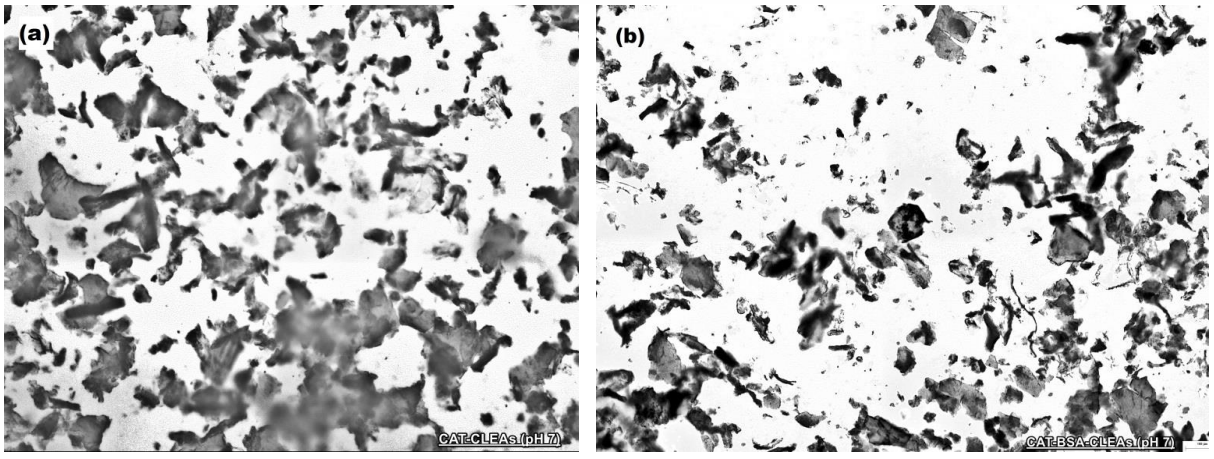


Fig. 3.1S – Particle size distribution for CAT-BSA-CLEAs and CAT-CLEAs prepared at pH 7.0. The particle diameter was obtained from microscopy images of CAT-CLEA (a) and CAT-BSA-CLEA (b) using the ImageJ software, where the frequency count was performed using a step size of 10 μm.

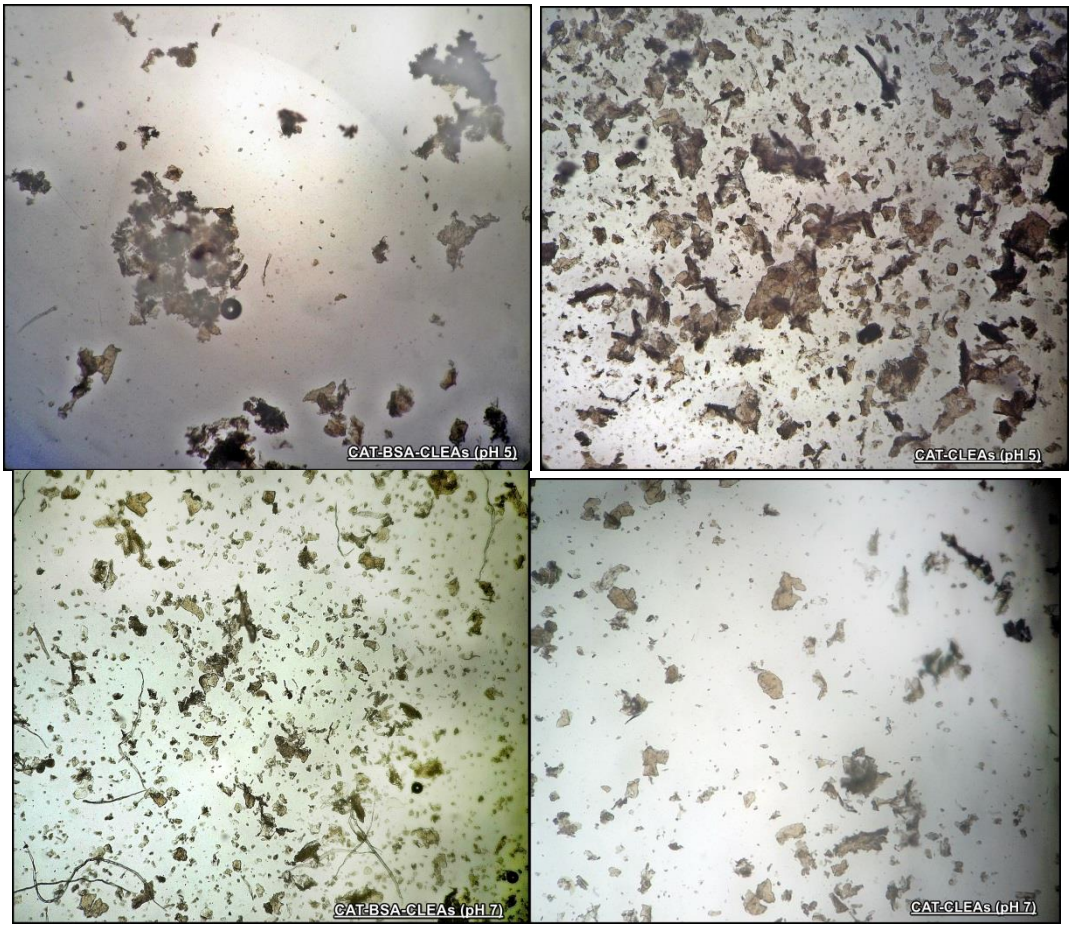


Fig. 3.2S - Microscopy images of CAT-BSA-CLEAs and CAT-CLEAs prepared and re-suspended at pH 5.0 (images of the top) and pH 7.0 (images at the bottom).

4. Enzymatic production of inverted sugar using SOY-CLEAs of invertase

Preface

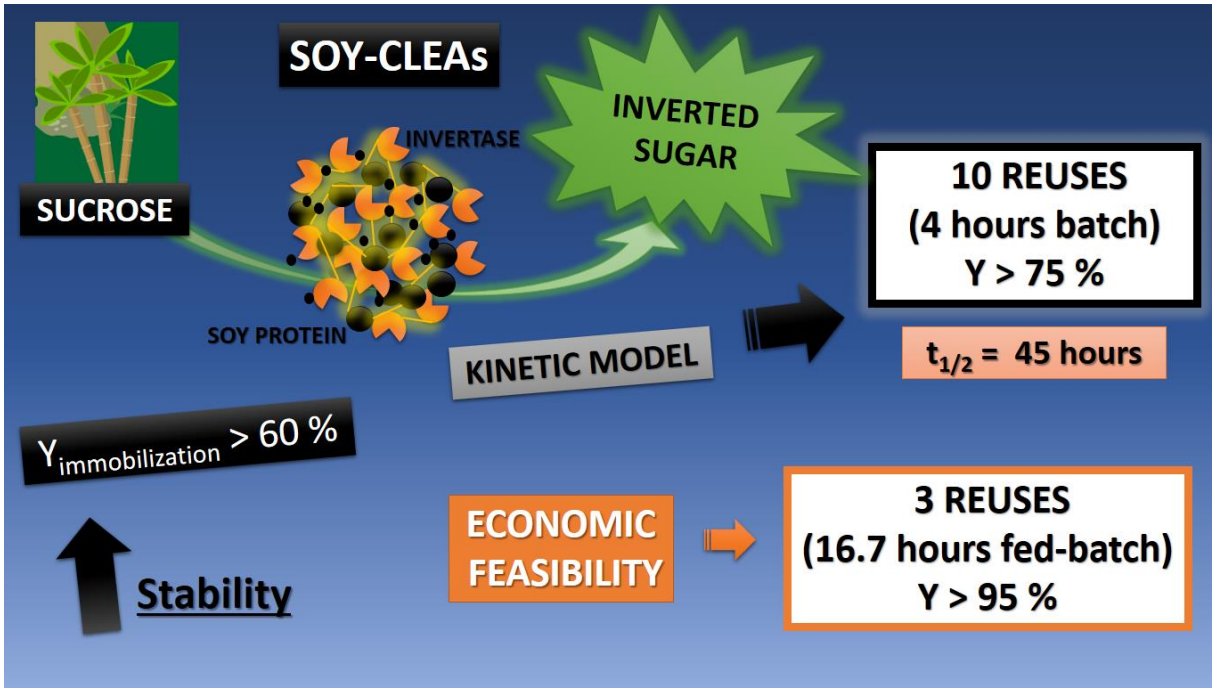
The application of enzymes in industrial processes is still limited by economic factors, a consequence of the unfeasibility of enzyme reuse in soluble form and its low stability in the presence of solvents and high temperatures. In order to overcome these problems, the enzymes have been immobilized using various techniques, such as entrapment into porous matrices, linkage to surfaces and aggregation/cross-linking (CLEAs). The CLEAs technique stand out for being a simple methodology, not requiring enzyme purity to be immobilized, and it can also be applied to enzyme mixtures.

In this study we evaluated the immobilization of invertase from S. cerevisiae by CLEAs technique, using soy protein as a feeder protein. Although several studies have reported the immobilization of this enzyme by different techniques, studies of economic feasibility of processes with immobilized enzymes are rarely reported. Therefore, this study has evaluated the economic feasibility process of sugar inversion catalyzed by immobilized invertase. The invertase immobilization by CLEAs technique showed high overall immobilization yields, with high stability of the immobilized enzyme in the sucrose inversion process. This study showed that the inversion of sucrose catalyzed CLEAs of invertase can be an economically feasible process. For this assessment, empirical models were fitted to the experimental data of enzymatic activity in the hydrolysis profiles and biocatalyst reuse in order to simulate different process conditions and thus propose a window of

economic viability. The results suggested that there is a window of economic viability using 100 g/l of sucrose only in feed batches when conversion reaches 95%.

This study had the collaboration of a scientific initiation student from DEQ-UFSCar, Maisa Bontorin Beltrame (Process FAPESP No 2014/21510-0). In the scientific initiation project, the conditions for the invertase immobilization were outlined using the aggregation/crosslinking technique, in addition to kinetic studies and inverted sugar production. The results of the scientific initiation project were used to improve the invertase immobilization protocol, as well as to achieve preliminary studies on economic feasibility and process design.

Graphical abstract



Highlights files

- Invertase was immobilized as CLEA with soy protein as feeder protein.
- CLEAs of invertase and soy protein showed to be stable after leaching assay.
- CLEAs of invertase and soy protein could be reused by ten 4h-cycles.
- Economic feasibility was estimated for batch and fed-batch sucrose inversion.
- A feasible window was obtained for three fed-batches using 100 $g.L^{-1}$ sucrose
- 3-Fed batch reactor and 100 $g.L^{-1}$ sucrose was the feasibility window.

Abstract

Enzyme immobilization is a widely known strategy used to increase the operational stability of enzymes, as well as to facility reuse and downstream process. Invertase was immobilized by the cross-linked enzyme aggregate (CLEA) methodology using soy protein as feeder molecules as an alternative to the commonly used expensive bovine serum albumin. The immobilized invertase retained around 30 % of the initial activity after the enzyme leaching assay. Maximum activity for the immobilized invertase was at 50.8 ^{o}C (5.8 degrees higher than the soluble invertase) and at pH 5.0 (similar to the soluble invertase). Michaelis-Menten constant (Km) was similar for soluble and immobilized invertase (145 and 130 mM, respectively). However, the catalytic efficiency (k_{cat}/K_m) of invertase did reduce approximately tenfold after immobilization, probably due to intraparticle diffusional delays. The immobilized invertase could be recycled tenfold in 4 h-batches of hydrolysis of sucrose at 40 ^{o}C and pH 6.0, maintaining the reaction conversion above 75%. The hydrolysis of sucrose catalyzed by immobilized invertase showed to be economically feasible in an operational window built based on economic metrics for a fed-batch process, with three intermittent feeds of sucrose to restore the substrate concentration at 100 g.L^{-1} when the conversion reaches 95 %. This work represents an advance in the field, because using a carrier-free and recyclable biocatalyst the specific productivity (gram of products per gram of biocatalyst per hour) of inverted sugar syrup was as high as those previously reported for invertase immobilized on solid carriers, which may dilute its volumetric activity and increase the cost of the biocatalyst.

Keywords: Invertase, soy protein, CLEAs, sucrose inversion, economic feasibily window.

4.1 Introduction

Inverted sugar is an equimolar mixture of fructose and glucose and it is of great interest in pharmaceutical and food industries. This sugar is a natural sweetener widely used for several purposes, such as in soft drink industries (HANOVER & WHITE, 1993; AIDER *et al*., 2007; ASHURST *et al*., 2017).

Inverted sugar can be produced by hydrolysis of sucrose. Industrially, the hydrolysis of sucrose is chemically carried out using HCl as catalyst at 75 ^{o}C (HEIDT *et al*., 1952). However, the product obtained contains several by-products (from glucose and fructose cyclization) (KOWALSKI *et al*., 2013), which prevents its use as raw material by pharmaceutical and food industries. Therefore, the inverted sugar syrup produced requires some purification steps, increasing the process costs. On the other hand, invertase (beta-fructofuranosidase, EC 3.2.1.26) can be used for hydrolyzing sucrose in equimolar amounts of glucose and fructose, without the formation of by-products. In order to make enzymatic processes industrially attractive, it is necessary to stabilize the biocatalyst in an active state, allowing it to be reused in the process and/or used for prolonged time in a continuous process (GUISÁN, 2006). Although there are several alternatives for the stabilization of enzymes, the most widely used technique is the immobilization of these molecules, either by reversible or irreversible bonding on solid supports (SANJAY & SUGUMAN, 2005), or encapsulation (ARRUDA & VITOLO, 1999) or crosslinking promoted by bifunctional reagents (TISCHER & KASCHE, 1999; ILLANES, 2008).

Cross-linked enzyme aggregates (CLEAs) have recently emerged as new, easy and versatile carrier-free immobilization. CLEAs consist of an immobilization method in which proteins are aggregated and precipitated by salts, organic solvents, and others, and after crosslinked by a bifunctional agent

(DOSCHER & RICHARDS, 1963; QUIOCHO & RICHARDS, 1964; CAO *et al*., 2000; SCHOEVAART *et al*., 2004; SHELDON, 2007). This new methodology allows preparing biocatalysts with lower costs and higher volumetric activity due to the absence of a solid matrix (SHELDON, 2011). Talekar *et al.* (2012) first proposed the immobilization of invertase from *S. cerevisiae* by aggregation in the presence of starch as a porogenic agent. The biocatalysts showed no diffusion problems and 100% recovered activity. However, this technique requires a second enzyme (alpha amylase) to break the starch into glucose and maltose, which increases the cost of immobilization.

Some works report the preparation of CLEAs with bovine serum albumin (BSA), hen egg or egg protein as a feeder protein (SHAH *et al*., 2006; CRUZ *et al*., 2012; TALEKAR *et al*., 2013; MAFRA *et al*., 2016), in order to prevent enzyme inactivation by excessive and distorted crosslinks, as well as enzyme leaching by co-aggregation with enzymes containing low lysine content (SHAH *et al*., 2006; TALEKAR *et al*., 2013; MAHMOD *et al*., 2016). However, the use of expensive inert proteins or polymers in the CLEA formulation can greatly increases the cost of immobilization.

This work aimed to immobilize invertase from *S. cerevisiae* (INV) by the CLEAs technique using soy protein (SOY) as inert spacer molecules. The use of SOY as feeder protein, instead of BSA, may reduce the cost of the immobilized biocatalyst, because BSA is approximately 180 times more expensive than SOY. The cross linker and soy protein concentrations were optimized to produce an active and stable biocatalyst. The best immobilized biocatalyst (hereafter termed as INV-SOY-CLEA) and the soluble invertase were characterized by the kinetic parameters and by both pH and temperature of maximum activities. The INV-SOY-CLEA was

evaluated in batch hydrolyses of sucrose and in operational stability (10 times). An operational window was built based on the economic feasibility of the enzymatic production of inverted syrup and validated in fed-batch hydrolyses with biocatalyst reuse.

4.2 Material and methods

4.2.1 Material

Soy protein and invertase from *Saccharomyces cerevisiae* (INV) were donated by Doremus ingredientes (Guarulhos, SP, Brazil) and LNF Latino Americana (Bento Gonçalves, RS, Brazil), respectively. Glutaraldehyde aqueous solution (25 % w/v) from Synth (Diadema, SP, Brazil), dimethoxyethane (DME) from Fluka (St. Louis, MO, USA), tert-butyl alcohol (TBA) from Vetec (Duque de Caxias, RJ, Brazil) and ammonium sulfate salt (AS) from Vetec (Duque de Caxias, RJ, Brazil) were used in CLEAs syntheses and used to evaluate their properties. Other reagents were of analytical grade.

4.2.2 Methods

4.2.2.1 Preparation of INV-CLEAs and INV-SOY-CLEAs

The preparation of CLEAs followed the method described by Mafra *et al.* (2016). An enzymatic solution containing protein concentration of 10 and 30 $mg.mL^{-1}$ was prepared dissolving INV powder in sodium phosphate buffer (100 mM, pH 7.0). Protein aggregation was induced by mixing 1 mL of the enzymatic solution and 1 mL of precipitant reagent (TBA, DME, or saturated AS solution). After 1 min of stirring at 3000 rpm in a vortex mixer (QL 901, Biomixer), glutaraldehyde was slowly

added to the final concentration of 25, 50 and 75 mM. When CLEAs of INV were prepared using a feeder protein (INV-SOY-CLEAs), soy protein was added to the enzyme solution at final concentrations of 10, 20, 30, 40, 50, and 60 $mg.mL^{-1}$.

After 3 h of crosslinking at 4 ^{o}C and 200 rpm in an incubator shaker (SL 221, Solab), the suspensions containing INV-CLEAs and INV-SOY-CLEAs were centrifuged at 10,000 × g for 10 min at 4 ºC (5810R, Eppendorf). CLEAs were recovered as pellets and washed with 100 mM sodium phosphate buffer (pH 7.0). After preparation, the enzyme was stored in the same buffer (2 mL) at 4 ºC for further use. The overall immobilization yield (*Y*) for INV-CLEAs and INV-SOY-CLEAs was calculated as follows:

$$Y(\%) = \frac{A_e(U)}{A_i(U)} \times 100 \quad (4.1)$$

Where A_e is the total activity of CLEAs; A_i is the total INV activity used for CLEAs production.

4.2.2.2 Biocatalyst evaluation after leaching

Yield of immobilization of all biocatalysts was also evaluated after a first leaching assay. The enzyme leaching assay was carried out at 30 ^{o}C (non-denaturing temperature) and pH 7.0 (100 mM sodium phosphate buffer), keeping the suspensions of CLEAs for 4 h at 250 rpm in an incubator shaker (SL 221, Solab). The suspensions containing immobilized INV were then centrifuged at 10,000 × g for 10 min at 4 ºC (5810R, Eppendorf). CLEAs were recovered as pellets and washed with 100 mM sodium phosphate buffer (pH 7.0).

The yield of activity after the assays of leaching (Y_L) was calculated as follows:

$$Y_L(\%) = \frac{A_a(U)}{A_i(U)} \times 100 \quad (4.2)$$

Where A_a is the total activity of the CLEAs of invertase measured after the leaching assay; A_i is the total INV activity used for CLEAs production.

4.2.2.3 Thermostability study

Thermostability of immobilized INV was evaluated at 40 ^{o}C and pH 6.0 (100 mM sodium phosphate buffer) for 4 h at 250 rpm, in an incubator shaker (SL 221, Solab). The retained activity (RA) was calculated as follows:

$$RA(\%) = \frac{A_T(U)}{A_i(U)} \times 100 \quad (4.3)$$

Where A_T is the total activity of the CLEAs of invertase measured after the stability assay; A_i is the total INV activity used for CLEAs production.

4.2.2.4 pH- and temperature-activity profiles

A factorial design followed by response surface analysis was used to evaluate the effect of temperature (from 24-66 ^{o}C; 33.9-76.2 ^{o}C) and pH (from 3.6-6.4 ; 3.6-6.4) on the soluble INV and INV-SOY-CLEAs activities (response variable). Table 4.1 shows the Rotatable Central Composite Design (RCCD) used. The twelve runs were carried out randomly. Statistica software (Statsoft, version 7.0) was used to analyze the experimental data, to generate the ANOVA (analysis of variance) data, and to build response surfaces. A second-order polynomial model (Eq. 4.4) was used to fit the data:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^{\,2} + \beta_{22} X_2^{\,2} + \beta_{12} X_1 X_2 \quad (4.4)$$

Where *Y* is the predicted response variable (activity); X_1 and X_2 are the coded independent variables representing the temperature and the pH, respectively; and β are empirical parameters.

4.2.2.5 Kinetic analysis

Kinetic parameters of soluble INV and INV-SOY-CLEA were estimated by measuring initial reaction rates using enzyme concentration of 36,700 $U.L^{-1}$ and sucrose concentrations in the range of 50 - 1,600 mM at 40°C and pH 6.0 (100 mM sodium phosphate buffer). The enzymatic activities were measured as described in section 4.2.2.9. In order to describe the effect of the substrate on INV activities, several models were used. Michaelis-Menten model (Eq. 4.5) was used to compare derivatives in the range without inhibition. The classical substrate inhibition model (Eq. 4.6) was used for soluble enzyme in the range with substrate inhibition and the Wu model (Eq. 4.7) was used for CLEAs of INV in the range with inhibition.

$$r_s = \frac{k_{cat} \cdot S}{K_m + S} \tag{4.5}$$

$$r_s = \frac{k_{cat} \cdot S}{K_m + S + \left(\frac{S^2}{K_i}\right)} \tag{4.6}$$

$$r_s = \frac{k_{cat} \cdot S}{\frac{K_m}{S} + 1 + \left(\frac{S}{K_i}\right)^w} \tag{4.7}$$

where, r_s is the specific reaction rate ($M.s^{-1}.mg_{INV}^{-1}$), k_{cat} is the turnover number (s^{-1}), K_m is the Michaelis-Menten constant (M); K_i is the inhibition constant (M); *S* is the substrate concentration (M) and *w* is an inactivation degree factor.

k_{cat} ,K_m, K_i and *w* values of soluble and immobilized INV were calculated from nonlinear regression fitting the models (Eq. 4.5-4.7) to the experimental data of *initial reaction rates* versus *sucrose concentrations*.

4.2.2.6 Inverted sugar production

The inverted sugar syrup was produced at 40 °C and pH 6.0 (100 mM sodium phosphate buffer), using 50 $g.L^{-1}$ of sucrose in a 0.5 L-batch reactor (thermostat circulating water, SL 152-Solab) containing 0.4 L-sucrose solution mechanically stirred at 300 rpm, in a mechanical stirrer (AM 20, Gehaka) equipped with a paddle impeller. The biocatalyst-to-substrate (w/w) ratio was optimized in prior studies (MAFRA *et al*., 2015). Samples of 0.5 mL were removed, diluted to a volume ratio of 1/1 in 0.2 M sodium hydroxide solution to stop the reaction, and quantified for the total reducing sugar (TRS) by the 3,5-dinitrosalicylic acid method, as well as for sucrose, fructose and glucose by the chromatographic methods.

4.2.2.7 Operational stability

The operational stability of INV-SOY-CLEA biocatalyst was evaluated in a 0.5 L-batch reactor thermostatically controlled by water circulation (SL 152, Solab), in which 2.2 mL of a CLEA suspension containing 73 mg of INV-SOY-CLEA (dry basis) was fed in the reactor containing 0.4 L of 50 $g.L^{-1}$ sucrose (in 25 mM phosphate buffer) at 40 °C and pH 6.0. After 4 h of reaction under 300 rpm mechanical stirring (AM 20, Gehaka), the bioreactor's stirrer was turned off and the biocatalyst was left to decant for about 2 h. Finally, the supernatant (invert sugar syrup) was removed with a peristaltic pump (MCP 404B V800, Ismatec) and the reactor was re-fed with sucrose solution at the same initial conditions. This process was repeated 10 times. Samples of the reaction medium (0.5 mL) were removed, diluted to a volume ratio of 1/1 in 0.2 M sodium hydroxide solution to stop the reaction, and quantified for sucrose, glucose and fructose.

4.2.2.8 Operational feasibility of sucrose hydrolysis

The methodology used to evaluate the operational feasibility of the sucrose hydrolysis was based on the metrics proposed by Tufvesson *et al.* (2013) and Lima-Ramos *et al.* (2014). According to the authors, three process metrics can be used to evaluate the process feasibility for each reuse: biocatalyst yield ($Y_{biocatalyst}$; $g_{product}.g_{biocatalyst}^{-1}$), product concentration (*[P]*; $g_{product}.L_{reactor}^{-1}$) and space-time yield (*STY*; $g_{product}.(L_{reactor}.h)^{-1}$). Depending on the sector of the industrial process, approximate threshold values are suggested for those metrics (TUFVESSON *et al*., 2013). Using the market price of inverted sugar syrup (50 wt.%) of 1.4 €/kg product (ALICEWEB, 2016), it is possible to classify this process as bulk chemical sector. In this case, the threshold values are (TUFVESSON *et al*., 2013): $Y_{biocatalyst}$ between 10^3 and 10^5 $g_{product}.g_{biocatalyst}^{-1}$,*[P]* >300 $g_{product}.L_{reactor}^{-1}$ and *STY* >10 $g_{product}.(L_{reactor}.h)^{-1}$. In order to determine the process feasibility window, 95% was chosen for the reactor yield ($Y_{reaction}$) (LIMA-RAMOS *et al*., 2014). Based on the substrate conversion, the reaction time was evaluated using the kinetic parameters of Michaelis-Menten estimated in section 4.2.2.5 (Eq. 4.8). First order enzyme inactivation model (Eq. 4.9) was also fitted to the reuse data determined according to section 4.2.2.7 and used to evaluate the feasibility window.

$$\frac{dS}{dt} = r = \frac{V_{max}.S}{K_m+S} = \frac{k_{cat}.C_{et}.S}{K_m+S} \quad (4.8)$$

$$\frac{dC_{et}}{dt} = -k_d \cdot t \quad (4.9)$$

where, r_s is the reaction rate ($M.min^{-1}$), k_{cat} is the turnover number (min^{-1}), K_m is the Michaelis-Menten constant (M), C_s is the substrate concentration (M), C_{et} is the enzyme concentration (M) and k_d is the inactivation constant (min^{-1}) and *t* is the time (min).

4.2.2.9 Validation of operational feasibility of sucrose hydrolysis

The first fed-batch of inverted sugar syrup was produced at 40 °C and pH 6.0 (100 mM sodium phosphate buffer), using 100 $g.L^{-1}$ of sucrose in a 0.5 L-batch reactor (thermostatic circulating water, SL 152-Solab) containing 0.4 L-sucrose solution mechanically stirred at 300 rpm, in a mechanical stirrer (AM 20, Gehaka) equipped with paddle impeller. After 4.7 and 9.9 hours, the amount of 40 g of powder sucrose was added into the reactor.

The ratio of biocatalyst-to-substrate (w/w) was optimized in prior studies (MAFRA *et al*., 2015). Samples of 0.5 mL were removed, diluted to a volume ratio of 1/1 in 0.2 M sodium hydroxide solution to stop the reaction, and quantified for total reducing sugar (TRS) by the 3,5-dinitrosalicylic acid method, as well as for sucrose, fructose and glucose by chromatographic methods.

4.2.2.9 INV activity assay

Activities of soluble and immobilized INV were determined at 30 ^{o}C using 200 mM sucrose solution prepared in 0.025 M acetate buffer, pH 4.8. The reaction was followed by measuring total reducing sugars (TRS) by the 3,5-dinitrosalicylic acid method (MILLER, 1959). One invertase unit was defined as the amount of enzyme that catalyzes the formation of 1 μmol of TRS per minute.

4.2.2.10 Protein concentration

Lowry's method (LOWRY *et al*., 1951) was used to measure protein concentration in the enzyme preparations. The protein content was spectrophotometrically quantified at 750 nm using bovine serum albumin (BSA) as protein standard.

4.2.2.11 Chromatographic methods

The concentrations of sucrose, glucose and fructose were determined using a Waters Model 410 HPLC equipped with a differential refractometer detector. The compounds were separated in a Sugar-Pak column at 80 ^{o}C, using Milli-Q water as the eluent at a flow rate of 1 mL.min^{-1}.

4.3 Results and discussion

4.3.1 Screening of parameters for immobilizing invertase by CLEA technique

A set of experiments were carried out to evaluate the main parameters of immobilizing invertase by the CLEA technique, *i.e*., invertase concentration (10 and 30 mg.mL^{-1} of enzyme solution), precipitant agent (*tert*-butyl alcohol - TBA, dimethoxyethane - DME, and saturated ammonium sulfate solution - AS), crosslinker concentration (25 and 75 mM), and feeder protein concentration (0, 20, 40, and 60 mg.mL^{-1} of enzyme solution).

The results showed immobilization yields higher than 11 % (entries 6 and 10 of Table 4.1S) using TBA and DME as precipitant agent. However, when saturated ammonium sulfate solution was used, all immobilization yields were less than 35 %.

In this set of experiments it was verified that a feeder protein (soy protein) exhibited an important role in the CLEA activity. All biocatalysts prepared in the absence of soy protein showed immobilization yields less than 21 %. When soy protein was used, it was observed that higher soy protein-to-invertase ratio (SOY/INV mass ratio), lower crosslinker-to-protein ratio (GLU/mg total) were required to prepare

active CLEAs. It is well-documented that the use of a lysine-rich feeder protein may assist the crosslinking, preventing loss of activity by leaching and may reduce mass diffusional delays in the inner enzyme layers within the CLEA particles (SHAH *et al*., 2006; DONG *et al*., 2010; AHUMADA *et al*., 2015; MAFRA *et al*., 2016). However, we should bear in mind that an excessive amount of feeder protein will excessively dilute the volumetric activity of the biocatalyst.

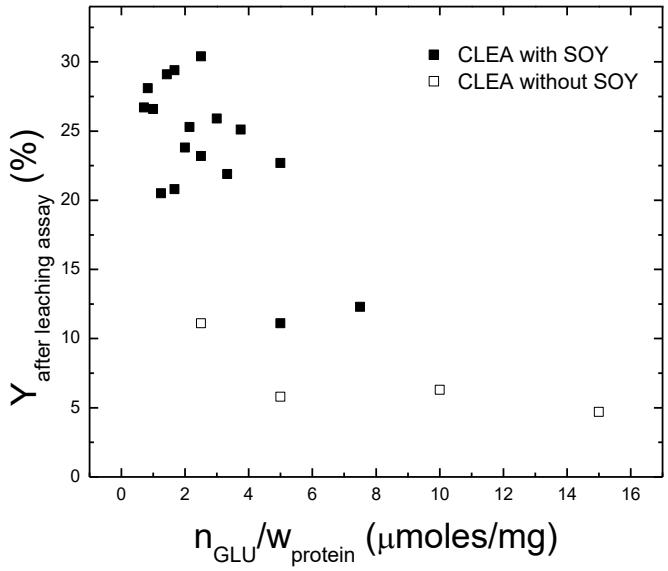
Therefore, a new set of experiments were performed to evaluate the influence of the crosslinker and feeder protein concentration in the CLEA activity and in its stability against enzyme leaching. In these experiments *tert*-butyl alcohol was chosen as precipitant agent and invertase concentration of 10 $mg.mL^{-1}$ of solution because the highest immobilization yields were achieved in these conditions.

4.3.2 Effect of crosslinker and feeder protein concentration

As stated previously, the best immobilization yields for crosslinked invertase in the absence or presence of soy protein were around 60 % (Table 4.2S, entries 1 and 10, respectively). However, all biocatalysts showed high activity decrease after the leaching assay. It was also observed that the higher the mass ratio of soy protein-to-invertase, the lower the ratio of glutaraldehyde-to-total protein to yield more active CLEAs after the leaching assay. On the other hand, even using higher amount of glutaraldehyde (5 to 15 μmol/mg of protein), the enzyme was poorly cross-linked in the absence of soy protein (immobilization yields after leaching assay less than 6 %). It is likely that the better activity retentions after the leaching assay for invertase cross-linked with soy protein (a maximum of 30 % of activity as shown in Figure 4.1 and Table 4.2S) are attributed to its high content of superficial lysine residues that helped the crosslinking.

The biocatalysts prepared according to experiments 17 (μmol GLU.mg^{-1} of 1.67 and SOY/INV mass ratio of 5, Table 4.2S) and 20 (μmol GLU.mg^{-1} of 1.43 and SOY/INV mass ratio of 6, Table 4.2S) were also evaluated for their stability in the process conditions for a multi-enzyme system aimed at the production of gluconic acid from sucrose (40 ^{o}C, pH 6.0, 100 mM sodium phosphate buffer, and 4 h reaction) (MAFRA *et al*., 2015). Both biocatalysts showed high stability, retaining 29.2 % (1,898 U.mL^{-1} or 63 U.mg^{-1} of total protein) and 27.5 % (1,787 U.mL^{-1} or 51 U.mg^{-1} of total protein) of activity, respectively. The biocatalyst prepared according to experiment 17 was chosen for further studies because its SOY/INV mass ratio is lower and consequently its volumetric activity is higher.

Figure 4.1 – Yield of immobilization after the leaching assay ($Y_{after\ leaching\ assay}$), at 30 ^{o}C pH 7.0 for 4 h shaking at 250rpm, for derivatives of invertase (INV) prepared by the CLEA technique as function of the ratio glutaraldehyde concentration (GLU)-to-amount of protein (INV and SOY protein).



The Feret's statistical geometric diameters were calculated using microscopy images of INV-SOY-CLEAs and ImageJ software. Figure 4.1S shows the histograms of particle size distribution using step size of 10 μm (Supplementary

data). The particle diameter for INV-SOY-CLEA ranged from 0 to 20 μm, with some clusters of ~ 40 μm. INV-SOY-CLEAs also were characterized as surface morphology by scanning electron microscopy (SEM), as shown in Figure 4.2S (Supplementary data). The SEM of invertase CLEAs with soy as a feeder protein showed a spherical structure.

4.3.3 Effects of pH and temperature on the enzymatic activity

The effects of pH and temperature on the invertase (soluble and CLEAs) activity were evaluated using statistical experimental design and surface analysis. The temperature effect was evaluated in the range of 66–24 ºC and 76.2–33.9 ºC for soluble and immobilized invertase, respectively. The pH effect was evaluated in the range of 6.4–3.6 for both biocatalysts (Table 4.1). Equation (4.2) was fitted to the experimental data of activities for soluble and immobilized invertase by multiple regressions using the Statistica 7.0 software, resulting in the mathematical model coefficients and the statistical parameter p-value shown in Table 4.2. Eliminating the non-significant ($p>0.1$) parameters from the model, the coefficients were obtained, as shown in Table 4.3.

The highest experimental values of enzymatic activity for soluble and immobilized invertase were found to be close to the central point condition, i.e., pH 5.0 and 45 ^{o}C for soluble invertase, and pH 5.0 and 50.8 ^{o}C for CLEAs of invertase. The pH and temperature values of maximum activity for invertases are commonly reported in the ranges between 4 and 7, and 40 and 55 ^{o}C, respectively (ARRUDA & VITOLO, 1999; HSIEH *et al*., 2000; TALEKAR, 2012).

The coefficients of determination (R-values) shown in Table 4.3 indicate that the model was well fitted to the experimental data of activities for soluble and immobilized invertase. Variance analysis shows that the calculated F values were at

least 6 times higher than the tabulated F values for a significance level of 10 %. These results indicated a good agreement between the experimental data and those predicted by the models, allowing the construction of the response surfaces, which are shown in Figure 4.2.

Table 4.1 – Experimental conditions and results of the statistical experimental design for the influence of pH and temperature on the activity of soluble and immobilized invertase cross-linked with soy protein (INV-SOY-CLEAs).

Experiment	Soluble invertase			INV-SOY-CLEAs		
	X_1 (T/^{o}C)	X_2 (pH)	Relative activity (%)	X_1 (T/^{o}C)	X_2 (pH)	Relative activity (%)
1	-1 (30)	-1 (4)	12	-1 (40)	-1 (4)	49
2	-1 (30)	1 (6)	28	-1 (40)	1 (6)	46
3	1 (60)	-1 (4)	47	1 (70)	-1 (4)	16
4	1 (60)	1 (6)	38	1 (70)	1 (6)	25
5	-1.41 (24)	0 (5)	27	-1.41 (33.9)	0 (5)	40
6	1.41 (66)	0 (5)	29	1.41 (76.2)	0 (5)	3
7	0 (45)	-1.41 (3.6)	76	0 (55)	-1.41 (3.6)	61
8	0 (45)	1.41 (6.4)	72	0 (55)	1.41 (6.4)	60
9	0 (45)	0 (5)	100	0 (55)	0 (5)	93
10	0 (45)	0 (5)	89	0 (55)	0 (5)	100
11	0 (45)	0 (5)	88	0 (55)	0 (5)	82

Table 4.2 – Coefficient values and statistical analysis for the activities of soluble invertase (INV) and immobilized invertase cross-linked with soy protein (INV-SOY-CLEAs).

	Soluble INV		**INV-SOY-CLEAs**	
	Coefficients	p-Value	Coefficients	p-Value
Mean	92.4	0.000*	91.7	0.000*
X_1 (L)	12.1	0.299	-26.6	0.003*
X_1 (Q)	-74.5	0.002*	-74.0	0.000*
X_2 (L)	0.240	0.982	1.1	0.830
X_2 (Q)	-28.2	0.073*	-34.6	0.002*
$X_1 . X_2$	-13.0	0.420	5.8	0.449

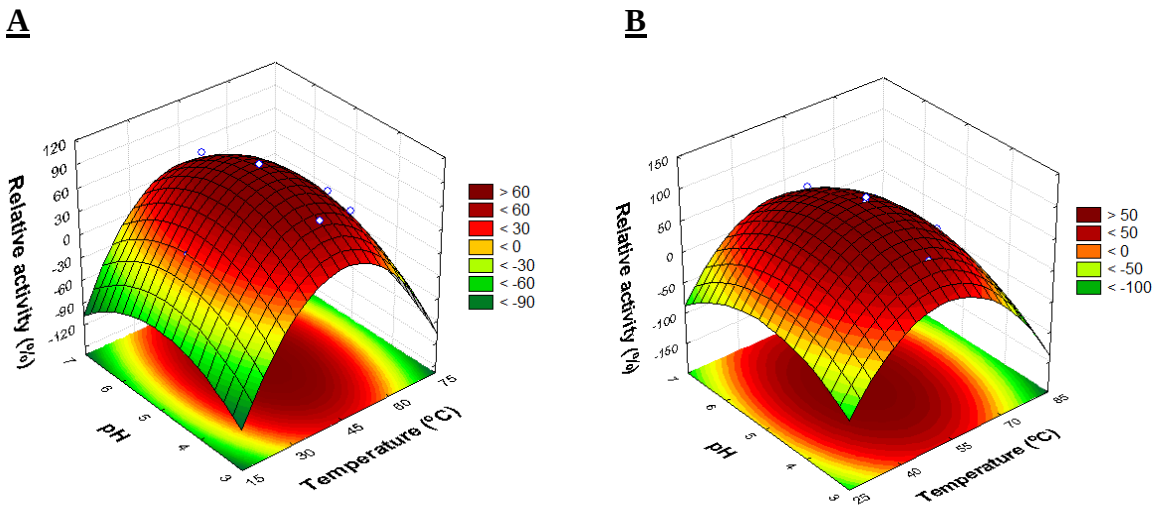
*Significant at 0.1 level.; X_1(T), X_2(pH).

Table 4.3 – Coefficient values and statistical analysis for the activities of soluble invertase (INV) and immobilized invertase cross-linked with soy protein (INV-SOY-CLEAs), after elimination of non-significant parameters.

	Soluble INV		INV-SOY-CLEAs	
	Coefficients	p-Value	Coefficients	p-Value
Mean	92.4	0.000	91.7	0.000
X_1 (L)	ND	ND	-26.6	0.001
X_1 (Q)	-74.5	0.000	-74.0	0.000
X_2 (L)	ND	ND	ND	ND
X_2 (Q)	-28.2	0.043	-34.6	0.000
$X_1 . X_2$	ND	ND	ND	ND
R-value	0.95		1.00	
F_{value}	20.2		76.1	
F_{calc}/F_{tab}	6		25	

R, coefficient of determination; X_1 (T), X_2 (pH).

Figure 4.2 – Surface response showing the pH and temperature effect on the activities of (A) soluble invertase (INV) and (B) CLEAs of invertase (INV-SOY-CLEAs).

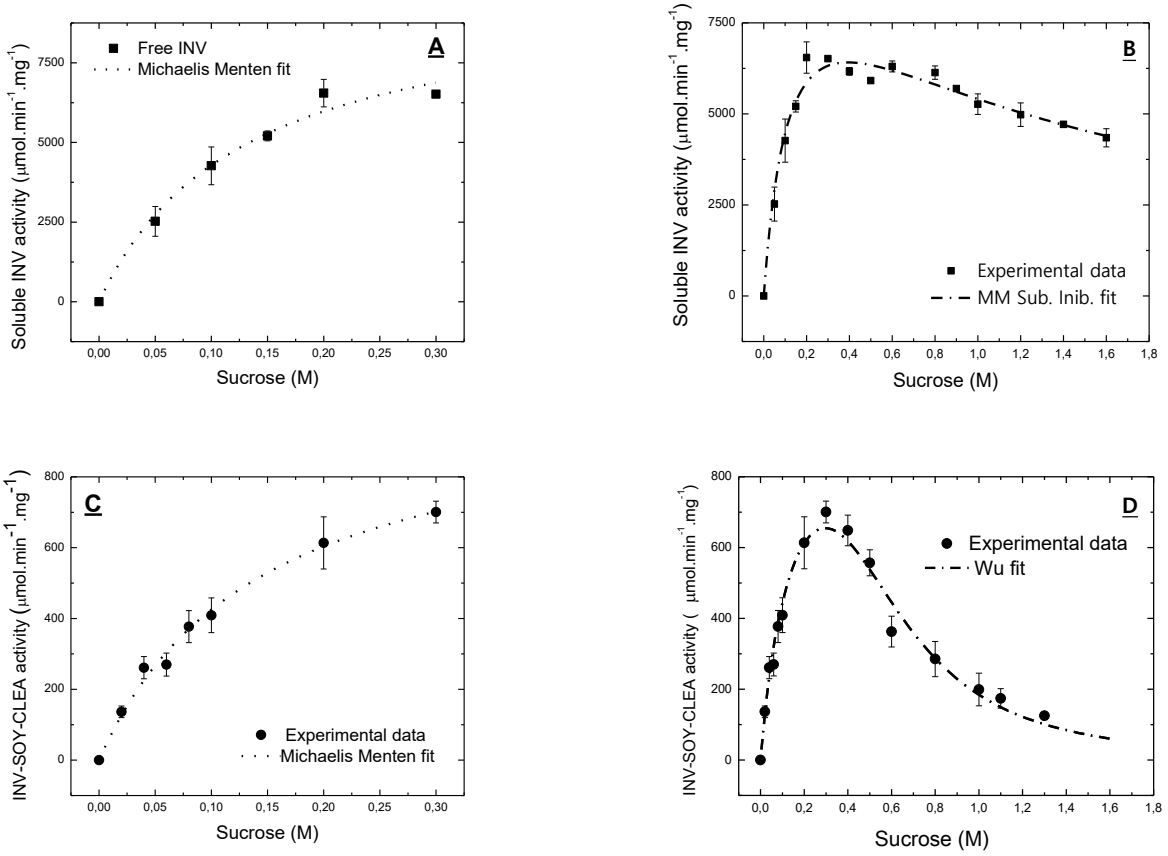


4.3.3 Kinetic parameters

The enzyme kinetics for the hydrolysis of sucrose was studied under the previously reported conditions (40°C and pH 6.0) for soluble invertase (MAFRA *et al.*, 2015). Figure 4.3 shows the effect of the substrate concentration on the kinetics

of soluble (MAFRA *et al*., 2015) and immobilized invertase evaluated using the initial rate method. Table 4.4 shows the fitted models and the estimated kinetic parameters. The Michaelis-Menten model fitted well to the initial rates for both enzyme forms up to a substrate concentration of 0.30 M. After that, while the kinetic for soluble invertase showed a typical behavior (BOWSKI *et al*., 1971), the reaction rates for INV-SOY-CLEAs decreased sharply, a finding not yet reported in the literature.

Figure 4.3 – Effect of sucrose concentration on the initial activities of invertase at 40°C and pH 6.0. Soluble invertase, INV: (A) Michaelis-Menten model fitted to experimental data in the range without inhibition; (B) Michaelis-Menten with substrate inhibition fitted in the range with and without inhibition. CLEAs of invertase (INV-SOY-CLEA): (C) Michaelis-Menten model fitted in the range without inhibition; (D) Wu model fitted in the range with and without inhibition.



Compared to the soluble invertase, the INV-SOY-CLEAs showed tenfold lower k_{cat} values. Commonly, the difference between the kinetic parameters for soluble and immobilized enzymes is not surprising (DALAL *et al*., 2007; TOUAHAR, 2014; LI *et al*., 2016; MAFRA *et al*., 2016). For the invertase immobilized by the CLEA technique cross-linked with soy protein (hereafter INV-SOY-CLEA), there may have been mass transfer problems for the substrate to access the enzyme active site, decreasing the turnover number (k_{cat}) (DALAL *et al*., 2007; VU & LE, 2008; VALERIO *et al*., 2013; MAFRA *et al*., 2016). On the other hand, the similarities between the K_m values for soluble and immobilized invertase may indicate that the immobilization by the CLEA technique did not alter the natural configuration of the enzyme active site, maintaining the same substrate affinity (POLAINA & MACCABE, 2007).

Table 4.4 – Kinetic parameters estimated for the sucrose hydrolysis at 40 ^{o}C and pH 6.0 catalyzed by soluble invertase (INV) and CLEAs of invertase (INV-SOY-CLEAs).

Parameters	Soluble	Soluble	INV-SOY-CLEA	INV-SOY-CLEA
K_m	130 ± 35 mM	130 mM[(a)]	145 ± 18 mM	145 mM[(a)]
K_I	-	1191 ± 109 mM	-	558 ± 31 mM
w	-	-	-	2.69± 0,28
k_{cat}[(b)]	(16 ± 1.9) x 10^3 s^{-1}	(18 ± 0.51) x 10^3 s^{-1}	(1.7 ± 0.11) x 10^3 s^{-1}	(1.8 ± 0.07) x 10^3 s^{-1}
V_{max}/K_m	1.8 x 10^2 s^{-1}	1.9 x 10^2 s^{-1}	1.2 x 10^2 s^{-1}	1.3 x 10^2 s^{-1}
k_{cat}/K_m	12 x 10^4 $M^{-1}.s^{-1}$	14 x 10^4 $M^{-1}.s^{-1}$	1.2 x 10^4 $M^{-1}.s^{-1}$	1.2 x 10^4 $M^{-1}.s^{-1}$
Model	$r = \frac{k_{cat}.[S]}{[S]+K_m}$	$r = \frac{k_{cat}.[S]}{[S]+K_m+\frac{[S]^2}{K_i}}$	$r = \frac{k_{cat}.[S]}{[S]+K_m}$	$r = \frac{k_{cat}}{1+\frac{K_m}{[S]}+\left(\frac{[S]}{K_i}\right)^w}$

[(a)] Fixed values.

[(b)] Assuming invertase molecular weight of 100 kDa (Aslam *et al*., 2013; Kulshrestha *et al.*, 2013) (Figure 4.3S).

The decrease in the reaction rate when sucrose concentration was increased was much higher for the immobilized invertase (INV-SOY-CLEA) than for

the soluble enzyme. This behavior affects the value of the *w* parameter of the Wu model, which agrees with the Michaelis-Menten model with substrate inhibition when *w* is equal to one. The low reaction rates for high sucrose concentration are probably due to intraparticle diffusional limitations in the immobilized enzyme since CLEAs show low porosity (SHAH *et al*., 2006; TALEKAR, 2012; TUKEL *et al*., 2013; MAFRA *et al*., 2016), even in the presence of feeder proteins. The reduction of the apparent K_I for the immobilized invertase can also be an indicative of intraparticle diffusional delays.

4.3.4 Production of inverted sugar syrup and operational stability

The hydrolysis of sucrose was carried out in a batch reactor at 40°C and pH 6.0 (100 mM sodium phosphate buffer) catalyzed by INV-SOY-CLEAs and soluble invertase using an invertase/sucrose ratio of 0.03 % (w/w), as previously determined by Mafra *et al.* (2015).

Figure 4.4 – Hydrolysis of sucrose (50 g.L^{-1}) catalyzed by INV-SOY-CLEA at 40°C and pH 6.0 in a batch reactor stirred at 300 rpm. (A) Time-conversion profiles; (B) conversions after each 4 h-batch for the reuse assays.

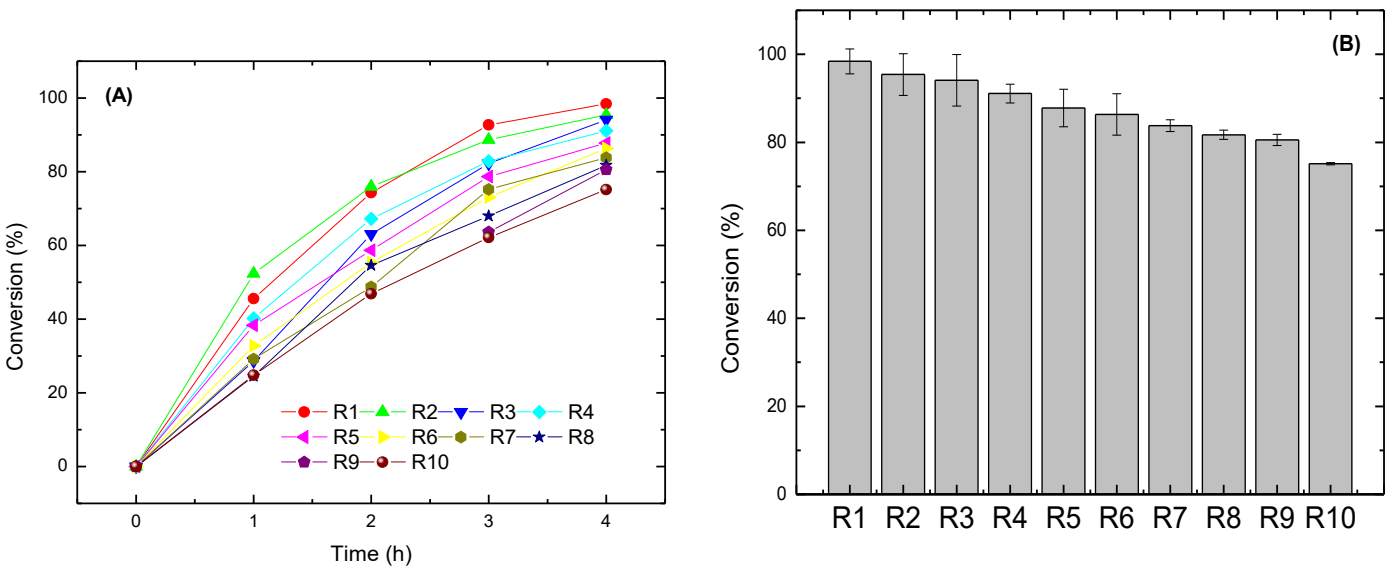


Figure 4.4-A shows the conversion profiles of sucrose, and Figure 4.4-B shows the conversions achieved in each 4 h-batch for the reuse assays. The biocatalyst showed a mild performance loss, maintaining the reaction conversion around 75 % after the tenth 4 h-batch of sucrose hydrolysis at 40 ^{o}C and pH 6.0 (Figure 4.4-B). The results showed that the CLEA methodology is a very promising technique for enzyme immobilization and stability. In fact, excellent reuse results have been previously reported for other enzymes, e.g., proteases, α-amylse, catalase, and combined xylanase and mannanase (TUKEL *et al*., 2013; BHATTACHARYA & PLETSCHKE, 2015; MAHMOD *et al*., 2016; NADAR *et al*., 2016).

Table 4.5 shows a brief investigation of previously reported operational stability of immobilized invertases on solid carriers and specific productivities of inverted sugar syrup, compared with our findings.

The specific productivities achieved in this work (70 – 99 $g_{product}.h^{-1}.g_{biocatalyst}^{-1}$) were around 21 times higher than one previously reported at temperatures in the range of 30 - 45 ^{o}C (SANJAY & SUGUMAN, 2005; GÓMEZ *et al*., 2006; REBROS *et al*., 2007; BAGAL-KESTWAL *et al*., 2011; RAJ *et al*., 2011). High specific productivity (64 $g_{product}.h^{-1}.g_{biocatalyst}^{-1}$) was also reported by (VALERIO *et al*., 2013), but in this case the authors used higher reaction temperature (i.e., 55 ^{o}C) and invertase immobilized on solid carrier that may have contributed to increase the cost of the biocatalyst, besides requiring a higher reactor volume to achieve high volumetric productivities. Generally, biocatalysts immobilized on solid supports can fill a significant useful volume of the reactor if high enzymatic load is required, thus reducing the volumetric productivity of the product of interest. The high specific productivity obtained in this work shows that the high volumetric activity of carrier-free

CLEAs is one of the main advantages of this immobilization technique (TALEKAR *et al*., 2013).

Table 4.5 – Productivity of inverted sugar and operational stability of immobilized invertases on solid carriers and CLEAs of invertase.

Carrier	Specific productivity ($g_{product}.h^{-1}.g_{biocatalyst}^{-1}$)	Initial sucrose (g/L)	Operational stability				Reference
			Reuse	Conversion up to (%)	Time of process	Temperature and pH	
Chitosan nanoparticles	64.0	80	69	53	30 min	55 ^{o}C and pH 4.5	Valerio *et al.* (2013)
Chitosan	6.0 x 10^{-3}	3 x 10^{-3}	20	70	10 min	37 ^{o}C and pH 4.5	Bagal-Kestwal *et al.* (2011)
New hydrogel comprising of methacrylic acid (MAAc) and N-vinyl pyrrolidone (N-VP) and ethyleneglycol dimethacrylate (EGDMA)	2.3	100	9	11	70 min	45 ^{o}C and pH 6.0	Raj *et al.* (2011)
LentiKats®	3.3	100	40	100	15 min	30 ^{o}C and pH 4.5	Rebros *et al.* (2007)
Montmorillonite	2.0	100	30	70	30 min	30 ^{o}C and pH 5.0	Sanjay & Suguman (2005)
Chitosan Carboxymethylcellulose-Chitin	17	68.4	10	98	4 h	30 ^{o}C and pH 4.5	Gómez *et al.* (2006)
Grafted alginate beads	0.2	19	20	80	30 min	60 ^{o}C and pH 5.0	Awad *et al.* (2013)
Montmorillonite K 10 activated by acid	16	50	30	75	40 min	Room temperature and pH 5.0	Sanjay & Sugunan (2008)
Free-carrier INV-SOY-CLEA	70.0[(a)]	50	10	75	4 h	40^{o}C and pH 6.0	Present work (batch process)
Free-carrier INV-SOY-CLEA	99.0[(b)]	100	3	95	16.7 h	40^{o}C and pH 6.0	Present work (fed-bath process)

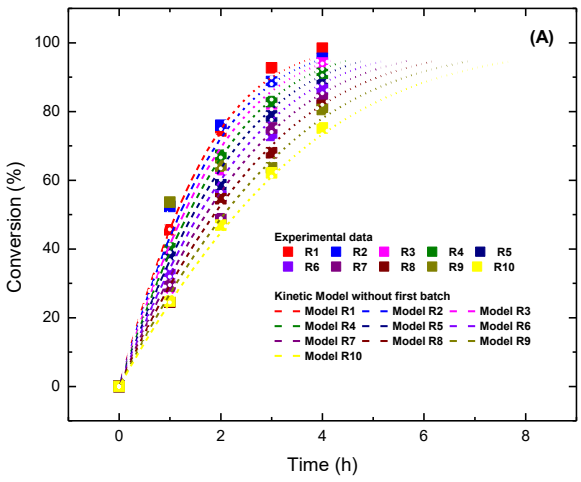
The specific productivity for INV-SOY-CLEAs was calculated as the ratio between concentration of product ($g_{product}.L^{-1}$) at the end of the first reuse (4 h[(a)], 16.7 h[(b)]) and concentration of biocatalyst ($g_{biocatalyst}.L^{-1}$) fed into the reactor. The mass of biocatalyst was taken as the total amount of protein (invertase and soy protein) used to prepare the INV-SOY-CLEA.

4.3.5 Economic assessment and validation

Lima-Ramos *et al.* (2014) and Tufvesson *et al.* (2013) have proposed a set of economic metrics to guide the assessment of biocatalytic processes. In this work, the economic metrics were used for the process design of the hydrolysis of sucrose using INV-SOY-CLEA as biocatalyst.

A first order inactivation model was fitted to the reuse assays using the Michaelis-Menten model, for which the parameters were previously fitted (Table 4.4). This inactivation model can reasonably describe the loss of activity of the INV-SOY-CLEA biocatalyst, as shown in Figure 4.5-A. The inactivation constant (k_d = 0.016 h^{-1}) resulted in a half-life of the biocatalyst around 45 h.

Figure 4.5 – Experimental data of reuse of INV-SOY-CLEA (R1 to R10) in the hydrolysis of 50 $g.L^{-1}$ sucrose solution in a batch reactor kept at 40 ^{o}C and pH 6.0. The curves represent the Michaelis-Menten model fitted to the experimental data (K_m = 145 ± 18 mM and k_{cat} = 1.7 ± 0.11·x 10^3 s^{-1}) combined to the first order equation of enzyme inactivation (k = 0.016 h^{-1}).



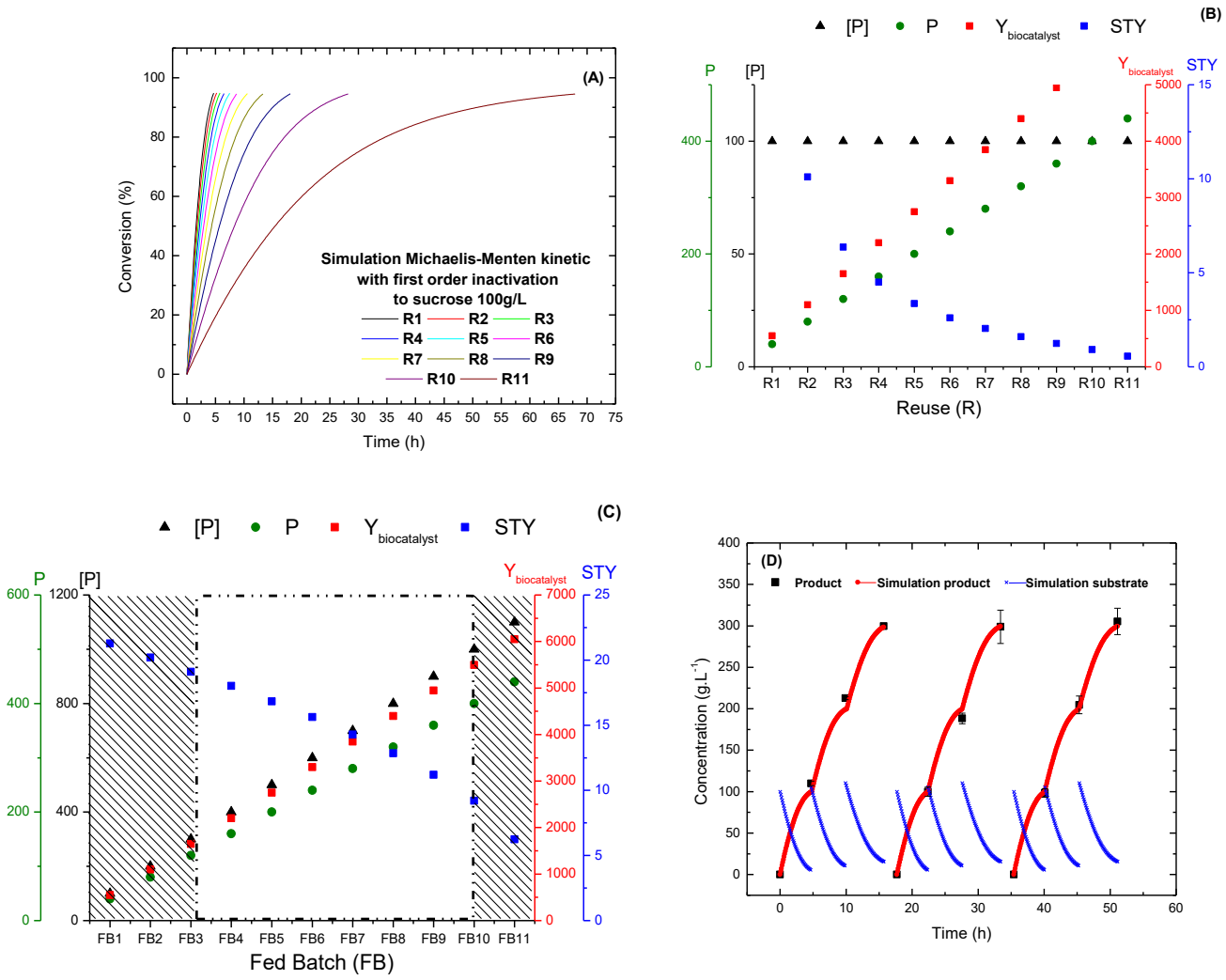
Based on: (1) economic feasibility window of minimum product concentration of 300 $g.L^{-1}$ (TUFVESSON *et al*., 2013; LIMA-RAMOS *et al*., 2014), (2) kinetic model of substrate inhibition (Table 4.4 and Figure 4.3) from substrate concentrations of ~ 0.4 M (137 $g.L^{-1}$), and (3) using the inactivation and kinetic

models combined, the behavior of the INV-SOY-CLEA was simulated in the reuse assays of sucrose hydrolysis at 40 ^{o}C and pH 6.0 until the complete loss of its enzymatic activity using batch or fed-batch processes. Feasibility windows were calculated using processes with 95% of conversion and an initial sucrose concentration of 100 g.L^{-1} (~ 0.3 M). The initial substrate concentration was based on the maximum reaction rate for this biocatalyst (Fig. 4.3-D). Figure 4.6-A shows simulation of reuse of INV-SOY-CLEA and the curve represent the Michaelis-Menten model fitted to the experimental data (K_m = 145 ± 18 mM and k_{cat} = 1.7 ± 0.11·x 10^3 s^{-1}) combined to the first order equation of enzyme inactivation (k = 0.016 h^{-1})

The process metrics used were as follow: product concentration [P], biocatalyst yield [$Y_{biocatalyst}$] and space time yield (STY). The comparison of these metrics with the threshold values proposed by Lima-Ramos *et al.* (2014) is shown in Figure 4.6-B,C, for a batch reactor and a fed-batch reactor (fed with 40 g sucrose at every 4 hours for a total reactional volume of 0.4 L). A feasibility window appears only in a fed-batch reactor between the feeding 3 and 10; in the eleventh feeding, the biocatalyst was completely inactivated during the process because the sucrose hydrolysis required around 70 h to reach 95% conversion (Figure 4.6-A). As previously reported by Lima-Ramos *et al.* (2014), the recovery of the final product from the reaction medium is a critical step and extends the process cost. A fed-batch can overcome the substrate inhibition and can contribute to increase specific productivity (XUE & WOODLEY, 2012; TUFVESSON *et al*., 2013; LIMA-RAMOS *et al*., 2014).

Figure 4.6-D shows the experimental validation of the first cycle of fed-batch within the feasibility window (FB3-Fig. 4.6-C). The results clearly show a similar behavior between simulation and experimental data.

Figure 4.6 – (A) Simulation of reuse of INV-SOY-CLEA (R1 to R11) in the hydrolysis of 100 $g.L^{-1}$ sucrose solution in a batch reactor. (B) Feasibility window using threshold values for process metrics and the data estimated from the fitted models. (C) Feasibility window using threshold values for process metrics and the data estimated from the fitted models for a fed batch reactor, in the hydrolysis of 100 $g.L^{-1}$ sucrose solution, fed of 40 g of sucrose when conversion reach 95%. (D) Validation of feasibility window estimated from the fitted models for a fed batch reactor. [P] is the product concentration in $g_{product}$ $L_{reactor}^{-1}$; $Y_{biocatalyst}$ is the biocatalyst yield in $g_{product}$ $g_{biocatalyst}^{-1}$; STY is space-time yield in $g_{product}$ $L_{reactor}^{-1}$ h^{-1}; P is the mass of product in $g_{product}$.



4.4 Conclusions

Soy protein showed to be a good alternative to replace expensive proteins as inert spacer molecules in the preparation of CLEAs. Invertase cross-

linked with soy protein yielded a stable biocatalyst to be used in the hydrolysis of sucrose. The immobilized biocatalyst INV-SOY-CLEA retained around 30% of activity without leaching, temperature of maximum activity 5.8 ^{o}C higher than that of the soluble enzyme, and it could be reused for ten 4h-cycles maintaining the conversion of sucrose above 75%. The catalytic efficiency (k_{cat}/K_m) of the INV-SOY-CLEA reduced around tenfold compared to the soluble invertase, probably due to intraparticle diffusional delays. On the other hand, the specific productivity of inverted syrup (70-99 $g_{product}.h^{-1}$. $g_{derivative}^{-1}$) was the highest when compared to that previously reported. The study of economic feasibility showed that the sucrose hydrolysis could be performed with a sucrose concentration of 100 $g.L^{-1}$ in a fed-batch reactor with 3 feedings of sucrose to restore the sucrose concentration when the conversion reaches 95 %.

4.5 Acknowledgements

This work was supported by the National Council for Scientific and Technological Development (CNPq) (grant numbers 141647/2013-2 and 402850/2013-0), the Federal Foundation CAPES and FAPESP (grant number 2014/21510-0). The authors thank LNF Latino Americana (Bento Gonçalves, Brazil) for the donation of the *S. cerevisiae* invertase, and the Postgraduate Program in Chemical Engineering of the Federal University of São Carlos (PPGEQ/UFSCar) where this work was undertaken. The authors thank Prof. Monica Lopes Aguiar from the Laboratory of Environmental Control (DEQ/UFSCar) for the microscopy images of CLEAs.

4.6 Reference

AHUMADA, K. *et al.* Production of combi-CLEAs of glycosidases utilized for aroma enhancement in wine. **Food and Bioproducts Processing**, v. 94, p. 555–560, 2015.

ALICEWEB. http://aliceweb.desenvolvimento.gov.br/ 2016.

ARRUDA, L.; VITOLO, M. Characterization of invertase entrapped into calcium alginate beads. **Applied Biochemistry and Biotechnology**, v. 81, n. 1, p. 23-33, 1999.

ASLAM, A.; HAQ, I.-U.; ALI , S. Purification and characterization of two invertases from mutant strain of *Saccharomyces cerevisiae*. **Pakistan Journal of Botany**, v. 45, n. 1, p. 285-291, 2013.

AWAD, G. E. A. *et al*. Production optimization of invertase by Lactobacillus brevis Mm-6 and its immobilization on alginate beads. **Carbohydrate Polymers**, v. 93, n. 2, p. 740–746, 2013.

BAGAL-KESTWAL, D. *et al*. Development of dip-strip sucrose sensors: Application of plant invertase immobilized in chitosan–guar gum, gelatin and poly-acrylamide films. **Sensors and Actuators B: Chemical**, v. 160, n. 1, p. 1026–1033, 2011.

BHATTACHARYA, A.; PLETSCHKE, B. I. Strategic optimization of xylanase–mannanase combi-CLEAs for synergistic and efficient hydrolysis of complex lignocellulosic substrates. **Journal of Molecular Catalysis B: Enzymatic**, v. 115, p. 140–150, 2015.

BOWSKI, L. *et al.* Kinetic modeling of the hydrolysis of sucrose by invertase. **Biotechnology and Bioengineering**, v. 13, n. 5, p. 641-656, 1971.

CAO, L. Q.; VAN RANTWIJK, F.; SHELDON, R. A. Cross-linked enzyme aggregates: A simple and effective method for the immobilization of penicillin acylase. **Organic Letters**, v. 2, n. 10, p. 1361-1364, 2000.

CRUZ, J. *et al.* Optimized preparation of CALB-CLEAs by response surface methodology: The necessity to employ a feeder to have an effective crosslinking. **Journal of Molecular Catalysis B: Enzymatic**, v. 80, p. 7–14, 2012.

DALAL, S.; KAPOOR, M.; GUPTA, M. N. Preparation and characterization of combi-CLEAs catalyzing multiple non-cascade reactions. **Journal of Molecular Catalysis B-Enzymatic**, v. 44, n. 3-4, p. 128-132, 2007.

DONG, T. *et al.* Preparation of cross-linked aggregates of aminoacylase from *Aspergillus melleus* by using bovine serum albumin as an inert additive. **Bioresource Technology**, v. 101, n. 16, p. 6569–6571, 2010.

DOSCHER, M. S.; RICHARDS, F. M. The Activity of an Enzyme in the Crystalline State: Ribonuclease S. **The Journal of Biological Chemistry,** v. 238, p. 2399 - 2406, 1963.

GUISÁN, J. M. **Immobilization of Enzymes and Cells**. 2006. ISBN 978-1-58829-290-2.

HANOVER, L. M.; WHITE, J. S. Manufacturing, composition, and applications of fructose. **The American Society for Clinical Nutrition**, v. 58, p. 724-732, 1993-11-01 1993.

HEIDT, L. J.; SOUTHAM, F. W.; SULLIVAN, E. A. Autocatalyzed Hydrolysis of Sucrose by Acid. **Journal of the American Chemical Society**, v. 74, n. 9, p. 2377-2378, 1952.

HSIEH, H.; LIU, P.; LIAO, W. Immobilization of invertase via carbohydrate moiety on chitosan to enhance its thermal stability. **Biotechnology Letters**, v. 22, n. 18, p. 1459-1464, 2000.

ILLANES, A. **Enzyme Biocatalysis - Principles and Applications**. Springer, 2008.

KOWALSKI, S. *et al.* 5-Hydroxymethyl-2-Furfural (HMF) – Heat-Induced Formation, Occurrence in Food and Biotransformation – a Review. **Polish Journal Of Food And Nutrition Sciences**, v. 63, n. 4, 2013.

KULSHRESTHA, S. *et al.* Invertase and its applications – A brief review. **Journal of Pharmacy Research**, v. 7, n. 9, p. 792–797, 2013.

GUISÁN, J. M. **Immobilization of Enzymes and Cells**, 2006.

GÓMEZ, L.; RAMÍREZ, H. L.; VILLALONGA, R. Immobilization of chitosan-invertase neoglycoconjugate on carboxymethylcellulose-modified chitin. **Preparative Biochemistry and Biotechnology**, v. 36, n. 3, p. 259-271, 2006.

LI, S. *et al.* Preparation and characterization of cross-linked enzyme aggregates (CLEAs) of recombinant thermostable alkylsulfatase (SdsAP) from *Pseudomonas sp.* S9. **Process Biochemistry**, v. 51, n. 12, p. 2084–2089, 2016.

LIMA-RAMOS, J.; TUFVESSON, P.; WOODLEY, J. M. Application of environmental and economic metrics to guide the development of biocatalytic processes. **Green Processing and Synthesis**, v. 3, n. 3, p. 195-213, 2014.

LOWRY, O. H. *et al.* Protein measurement with the folin phenol reagent. **Journal of Biological Chemistry**, v. 193, n. 1, p. 265-275, 1951.

MAFRA, A. C. O. *et al.* Gluconic acid production from sucrose in an airlift reactor using a multi-enzyme system. **Bioprocess and Biosystems Engineering**, v. 38, n. 4, p. 671-680, 2015.

MAFRA, A. C. O. *et al.* Diffusion effects of bovine serum albumin on cross-linked aggregates of catalase. **Journal of Molecular Catalysis B: Enzymatic**, v. 133, p. 107–116, 2016.

MAHMOD, S. S. *et al.* Optimizing the preparation conditions and characterization of a stable and recyclable cross-linked enzyme aggregate (CLEA)-protease. **Bioresources and Bioprocessing**, v. 3, n. 1, p. 1, 2016.

MILLER, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry**, v. 31, p. 426-428, 1959.

NADAR, S. S. *et al.* Macromolecular cross-linked enzyme aggregates (M-CLEAs) of α-amylase. **International Journal of Biological Macromolecules**, v. 84, p. 69–78, 2016.

POLAINA, J.; MACCABE, A. P. **Industrial Enzymes - Structure, Function and Applications**. Springer, 2007. 642 ISBN 978-1-4020-5377-1.

QUIOCHO, F. A.; RICHARDS, F. M. Intermolecular cross linking of a protein in the crystalline state: carboxypeptidase-A. **Proceedings of the National Academy of Sciences of the United States of America**, v. 52, n. 3, p. 833-9, 1964.

RAJ, L. *et al.* Kinetics study of invertase covalently linked to a new functional nanogel. **Bioresource Technology**, v. 102, n. 3, p. 2177–2184, 2011

REBROS, M. *et al.* Hydrolysis of sucrose by invertase entrapped in polyvinyl alcohol hydrogel capsules. **Food Chemistry**, v. 102, n. 3, p. 784–787, 2007.

SANJAY, G.; SUGUMAN, S. Invertase immobilized on montmorillonite: reusability enhancement and reduction in leaching. **Catalysis Communications**, v. 6, n. 1, p. 81–86, 2005.

SANJAY, G.; SUGUNAN, S. Acid activated montmorillonite: an efficient immobilization support for improving reusability, storage stability and operational stability of enzymes. **Journal of Porous Materials**, v. 15, n. 3, p. 359-367, 2008.

SCHOEVAART, R. *et al*. Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). **Biotechnology and Bioengineering**, v. 87, n. 6, p. 754-762, 2004.

SHAH, S.; SHARMA, A.; GUPTA, M. N. Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. **Analytical Biochemistry**, v. 351, n. 2, p. 207–213, 2006.

SHELDON, R. A. Enzyme Immobilization: The Quest for Optimum Performance. **Advanced Synthesis & Catalysis**, v. 349, n. 8-9, p. 1289-1307, 2007.

SHELDON, R. A. *et al*. Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs). **Applied Biochemistry and Biotechnology**, v. 92, n. 3, p. 467-77, 2011

TALEKAR, S. *et al.* Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). **The Royal Society of Chemistry**, v. 3, p. 12485 - 12511, 2013.

TALEKAR, S., SHAH, V. , PATIL, S. , NIMBALKAR, M. Porous cross linked enzyme aggregates (p-CLEAs) of Saccharomyces cerevisiae invertase. **The Royal Society of Chemistry**, v. 2, p. 1575 - 1579, 2012.

TISCHER, W.; KASCHE, V. Immobilized enzymes: crystals or carriers? **Trends in Biotechnology**, v. 17, n. 8, p. 326-335, 1999.

TOUAHAR, I. E. *et al.* Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals. **Applied Microbiology and Biotechnology** , v. 481, p. 90–99, 2014.

TUFVESSON, P. *et al.* Advances in the Process Development of Biocatalytic Processes. **Organic process research and development**, v. 17, n. 10, p. 1233 - 1238, 2013.

TUKEL, S. S. *et al.* Preparation of crosslinked enzyme aggregates (CLEA) of catalase and its characterization. **Journal of Molecular Catalysis B-Enzymatic**, v. 97, p. 252-257, 2013.

VALERIO, S. G. *et al.* High operational stability of invertase from Saccharomyces cerevisiae immobilized on chitosan nanoparticles. **Carbohydrate Polymers**, v. 92, n. 1, p. 462-468, 2013.

VU, T. K. H.; LE, V. V. M. Biochemical Studies on the Immobilization of the Enzyme Invertase (EC.3.2.1.26) in Alginate Gel and its Kinetics. **ASEAN Food Journal**, v. 15, n. 1, p. 73-78, 2008.

XUE, R.; WOODLEY, J. Process technology for multi-enzymatic reaction systems. **Bioresource Technology**, v. 115, p. 183-195, 2012.

4.7 Supplementary data

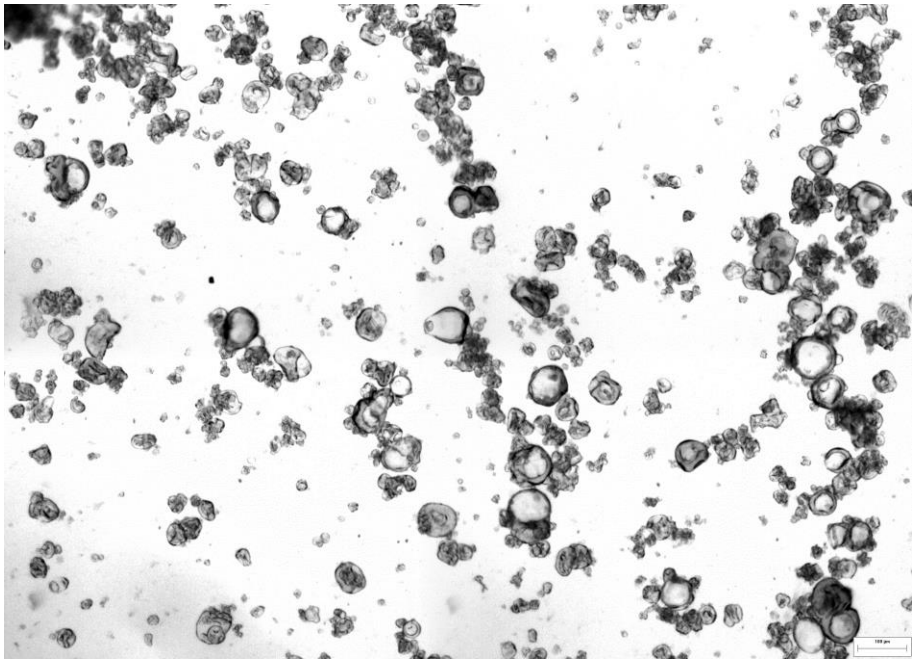
Table 4.1S – Yield of immobilization (Y) of invertase (INV) by the CLEA technique as function of precipitant agent (tert-butyl alcohol – TBA, dimethoxyethane – DME, and saturated ammonium sulfate solution – AS), glutaraldehyde concentration (GLU), and soy protein concentration (SOY).

Assay	GLU (mM)	INV (mg_{prot}/mL)	SOY (mg_{prot}/mL)	SOY/INV mass ratio	µmol Glu/mg total protein	YI (%) TBA	YI (%) DME	YI (%) SA
1	25	10	0	0	5.0	23.0 ± 0.34	6.7 ± 0.18	0
2	75	10	0	0	15.0	20.8 ± 0.31	21.1 ± 0.58	0
3	25	30	0	0	1.7	10.4 ± 0.16	15.9 ± 0.44	0
4	75	30	0	0	5.0	18.9 ± 0.28	14.0 ± 0.39	0
5	25	10	20	2	1.7	46.2 ± 0.69	15.5 ± 0.43	0
6	75	10	20	2	5.0	65.49 ± 0.98	11.87 ± 0.33	0
7	25	30	20	0.67	1.0	34.2 ± 0.51	14.5 ± 0.40	0
8	75	30	20	0.67	3.0	34.8 ± 0.52	11.7 ± 0.32	0
9	25	10	40	4.0	1.0	33.9 ± 0.51	33.8 ± 0.93	17.15 ± 0.67
10	75	10	40	4.0	3.0	62.3 ± 0.93	63.3 ± 1.75	34.91 ± 1.36
11	25	30	40	1.33	0.7	22.65 ± 0.34	43.29 ± 1.19	3.70 ± 0.14
12	75	30	40	1.33	2.1	41.7 ± 0.63	27.6 ± 0.76	4.14 ± 0.16
13	25	10	60	6	0.7	45.62 ± 0.68	28.51 ± 0.79	5.45 ± 0.21
14	75	10	60	6	2.1	41.55 ± 0.62	29.26 ± 0.81	12.42 ± 0.48
15	25	30	60	2	0.55	29.21 ± 0.44	43.29 ± 1.19	4.14 ± 0.16
16	75	30	60	2	1.7	47.46 ± 0.71	43.88 ± 1.21	1.56 ± 0.06

Table 4.2S– Yield of immobilization (Y) and yield of immobilization after the assay of stability to leaching ($Y_{after\ leaching\ assay}$) of invertase (INV) by the CLEA technique as function of glutaraldehyde concentration (GLU) and SOY/INV ratios (w/w).

Assay	SOY(mg_{prot}/mL)	SOY/INV ratio (w/w)	[GLU] mM	μmol GLU/$mg_{protein}$	Y(%)	$Y_{after\ leaching\ assay}$ (%)[a]
1			25	5.0	62.2 ± 6.9	5.8 ± 0.9
2	0	0	50	10.0	55.7 ± 0.4	6.3 ± 0.8
3			75	15.0	54.0 ± 3.2	4.7 ± 0.6
4			25	2.5	46.4 ± 3.7	11.1 ± 3.4
5	10	1	50	5.0	32.3 ± 3.1	11.1 ± 0.1
6			75	7.50	40.0 ± 4.9	12.3 ± 0.1
7			25	1.67	44.7 ± 6.9	20.8 ± 0.4
8	20	2	50	3.33	40.1 ± 4.0	21.9 ± 3.1
9			75	5.0	42.6 ± 6.8	22.7 ± 2.4
10			25	1.25	61.0 ± 3.6	20.5 ± 2.4
11	30	3	50	2.5	49.6 ± 7.7	23.2 ± 2.0
12			75	3.75	41.8 ± 2.1	25.1 ± 2.1
13			25	1.0	46.0 ± 0.8	26.6 ± 6.6
14	40	4	50	2.0	40.2 ± 3.0	23.8 ± 1.0
15			75	3.0	44.2 ± 3.9	25.9 ± 1.4
16			25	0.83	47.0 ± 4.2	28.1 ± 5.1
17	50	5	50	1.67	47.2 ± 2.1	29.4 ± 2.3
18			75	2.5	33.8 ± 3.2	30.4 ± 0.8
19			25	0.71	47.7 ± 8.0	26.7 ± 3.7
20	60	6	50	1.43	49.2 ± 1.2	29.1 ± 5.9
21			75	2.14	44.7 ± 5.5	25.3 ± 2.8

[a]Assay of stability to leaching were evaluated at 30 °C and pH 7.0 (100 mM sodium phosphate buffer) for 4 h at 250 rpm.



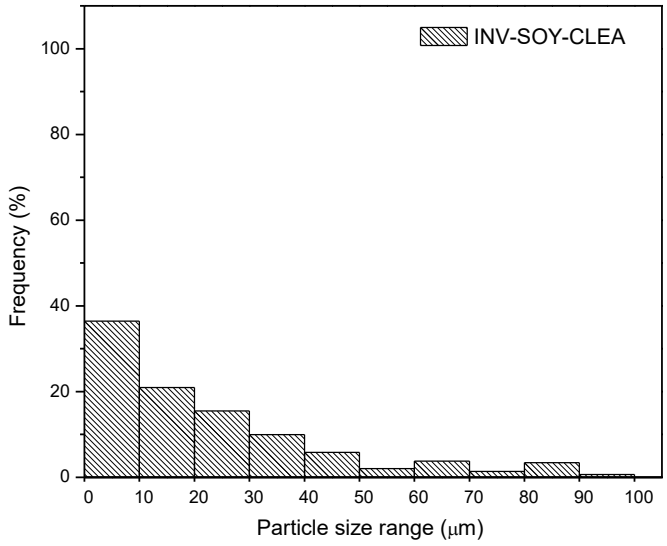


Fig. 4.1S – Particle size distribution for INV-SOY-CLEA prepared at pH 7.0. The particle diameter distribution was obtained from microscopy image of INV-SOY-CLEA using the ImageJ software, where the frequency count was performed using a step size of 10 µm.

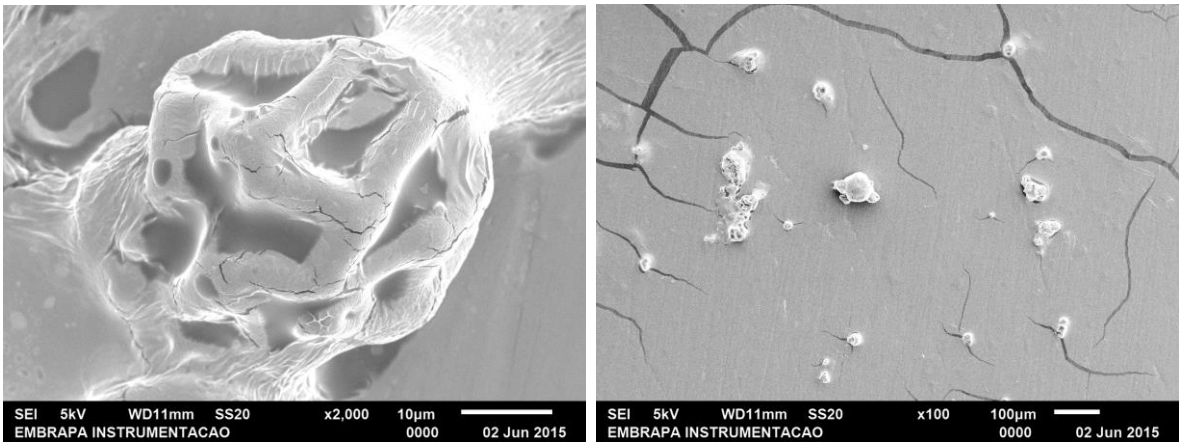


Fig. 4.2S – SEMs of CLEAs of invertase with soy as feeder protein. Micrographs were taken on a JSM 6510/JEOL (5KV) SEM instrument.

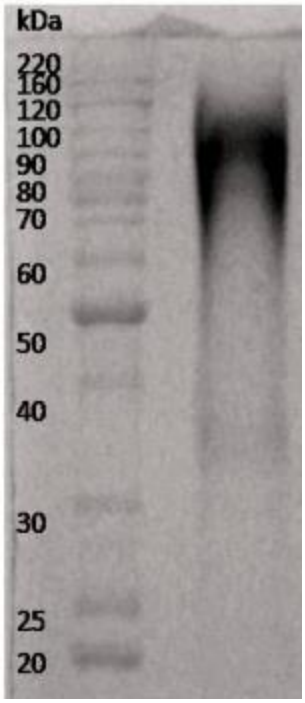


Fig. 4.3S – Characterization of invertase from *Saccharomyces cerevisiae* by SDS PAGE on right; Marker protein at the left in kD

5. Combi-CLEAs of glucose-oxidase and catalase for multiconversion of glucose to gluconic acid process in a pneumatic reactor

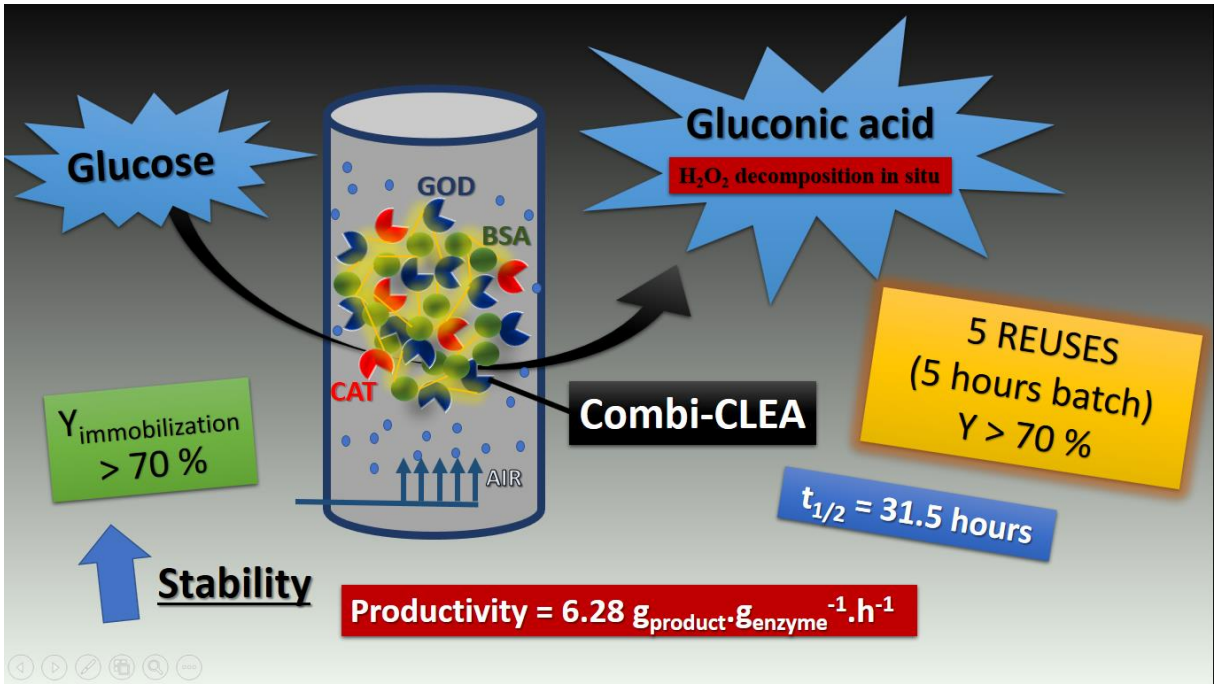
Preface

Multi-enzymatic processes are considered an innovative method for the production of complex compounds of industrial interest. Since a mixture of enzymes catalyzes various reactions without production of intermediates and the purification process of the final product can be eliminated, it can also be considered an application of the intensification process. Consequently, the application of the multi-enzymatic process leads to reduced operational costs and increases the productivity and process yield.

In this study the co-immobilization of glucose oxidase and catalase by Combi-CLEAs technique was evaluated, using bovine serum albumin as feeder protein. Although some studies have reported the immobilization of both enzymes, studies of reusability using Combi-CLEAs are not often reported in the literature. Thus, the present work has evaluated the reusability of the glucose oxidase and catalase co-immobilized by Combi-CLEAs in the production of gluconic acid from glucose. The Combi-CLEAs showed high activity after immobilization (~80 %) and stability for both enzymes. The Combi-CLEAs were prepared by co-precipitation and crosslinking of glucose-oxidase, catalase and bovine serum albumin (BSA). The effects of precipitant agents (organic and inorganic salts), crosslinker (glutaraldehyde) and feeder protein (BSA) concentrations were investigated for the activities of each enzyme. The results showed that Combi-CLEAs of glucose-oxidase

and catalase could be used in a batch process of glucose-to-gluconic acid using a pneumatic reactor, yielding around 100% glucose conversion. Under the operational conditions, the half-life of the biocatalyst was 31.5 hours.

Graphical abstract



Highlights files

❖ Glucose-oxidase and catalase were co-immobilized as CLEA with BSA as feeder protein.

❖ Combi-CLEAs of glucose-oxidase, catalase and BSA showed to be stable after stability assay for both enzymes.

❖ Combi-CLEAs of glucose-oxidase, catalase and BSA efficiently catalyzed the conversion of glucose to gluconic acid in a pneumatic reactor.

❖ Combi-CLEAs of glucose-oxidase, catalase and BSA could be reused by four 5h-cycles, yielding glucose conversion > 70 %.

Abstract

Gluconic acid (GA) is a multifunctional carbonic acid, which has been widely used in chemical industries, such as detergents, leather, photographic, textile, and especially in food and pharmaceutical industries. Multi-enzymatic process is advantageous to produce gluconic acid, since the mixture of several enzymes catalyzes the reactions producing few or no intermediates. The use of a multi-enzyme system immobilized by combined cross-linked enzyme aggregates (Combi-CLEAs) has been recently reported as a promising technique. In this work, combined cross-linked aggregates of catalase from bovine liver and glucose-oxidase from *Aspergillus niger* were prepared, evaluating the effect of precipitant and crosslinking agents, as well as bovine serum albumin (BSA) as feeder protein on enzyme immobilization yield and thermal stability of each enzyme. Combi-CLEAs prepared using dimethoxyethane as precipitant, 25 mM glutaraldehyde and mass ratio BSA/enzymes of 5.45 (w/w) were chosen for further studies, by evaluating their activities and stabilities at 40 ^{o}C, pH 6.0, and 250 rpm for five hours. Combi-CLEAs were used in GA production in a pneumatic reactor operated at 40 ^{o}C, pH 6.0 and 10 vvm of aeration, using 26 g.L^{-1} glucose as substrate. Results showed conversion of 100 % and kinetic profile very similar to the process using free enzymes. The reusability of the Combi-CLEAs was also studied in ten 5h-batches of GA production. The operational half-life was determined from kinetic profiles and first order inactivation model, reaching 31.50 hours. Combi-CLEAs of glucose-oxidase and catalase showed to be a robust biocatalyst for applications in the production of gluconic acid from glucose.

Keywords: Glucose-oxidase, catalase, BSA, CLEAs, gluconic acid, glucose, reusability.

5.1 Introduction

Gluconic acid (GA) is a weak organic acid, characterized as a white powder, odorless and crystalline. The GA is very soluble in water, slightly soluble in ethanol, and insoluble in organic solvents. A solution of 50 % (w/v) GA, at 20 °C, has a pH of 1.82 and density of 1.23 $g.cm^{-3}$. The anion gluconate is Ca^{2+}, Fe^{2+}, Al^{3+}, K^{+} and Na^{+} chelating, yield calcium (anhydrous and monohydrate), sodium and potassium (anhydrous) gluconates (BROOKES & NEVILLE, 2005). The low corrosive capacity and good complexation with metal ions allow its application in food, pharmaceutical and textile industries (RAMACHANDRAN *et al*., 2006; SINGH & KUMAR, 2007; PURANE *et al*., 2012).

The glucose oxidation using enzymes as biocatalysts has been a very viable method, allowing up to 100% of glucose conversion to gluconic acid (GA). The literature reports that the enzyme glucose oxidase (β-D-glucose: oxygen-reductase 1, GOD, EC 1.1.3.4) is able to produce GA (NAKAO *et al*., 1991, 1997, 2000b). GOD is an oxidoreductase (BANKAR *et al*., 2009; WITT *et al*., 2000) that exhibits Bi-Bi Ping-Pong kinetic mechanism, as described by Leskovac *et al*. (2005). The GOD catalyzes the oxidation reaction of β-D-glucose to β-D-glucolactone, using molecular oxygen as electron acceptor. β-D-glucolactone is further hydrolyzed spontaneously (or catalyzed by gluconolactonase) to gluconic acid, releasing hydrogen peroxide (H_2O_2) as byproduct. The GOD catalysis requires the coenzyme FAD, which accepts hydrogen, forming $FADH_2$, which is sequentially regenerated to FAD. Hydrogen peroxide is GOD inhibitor, requiring its elimination from the reaction medium. H_2O_2 can also be decomposed by the action of light, as well as by enzymatic catalysis. The decomposition of H_2O_2 to water and oxygen can be catalyzed by the enzyme catalase (CAT, E.C.1.11.16) (NAKAO *et al*., 1997). CAT catalyzes the H_2O_2

decomposition following two steps. In the first step of the reaction, a H_2O_2 molecule oxidizes the ion Fe^{3+} in the prosthetic group, with the condensation of one molecule of water. In the second step, a second H_2O_2 molecule reduces the prosthetic group, which was oxidized in the first step ($O\text{-}Fe^{4+}$), generating Fe^{3+} and releasing H_2O and O_2 (SCANDALIOS, GUAN & POLIDOROS, 1997; SWITALA & LOEWEN, 2002; ADÁNYI *et al.*, 2007).

A mixture of several enzymes catalyzes reactions without producing the intermediates, allowing the process intensification and the elimination or simplification of the purification step (XUE; WOODLEY, 2012; BA *et al.* 2013; TOUAHAR *et al.* 2014). Godjevargova *et al.* (2004) proposed the GA production through glucose oxidation using an ion exchange membrane reactor where GOD and CAT were immobilized. They obtained yield and productivity of about 90 % and 0.20 $g.L^{-1}.h^{-1}$, respectively, using 1 $g.L^{-1}$ glucose. Silva *et al.* (2011) produced GA from sucrose using invertase, GOD and CAT retained in a membrane reactor with recirculation, obtaining 85 % yield. Mafra *et al*. (2014) reported a soluble multi-enzyme system composed by invertase, GOD and CAT for the production of GA from sucrose, which allowed obtaining conversion and yield of 100%. The oxygen transfer rate is a key parameter in this kind of reaction. Several works report proposals of combined processes, e.g., airlift or bubble column bioreactors, GOD co-immobilized with MnO_2, precipitation of GA as calcium gluconate, and thereby increase the oxygen transfer rate, as well as decrease GOD inhibition by oxygen peroxide and gluconic acid (NAKAO *et al*., 1991, 1997, 2000b; BAO *et al*., 2000, 2001a,b, 2003, 2004).

The use of the multi-enzyme system in the immobilized form could contribute to reduce operational costs, because if the combined biocatalyst is thermal

and mechanically stable it can be recovered and reused in batch processes or used for longer time in continuous processes (XUE; WOODLEY, 2012). Carrier-free immobilized enzymes by aggregation and crosslinking (CLEAs) have been studied as a promising technique for co-immobilization of two or more enzymes (Combi-CLEAs) (SHELDON, 2007; TALEKAR *et al*., 2013; DALAL *et al*., 2007). The crosslinking of enzyme aggregates is a simple methodology for enzyme immobilization based in the non-denaturing precipitation of the enzyme protein and its further crosslinking with bifunctional reagents, not requiring the previous purification of the enzyme preparation (CAO, 2006).

This work focused on the preparation of Combi-CLEAs of glucose oxidase from *Aspergillus niger* and catalase from bovine liver and its application in gluconic acid production. The Combi-CLEAs were prepared by co-precipitation and crosslinking of GOD, CAT and bovine serum albumin (BSA). The effects of precipitant agents (organic and inorganic salts), crosslinker (glutaraldehyde) and feeder protein (BSA) concentrations on the activities of each enzyme were investigated. Additionally, the stability of soluble enzymes and derivatives were studied at 40^{o}C and pH 6.0. Combi-CLEAs were used in GA production in a pneumatic reactor. The reusability of Combi-CLEAs was also studied in ten 5h-batches of GA production.

5.2 Material and methods

5.2.1 Material

Aspergillus niger glucose oxidase (GOD, EC 1.1.3.4) was donated by Granotec (Curitiba, PR, Brazil), bovine liver catalase (CAT; EC:1.11.1.6) and bovine

serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO, EUA). For Combi-CLEAs and Combi-BSA-CLEAs syntheses and for the evaluation of their properties, dimethoxyethane (DME) from Fluka (St. Louis, MO, EUA), tert-butyl alcohol (TBA) and ammonium sulfate salt (AS) from Vetec (Duque de Caxias, RJ, Brazil), and glutaraldehyde aqueous solution (25 % w/v) from Sinth (Diadema, SP, Brazil) were used. Other reagents were of analytical grade.

5.2.2 Methods

5.2.2.1 GOD and CAT activity assays

Activities of soluble GOD and GOD in the Combi-CLEAs were determined by oxygen consumption (initial rates) in the oxidation of a 55 mM glucose solution at 30 °C and pH 6.0 (50 mM sodium phosphate buffer).

Activities of soluble CAT and CAT in the Combi-CLEAs were determined by oxygen production (initial rates) in the oxidation of a 55 mM H_2O_2 solution at 30 °C and pH 6.0 (50 mM sodium phosphate buffer).

A volume of 1 mL of a solution of GOD/CAT or 2 mL of a suspension of Combi-CLEAs was added to 200 mL of substrate in a stirred and thermostatically controlled reactor. A sterilizable amperometric electrode (Model InPro 6800, Mettler Toledo), linked to a silicone membrane (Model InProT96, Mettler Toledo) and mounted in the middle of circular section of the reactor, was used to measure the change in oxygen concentration through the time. The oxygen concentration signal was recorded by a data acquisition system at 1 second intervals, for 3 min. The initial rate of oxygen decomposition was calculated from the slope of the curve oxygen concentration versus time.

One GOD and CAT units were defined as the amount of enzyme that catalyzes the decomposition or production of 1 μmol of O_2 per minute, respectively.

5.2.2.2 Protein concentration

Lowry (1951) method was used to measure protein concentration in the preparation of enzymes. The protein content was spectrophotometrically quantified at 750 nm using bovine serum albumin (BSA) as protein standard.

5.2.2.3 Combi-CLEA preparation

5.2.2.3.1 Selection of the precipitant agent

Dimethoxyethane, tert-butyl alcohol and saturate ammonium sulfate were evaluated as precipitant agent. 1 mL of each precipitant was slowly added in 1 mL of sodium phosphate buffer (100 mM, pH 7.0) prepared with 10 mg of GOD protein and 1 mg of CAT protein. All preparations were made with ice-cold solutions (~ 10 ºC). A set of Eppendorf tubes (2 mL) was stirred at 3000 rpm in a vortexer (QL 901, Biomixer), for 1 min. After 3 h of aggregation/precipitation at 4 ^{o}C and 200 rpm with the Eppendorf tubes lying in an incubator shaker (SL 221, Solab), the aggregate suspensions were centrifuged at 10,000 × g for 10 min at 4 ºC (5810R, Eppendorf Centrifuge). The supernatant was removed, the precipitate was re-suspended in 2 mL of sodium phosphate buffer (100 mM, pH 7.0). Comi-CLEAs were stored at 4 ^{o}C for measurement of GOD and CAT activities as described in enzymes activities assay section.

5.2.2.3.2 Evaluation of BSA and glutaraldehyde concentrations in Combi-CLEAs preparations

Combined cross-linked enzyme aggregates (Combi-CLEAs) were prepared dissolving 10 mg of GOD protein and 1 mg of CAT protein in 1 mL of

sodium phosphate buffer (100 mM, pH 7.0). Next, 1 mL of precipitant (previously selected) was added for protein aggregation. After 1 min of mixing at 3000 rpm in a vortexer (QL 901, Biomixer), glutaraldehyde was slowly added to the final concentration of 25, 50, 100 and 200 mM. When Combi-CLEAs were prepared using feeder protein, bovine serum albumin (BSA) was added in the enzyme solution at concentrations of 15, 30 and 60 mg/mL before the addition of the precipitant reagent. All preparations were made with ice-cold solutions (~ 10 ºC). After 3 h of crosslinking at 4 ^{o}C and 200 rpm in an incubator shaker (SL 221, Solab), the suspensions containing Combi-CLEAs with and without BSA were centrifuged at 10,000 × g for 10 min at 4 ºC (5810R, Eppendorf Centrifuge). Combi-CLEAs were recovered as pellets and washed twice with 100 mM sodium phosphate buffer (pH 7.0). After preparation, the Combi-CLEAs were stored in the same buffer (2 mL) at 4 ºC for further use. The GOD and CAT activities were calculated as described in the enzymes activity assay section and the immobilization yield (*Y*) was calculated separately as follows:

$$Y(\%) = \frac{A_e(U)}{A_i(U)} \times 100 \quad (5.1)$$

Where A_e is the total activity of GOD or CAT in Combi-CLEAs; A_i is the total GOD or CAT activity used for Combi-CLEAs production.

5.2.2.4 Stability of Combi-CLEAs

Thermostability of soluble and immobilized biocatalyst were evaluated at 40 ^{o}C and pH 6.0 (100 mM sodium phosphate buffer) for 5 h at 250 rpm, in an incubator shaker (SL 221, Solab). For all assays, the initial activity was taken as 100 %. The retained activity was calculated as follows:

$$RA(\%) = \frac{A_T(U)}{A_i(U)} \times 100 \quad (5.2)$$

Where A_T is the total activity of the Combi-CLEAs of GOD or CAT measured after the stability assay; A_i is the total GOD or CAT activity used for CLEAs production.

5.2.2.5 Gluconic acid production and operational stability

Production of GA from glucose was performed in a pneumatic reactor of 0.1 L volume. Three batch experiments with initial glucose concentrations of 26 $g.L^{-1}$ were performed at 40 ^{o}C and pH 6.0 (controlled by the addition of 0.35 M calcium carbonate suspension). A specific air flow rate of 10 vvm (1 $L.min^{-1}$), controlled by a mass flow meter (Model GFC37, Aalborg), was used to ensure an excess of dissolved oxygen. Samples were removed at 1-h intervals to determine the glucose and GA concentrations. The optimum ratios for the enzymes (GOD and CAT)/substrate (glucose) were 4 and 0.04 wt %, respectively, determined as described by Mafra *et al*. (2015).

5.2.2.8 Kinetic of Combi-CLEAs

Based on the conversion of glucose, the reaction time was evaluated using the kinetic parameters (K_m and k_{cat}) estimated using the integrated Michaelis-Menten model (Eq. 5.3). First order enzyme inactivation model was also fitted to the reuse data to determine the half-life time (Eq. 5.4).

$$C_{S0} + K_m \cdot (lnC_{S0} - lnC_S) - C_{et} \cdot t \cdot k_{cat} = C_S \quad (5.3)$$

$$\frac{dC_{et}}{dt} = -k_d . t \quad (5.4)$$

where, k_{cat} is the turnover number (min^{-1}), K_m is the Michaelis-Menten constant (M), C_s is the substrate concentration (M), C_{et} is the enzyme concentration (M) and k_d is the inactivation constant (min^{-1}) and t is the time (min).

5.2.2.9 SEM

The surface morphology of Combi-CLEAs was obtained by scanning electron microscopy (SEM). Micrographs were taken on a JSM 6510/JEOL (10KV) SEM instrument.

5.3 Results and discussion

5.3.1 Selection of conditions for preparing the Combi-CLEAs

Different variables were analyzed to determine the best conditions for preparing GOD-CAT Combi-CLEAs: precipitant agent, concentration of glutaraldehyde; and feeder protein (BSA). Combi-CLEA evaluation was based on the immobilization yield of glucose-oxidase (GOD) and catalase (CAT).

5.3.1.1 Selection of the precipitant agent

Enzyme aggregates are produced by changing the hydration state of the enzyme molecules or by changing the electrostatic constant of the solution by adding a suitable aggregation agent (SCHOEVAART *et al.*, 2004). Different precipitants were tested to precipitate 10 mg of GOD with 1 mg of CAT: dimethoxyethane (DME), tert-butyl alcohol (TBA) and saturate ammonium sulfate solution (AS). After precipitation, the aggregates were centrifuged, supernatant was removed and the precipitate was re-suspended in sodium phosphate buffer (100 mM, pH 7.0). The activities after precipitation were measured for each enzyme. Table 5.1 shows the results in percentage of initial activity. DME yielded the best precipitation for GOD and CAT, yielding 98.3 and 87.6 % of the initial activity respectively. Similar results were obtained by Schoevaart *et al.* (2004), precipitating lipase, laccase, trypsin, phytase, glucose-oxidase, galactose-oxidase and β-galactosidase. Mafra *et*

al. (2016) evaluated different precipitants for precipitation of bovine liver catalase (20 mg) and showed very similar results with the same precipitants used in this work.

Table 5.1 – Effect of the precipitant agent on the activities of GOD (glucose-oxidase) and CAT (catalase) after precipitate re-suspension.

Precipitant agent	Precipitate activity GOD (%)	Precipitate activity CAT (%)
DME	98.3 ± 9.34	87.6 ± 8.65
TBA	80.6 ± 7.08	77.4 ± 6.88
AS	5.62 ± 0.93	90.2 ± 9.11

5.3.1.2 Selection of crosslinking conditions

Immobilization of multimeric enzymes by CLEAs has been reported as a successful technique, as in the case of laccase (CABANA *et al*., 2007), bovine liver catalase (WILSON *et al*., 2004; MAFRA *et al*., 2016) and Penicillin G Acylase (WILSON *et al*., 2006; SHAH *et al*., 2006). However, as far as we know, this is the first work concerning the co-immobilization of GOD and CAT by Combi-CLEAs technique. Combi-CLEAs of glucose-oxidase and catalase were precipitated with DME and crosslinked with glutaraldehyde (GLU) concentration ranging from 25 to 200 mM. GOD and CAT activities were evaluated as shown in Figure 5.1-A,B. Activities of GOD and CAT in Combi-CLEAs increased with increasing GLU concentration, with the maximum activity (505.9±45.5 and 3806.6±342.4 U/mL, respectively) obtained at 50 mM concentration (without BSA addition) (Tables 5.1S and 5.2S). For both enzymes, with further increase in the GLU concentration, the activity began to decrease. This activity reduction could be attributed to excessive crosslinking, resulting in the loss of enzyme flexibility, hindering the activity of the

catalytic site (SHAH *et al*., 2006; REHMAN *et al*., 2016). On the other hand, this reduction may be caused by strong diffusional limitations by the unwanted crosslinking (SHAH *et al*., 2006; AHUMADA *et al*., 2015; REHMAN *et al*., 2016; MAFRA *et al*., 2016).

5.3.1.3 Selection of BSA concentration

It has been reported that co-aggregation with BSA may improve the stability of CLEAs by favoring a more efficient crosslinking of the enzyme molecules (DONG *et al*., 2010; AYHAN *et al*., 2011; MAFRA *et al*., 2016). Therefore, glutaraldehyde and BSA concentration were evaluated regarding GOD and CAT activities in the Combi-CLEAs (Figure 5.1-A,B and Tables 5.1S and 5.2S).

Independent of GLU/Enzymes ratios, the addition of BSA increased or preserved the immobilization *Yields* of GOD and CAT compared with the Combi-CLEAs without BSA. The co-aggregation with BSA increased substantially the GOD and CAT activities (Figure 5.2-A), as previously reported (CABANA *et al.*, 2007; SHAH *et al*., 2006; DONG *et al.*, 2010). This increase may be explained by the increase in the crosslinking efficiency due to increase in the amount of lysine residues by the addition of BSA (SHAH *et al*., 2006; AHUMADA *et al*., 2015). It can be observed that the increase of the ratio GLU/total protein did not increase the immobilization yield. In fact, the best GLU/total protein ratio of 0.70 µmol/mg (25 mM GLU and 60 mg BSA) and the worst of 36.36 µmol/mg (200 mM GLU and without BSA) was obtained for both enzymes. The concentrations of enzyme plus feeder protein and GLU must be carefully studied to obtain a suitable CLEA. Migneault *et al*. (2004) emphasize that low concentration of enzyme plus feeder protein and high GLU tend to induce intramolecular crosslinking of relevant enzyme residues, justifying the results of this work.

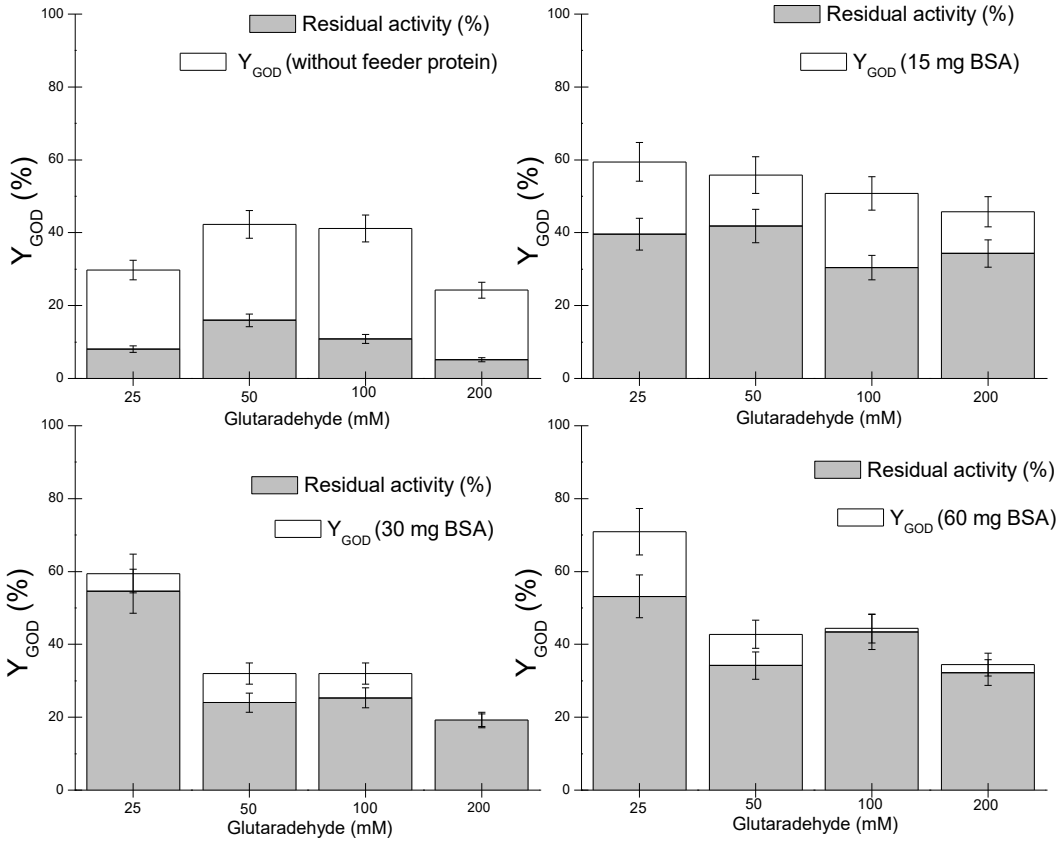
5.3.1.4 Thermo-stability of Combi-CLEAs

Stability was the main variable to evaluate the preparation of Combi-CLEAs of GOD and CAT, since the aim of this work was its use/reuse in GA production. For this, all the Combi-CLEAs and soluble enzymes were incubated at pH 6.0, 40 ^{o}C, shaked at 250 rpm for 5 hours (Figure 5.1-A,B, gray bars). These conditions were previously (MAFRA *et al*., 2015) for soluble GOD and CAT as non-denaturing conditions: ~98 % of their initial activities (GOD = 1,200 U/mL and CAT = 7,870 U/mL) were preserved. As shown in Figure 5.1-A in gray bars and Table 5.1S, the increase of GOD activities after the stability test of Combi-CLEAs with BSA, is ranged 3 to 5 times. Other authors have also reported the increase of stability in CLEAs with feeder protein (CABANA *et al.*, 2007; SHAH *et al*., 2006; AHUMADA *et al*., 2015; MAFRA *et al.*, 2016). Figure 5.2-A,B shows that 60 mg of BSA and 25 mM of glutaraldehyde enabled preparing a Combi-CLEA of GOD/CAT with the best combined yield, after the stability test. This biocatalyst was able to retain more than 50% and 60 % of GOD and CAT activities after the stability test, respectively. Therefore, this biocatalyst was further used.

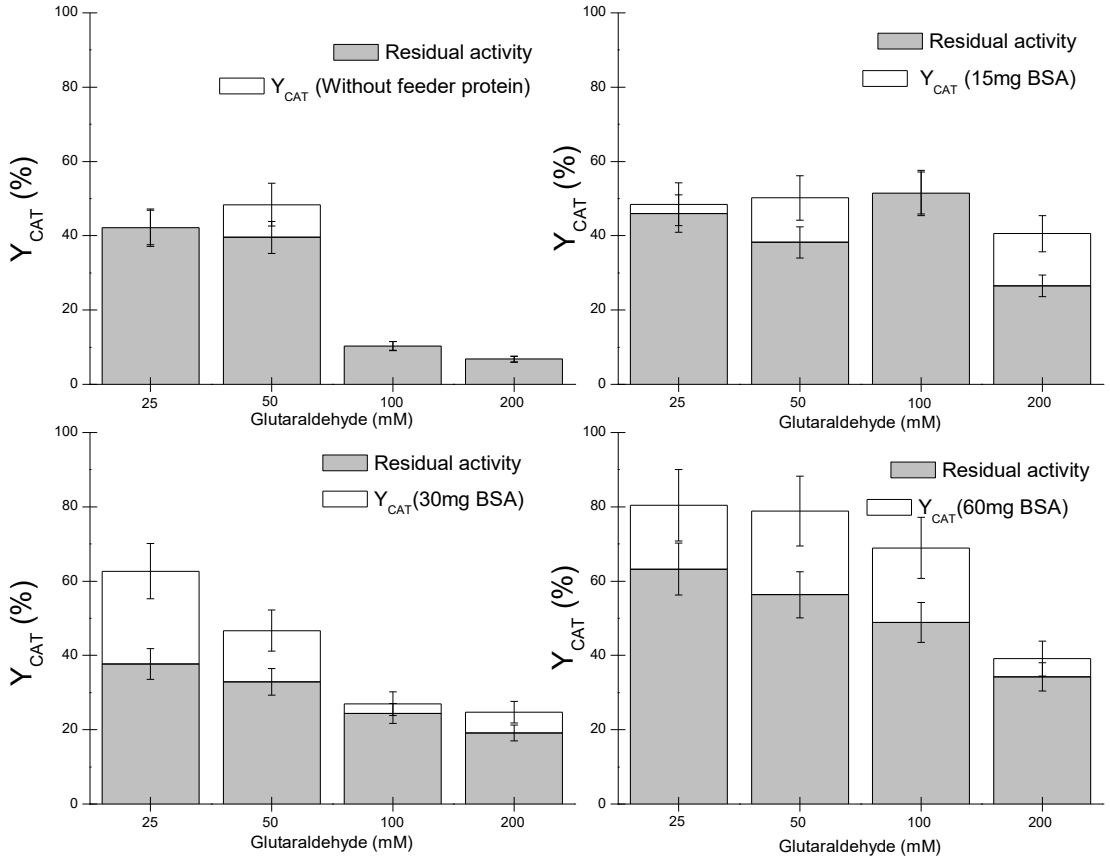
According to Schoevaart *et al.* (2004) particle shape and size is the major unexplored property of CLEAs, and the final aggregate size is product of the ratio between velocity of nucleation and growth of CLEAs. Scanning electron microscopy (SEM) showed a very uniform structure of the aggregates (Figure 5.1S). The Feret's statistical geometric diameters were calculated using microscopy images and ImageJ software. The histogram of particle size distribution using step size of 10 µm is showed in Figure 5.2S. The most of particle of Combi-CLEAs of GOD/CAT had diameter up to 10 µm.

Figure 5.1 – (White) Immobilization yield of GOD (A) and CAT (B) in the Combi-CLEAs in the presence and absence of BSA as feeder protein. (Black) Residual activities of the GOD in the Combi-CLEAs after 5h at 40 ^{o}C and pH 6.0.

(A)



(B)


Residual activity
Y CAT (Without feeder protein)
Residual activity
Y CAT (15mg BSA)
Residual activity
Y CAT(30mg BSA)
Residual activity
Y CAT(60mg BSA)
Y CAT (%)
Glutaraldehyde (mM)
0
20
40
60
80
100
25
50
100
200

5.3.2 GA production

GA production was carried out in a pneumatic reactor with a volume of 0.1 L, operated at 10 vvm, 40 ^{o}C, and pH 6.0, as shown in Figure 5.2 in the first batch. The pH was controlled by the addition of calcium carbonate suspension 0.35 M to neutralize the GA produced. The experiments were carried out using Combi-CLEAs GOD/CAT with 847.7 ± 76.3 U.mL^{-1} and 6329.2 ± 547.5 U.mL^{-1}. It is well known that the addition of catalase in excess to the immobilized system reduces the inactivation effects since hydrogen peroxide is a substrate of the catalase (GODJEVARGOVA *et al.* 2004). In a previous work, it was determined that the optimum ratio for the enzymes/substrate (glucose) was 4 and 0.04 w/w (%), for GOD and CAT, respectively (MAFRA *et al.*, 2015). Thus, the Combi-CLEAs were produced with 10-fold more activity than necessary to maintain the H_2O_2 concentration below 35 mM throughout the reaction and prevent the inhibition of CAT by its substrate (MAFRA *et al.*, 2015, 2016).

Figure 5.2 – Gluconic acid production from 26 g.L^{-1} glucose solution catalyzed by the Combi-CLEAs GOD/CAT in a pneumatic reactor kept at constant 40 ^{o}C and pH 6.0. (A) Time-conversion profiles; (B) conversions after each 5 h-batch for the reuse assays.

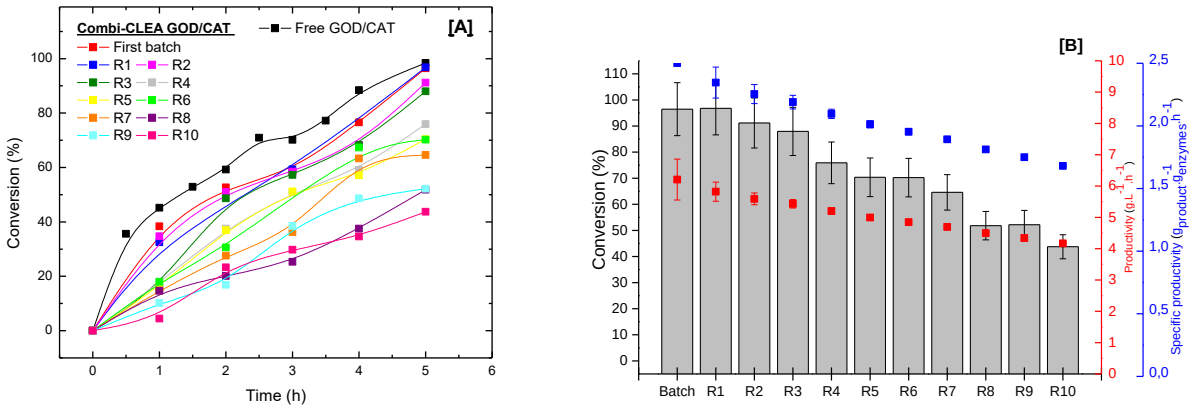


Table 5.2 – Productivity of gluconic acid catalyzed by GOD/CAT immobilized by different methods.

Immobilized catalyst and bioreactor	GA productivity ($g.L^{-1}.h^{-1}$)	Specific productivity ($g_{product}.h^{-1}.g_{biocatalyst}^{-1}$)	Reuses	Time of process (h)	Reference
GOD immobilized within calcium alginate beads and palladium particles (airlift reactor)	3.84	1.12	-	5	Nakao *et al.* (1997)
GOD immobilized on anion-exchange membrane (membrane reactor)	0.16	59.7	-	6.7	Hestekin *et al.* (2002)
GOD/CAT immobilized on anion-exchange membrane (membrane reactor)	0.18	0.0542	-	6	Godjevargova *et al.* (2004)
Immobilized *Aspergillus niger* (fermenter)	1.4	0.31	-	50	Mukhopadhyay *et al.* (2005)
INV/GOD/CAT confined in an ultrafiltration membrane reactor	1.42	0.4	-	1	Silva *et al*. (2011)
Combi-CLEA GOD/CAT (pneumatic reactor)	4.17	1.7	10	55	Present work (reactor airlift)

The conversion of glucose to gluconic acid obtained in the first batch was 100 % (Figure 5.2), with specific productivity of 2.50 $g_{product}.g_{enzyme}^{-1}.h^{-1}$. Few works have proposed the immobilization of GOD and CAT due to the difficulty of combining the immobilization conditions to both enzymes, as shown in Table 5.2. Godjevargova *et al.* (2004) proposed the co-immobilization of GOD plus CAT on copolymer membrane, recovering almost 70 % of GOD activity (similar of this work). Also, high amounts of gluconic acid were obtained, reaching 95% yield and specific productivity of 0.0542 $g_{product}.g_{enzyme}^{-1}.h^{-1}$. Silva *et al*. (2011) evaluated the oxidation of glucose in a batch process using free GOD and CAT confined in an ultrafiltration membrane, obtaining specific productivity of 0.4 $g_{product}.g_{enzyme}^{-1}.h^{-1}$ and yield of 100 %. As can be seen, the system utilizing Combi-CLEAs GOD/CAT exhibited the highest specific productivity (around 6 to 46 times higher than those previously reported).

5.3.3 Reusability

The advantage of immobilized enzymes is the separation from the reaction medium for reuse. The reusability of Combi-CLEAs was examined in ten 5-hour cycles, with each cycle run in one day (total of 10 days), as shown in Figure 5.2-A,B. It is noteworthy that the reusability of CLEAs or Combi-CLEAs is rarely reported in the literature; only reusability for activity measurement assays are commonly reported (DALAL *et al*., 2007; TUKEL *et al*., 2013; JUNG *et al*., 2013; CHMURA *et al*., 2013). After each cycle, the Combi-CLEAs were separated from the reaction medium by decantation and the medium was removed by pumping. The yield of each batch was well-maintained (> 70 %) up to five 5h-cycles (Figure 5.2-B), even in the

presence of probable loss of enzyme by leaching or very low thermal inactivation at the reaction conditions (40 ^{o}C and pH 6.0).

5.3.4 Kinetic parameters

Soluble and Combi-CLEA GOD/CAT prepared in this work were kinetically characterized. An integrated Michaelis-Menten model was fitted to the experimental data of the first batch of consumption of glucose vs. time catalyzed by a mixture of soluble GOD/CAT and Combi-CLEA GOD/CAT (showed in Figure 5.3) at 40 ^{o}C and pH 6.0. Apparent global parameters of K_m and k_{cat}, were fitted, since there are two reactions occurring simultaneous in the gluconic acid production. The values of kinetic parameters and R^2 of soluble and immobilized GOD/CAT are presented in Table 5.3.

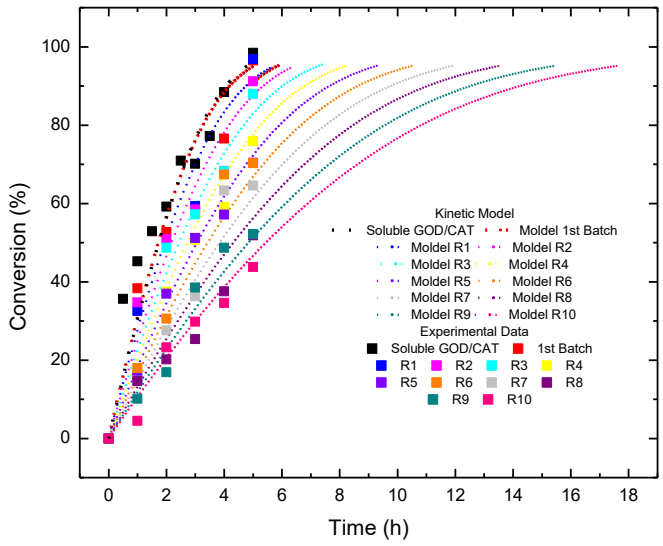
Table 5.3 – Kinetic parameters of soluble and immobilized GOD/CAT fitting an integrated Michaelis-Menten model to the experimental data

GOD/CAT	**$k_{cat,app}$ (h^{-1})**	**$K_{m,app}$ (mM)**	**R^2**
Soluble	$1.16 \pm 0.12 \times 10^{4}$	51.11 ± 13.44	0.95
Combi-CLEAs	$4.37 \pm 0.43 \times 10^{2}$	65.78 ± 13.40	0.90

$K_{m,app}$ values of soluble and Combi-CLEAs GOD/CAT were estimated as 51.11 ± 13.44 mM and 65.78 ± 13.40 mM glucose, respectively. The similar $K_{m,app}$ value of the soluble and Combi-CLEAs GOD/CAT could may indicate the absence of conformational changes in the tridimensional structure of the enzymes, during the immobilization process. The second reaction of hydrogen peroxide breaking by CAT is faster than the first reaction of oxidation of glucose by GOD. So, it could be inferred that the apparent constant represents the occurrence of the first reaction (oxidation of glucose by GOD). Then the obtained $K_{m,app}$ can be compared with K_m reported for

Aspergillus niger glucose-oxidase soluble and immobilized in the literature, which were similar and of the same order of magnitude (CONSTANTINIDES *et al*. 1973; SZAJANI *et al*. 1987; BHATTI *et al.* 2006; WANG *et al.* 2010; COURJEAN AND MANO, 2011).

Figure 5.3 – Experimental data of reuse of Combi-CLEAs GOD/CAT (R1 to R10) in the gluconic acid production from glucose in a batch reactor kept at 40 ^{o}C and pH 6.0. The curves represent the Michaelis-Menten integrated model fitted to the experimental data of free GOD/CAT ($K_{m,app}$ = 51.11 ± 13.44 mM and $k_{cat,app}$ = 1.16·± 0.12 x 10^4 h^{-1}), and Combi-CLEA GOD/CAT ($K_{m,app}$ = 65.78 ± 13.40 mM and $k_{cat,app}$ = 4.37·± 0.43 x 10^3 h^{-1}) combined to the first order equation of enzyme inactivation (k_d = 0.022 h^{-1}).



The apparent turnover number ($k_{cat,app}$) obtained for the Combi-CLEAs GOD/CAT was only 4% of $k_{cat,app}$ of soluble GOD/CAT. Reduction on k_{cat} parameter is commonly observed for immobilized enzymes (DALAL *et al*., 2007; TOUAHAR, 2014; LI *et al*., 2016; MAFRA *et al*., 2016). This result should be attributed to the difficult to substrate and products diffusion into CLEAs (MAFRA *et al*., 2016).

5.3.5 Operational half-life

Half-life of the biocatalyst is a parameter related with the economic feasibility of the bioprocess (LIMA-RAMOS *et al.,* 2014). According to Talekar *et al.* (2013), in classical experiments of measurement of decay activity versus time, CLEAs could appear to be much less sensitive to the decay of enzyme activity, due to the mass transfer limitations. These limitations may affect the measurement of the initial activity when all enzyme molecules are totally active, causing a false low activity at the beginning of the experiment. And during the half-life experiment, the enzymes that are being inactivated are replaced by those that were impossible to measure, thus falsely creating an impression of stabilization. To avoid such misinterpretation, Talekar *et al.* (2013) suggest the evaluation of productivity (units/kg of the product).

Therefore, we have chosen to evaluate the Combi-CLEA in several respects regarding stability: activity after stability (Figure 5.1), productivity and reusability (Figure 5.2-B) and half-live. To evaluated the half-life, the apparent parameters ($K_{m,app}$ and $k_{cat,app}$) fitted to the experimental data of the first batch of glucose conversion catalyzed by Combi-CLEA GOD/CAT were fixed, and a first order equation of enzyme inactivation (k_d) was fitted to the experimental data of reuse to determine the operational half-life (Figure 5.3). The estimated half-life, 31.50 h, was similar in magnitude to the CLEAs half-lives reported previously (KUMAR *et al*., 2012; KIM *et al.,* 2013).

5.4 Conclusions

Aspergillus niger GOD and bovine liver CAT were first immobilized using the Combi-CLEAs technique. The immobilization yields of Combi-CLEAs

showed promising yields (> 70 %). The Combi-CLEAs exhibited good stability under harsh temperature and pH conditions. The evaluation of Combi-CLEAs in gluconic acid production in a pneumatic reactor showed conversion of 100 %, and it was possible to reuse the biocatalyst five times, maintaining conversions above 80 %. This biocatalyst showed an operational half-life of 31.50 hours.

5.5 Acknowledgements

This work was supported by the National Council for Scientific and Technological Development (CNPq) (grant numbers 141647/2013-2 and 402850/2013-0), and the Federal Foundation CAPES. The authors thank Granotec for the donation of *Aspergillus niger* glucose oxidase. The authors also thank Embrapa Instrumentation (São Carlos, SP, Brazil) for granting the use of SEM equipment, Prof. Monica Lopes Aguiar from the Laboratory of Environmental Control (DEQ/UFSCar) for the microscopy images of CLEAs and Prof. Alberto Colli Badino from Laboratory of Fermentation (DEQ/UFSCar) for the pneumatic reactors and oxygen measuring equipment used in this study.

5.6 Reference

ADÁNYI, N. *et al.* Hydrogen Peroxide Producing and Decomposing Enzymes: their Use in Biosensors and other Applications. **Industrial Enzymes**, p. 441-459, 2007.

AHUMADA, K. *et al.* Production of combi-CLEAs of glycosidases utilized for aroma enhancement in wine. **Food and Bioproducts Processing**, v. 94, p. 555–560, 2015.

AYHAN, F.; DOĞAÇ, Y. I.; AYHAN, H. Cross-Linked Glucose Oxidase Aggregates: Synthesis and Characterization**. Hacettepe Journal of Biology and Chemistry**, v. 39, n. 3, p. 241 - 251, 2011.

BA, S. *et al.* Synthesis and characterization of combined cross-linked laccase and tyrosinase aggregates transforming acetaminophen as a model phenolic compound in wastewaters. **Science of The Total Environment**, v. 487, p. 748–755, 2014.

BANKAR, S. *et al.* Glucose oxidase - An overview. **Biotechnology Advances**, v. 27, n. 4, p. 489-501, 2009.

BAO, J. *et al.* Average and local oxygen transfer properties in bubble column with axial distribution of immobilized glucose oxidase gel beads. **Chemical Engineering Science**, v. 55, n. 22, p. 5405–5414, 2000.

BAO, J. *et al.* A kinetic study on air oxidation of glucose catalyzed by immobilized glucose oxidase for production of calcium gluconate. **Biochemical Engineering Journal**, v. 8, n. 2, p. 91–102, 2001a.

BAO, J. *et al*. Competitive inhibition by hydrogen peroxide produced in glucose oxidation catalyzed by glucose oxidase. **Biochemical Engineering Journal**, v. 13, n. 1, p. 69–72, 2003.

BAO, J. *et al.* Optimal operation of an integrated bioreaction–crystallization process for continuous production of calcium gluconate using external loop airlift columns. **Biochemical Engineering Journal,** v. 56, n. Issues 21–22, p. 6165–6170, 2001b.

BAO, J. *et al.* A kinetic study on crystallization of calcium gluconate in external loop airlift column and stirred tank for an immobilized glucose oxidase reaction with crystallization. **Biochemical Engineering Journal**, v. 15, n. 3, p. 177–184, 2003.

BROOKES, G. C.; NEVILLE, K. B. **An analysis of labeling requirements, market dynamics and cost implications**. The Global GM Market — implications for the European food chain. UK: PG Economics Limited, 2005.

CABANA, H.; JONES, J. P.; AGATHOS, S. N. Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. **Journal of Biotechnology**, v. 132, n. 1, p. 23–31, 2007.

CAO, L. **Carrier-bound Immobilized Enzymes**. Wiley. p.578, 2006.

CHMURA, A. The combi-CLEA approach: enzymatic cascade synthesis of enantiomerically pure (S)-mandelic acid. **Tetrahedron: Asymmetry**, v. 24, p. 1225–1232, 2013.

CONSTANTINIDES, A.; VIETH, W.R.; FERNANDES, P.M. Characterization of glucose oxidase immobilized on collagen. **Molecular and Cellular Biochemistry**. v.1, p. 127-133, 1973.

COURJEAN, O.; MANO, N. Recombinant glucose oxidase from Penicillium amagasakiense for efficient bioelectrochemical applications in physiological conditions. **Journal of Biotechnology**, v.151, p. 122-129, 2011.

DALAL, S.; KAPOOR, M.; GUPTA, M. N. Preparation and characterization of combi-CLEAs catalyzing multiple non-cascade reactions. **Journal of Molecular Catalysis B-Enzymatic**, v. 44, n. 3-4, p. 128-132, 2007.

DONG, T. *et al.* Preparation of cross-linked aggregates of aminoacylase from Aspergillus melleus by using bovine serum albumin as an inert additive. **Bioresource Technology**, v. 101, n. 16, p. 6569–6571, 2010.

GODJEVARGOVA, T.; DAYAL, R.; TURMANOVA, S. Gluconic acid production in bioreactor with immobilized glucose oxidase plus catalase on polymer membrane adjacent to anion-exchange membrane. **Macromolecular Bioscience**, v. 4, n. 10, p. 950-956, 2004.

HEINRICH, M.; REHM, H. J. Formation of gluconic acid at low pH-values by free and immobilized Aspergillus niger cells during citric acid fermentation. **European journal of applied microbiology and biotechnology**, v. 15, n. 2, p. 88-92, 1982.

HESTEKIN, J. *et al.* Electrochemical enhancement of glucose oxidase kinetics: gluconic acid production with anion exchange membrane reactor. **The Journal of Applied Electrochemistry**, v. 32, p. 1049–1052, 2002.

JUNG, D. *et al.* One-pot bioconversion of sucrose to trehalose using enzymatic sequential reactions in combined cross-linked enzyme aggregates. **Bioresource Technology**, v. 130, p. 801–804, 2013.

KIM, D.; KIM, H. Continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an oxidoreductase of *Zymomonas mobilis* and inulinase. **Biotechnology and Bioengineering**, v. 39, n. 3, p. 336-342, 1992.

KIM, M. H. *et al*. Immobilization of formate dehydrogenase from *Candida boidinii* through cross-linked enzyme aggregates. **Journal of Molecular Catalysis B: Enzymatic**, v. 97, p. 209 - 214, 2013.

KUMAR, V. V. *et al*. Preparation and characterization of porous cross linked laccase aggregates for the decolorization of triphenyl methane and reactive dyes. **Bioresource Technology**, v. 119, p. 28 - 34, 2012.

LI, S. et al. Preparation and characterization of cross-linked enzyme aggregates (CLEAs) of recombinant thermostable alkylsulfatase (SdsAP) from *Pseudomonas sp*. S9. **Process Biochemistry**, v. 51, n. 12, p. 2084–2089, 2016.

LIMA-RAMOS, J.; TUFVESSON, P.; WOODLEY, J. M. Application of environmental and economic metrics to guide the development of biocatalytic processes. **Green Processing and Synthesis**, v. 3, n. 3, p. 195-213, 2014.

LIU, J.; CUI, Z. Optimization of operating conditions for glucose oxidation in an enzymatic membrane bioreactor. **Journal of Membrane Science**, v. 302, n. 1–2, p. 180–187, 2007.

LOWRY, O. H. *et al.* Protein measurement with the folin phenol reagent. **Journal of Biological Chemistry**, v. 193, n. 1, p. 265-275, 1951.

MAFRA, A. C. O. *et al.* Gluconic acid production from sucrose in an airlift reactor using a multi-enzyme system. **Bioprocess and Biosystems Engineering**, v. 38, n. 4, p. 671-680, 2015.

MIRÓN, J. *et al.* A mathematical model for glucose oxidase kinetics, including inhibitory, deactivant and diffusional effects, and their interactions. **Enzyme and Microbial Technology**, v. 34, n. 5, p. 513–522, 2004.

MUKHOPADHYAY, A. R. *et al.* Production of gluconic acid from whey by free and immobilized *Aspergillus niger*. **The International Dairy Journal**, v.15, p. 299 – 303, 2005.

NAKAO, K. *et al.* Measurement of oxygen transfer properties using oxidation of glucose with air catalyzed by glucose oxidase. **Kagaku Kogaku Ronbunshu**, v. 17, p. 873-881, 1991.

NAKAO, K. *et al.* Production of gluconic acid with immobilized glucose oxidase in airlift reactors. **Chemical Engineering Science**, v. 52, n. 21-22, p. 4127-4133, 1997.

NAKAO, K. *et al.* Measurement and prediction of axial distribution of immobilized glucose oxidase gel beads suspended in bubble column. **Journal of Chemical Engineering of Japan**, v. 33, n. 5, p. 721-729, 2000b.

PURANE, N. K. *et al.* Gluconic Acid Production from Golden Syrup by Aspergillus niger Strain Using Semiautomatic Stirred-Tank Fermenter. **Journal of Microbial & Biochemical Technology**, v. 4, n. 4, p. 092 - 095, 2012.

RAMACHANDRAN, S. *et al.* Gluconic acid: Properties, applications and microbial production. Food Technology and Biotechnology, v. 44, n. 2, p. 185-195, 2006.

REHMAN, S. *et al*. Cross-linked enzyme aggregates (CLEAs) of Pencilluim notatum lipase enzyme with improved activity, stability and reusability characteristics. **International Journal of Biological Macromolecules**, v. 91, p. 1161–1169, 2016.

SCANDALIOS, J. G. **Oxidative stress and the molecular biology of antioxidant defenses**. Plainview, N.Y.: Cold Spring Harbor Laboratory Press, p. 890, 1997

SCHOEVAART, R. *et al.* Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). **Biotechnology and Bioengineering**, v. 87, n. 6, p. 754-762, 2004.

SHAH, S.; SHARMA, A.; GUPTA, M. N. Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. **Analytical Biochemistry**, v. 351, n. 2, p. 207–213, 2006.

SHELDON, R. A. Enzyme Immobilization: The Quest for Optimum Performance. **Advanced Synthesis & Catalysis**, v. 349, n. 8-9, p. 1289-1307, 2007.

SILVA, A. R.; TOMOTANI, E. J.; VITOLO, M. Invertase, glucose oxidase and catalase for converting sucrose to fructose and gluconic acid through batch and membrane-continuous reactors. **Brazilian Journal of Pharmaceutical Sciences**, v. 47, n. 2, p. 399-407, 2011.

SILVEIRA, M. M. *et al.* Bioconversion of glucose and fructose to sorbitol and gluconic acid by untreated cells of *Zymomonas mobilis*. **Journal of Biotechnology**, v. 75, n. Issues 2–3, p. 99–103, 1999.

SINGH, O. V.; KAPUR, N.; SINGH, R. P. Evaluation of agro-food byproducts for gluconic acid production by *Aspergillus niger* ORS-4.410. **World Journal of Microbiology and Biotechnology**, v. 21, n. 4, p. 519-524, 2005.

SINGH, O. V.; KUMAR, R. Biotechnological production of gluconic acid: future implications. **Applied Microbiology and Biotechnology**, v. 75, n. 4, p. 713-722, 2007.

SWITALA, J.; LOEWEN, P. Diversity of properties among catalases. **Archives of Biochemistry and Biophysics**, v. 401, n. 2, p. 145-154, 2002.

SZAJANI, B.; MOLNAR, A.; KLAMAR, G.; KALMAN, M. Preparation, characterization, and potential application of an immobilized glucose oxidase. **Applied Biochemistry. Biotechnology**, v.14, p. 37 - 47, 1987.

TALEKAR, S. *et al.* Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). **The Royal Society of Chemistry**, v. 3, p. 12485 - 12511, 2013.

TOUAHAR, I. E. et al. Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals. **Applied Microbiology and Biotechnology** , v. 481, p. 90–99, 2014.

TUKEL, S. S. *et al*. Preparation of crosslinked enzyme aggregates (CLEA) of catalase and its characterization. **Journal of Molecular Catalysis B-Enzymatic**, v. 97, p. 252-257, 2013.

WANG, Q. *et al*.. New insights into the effects of thermal treatment on the catalytic activity and conformational structure of glucose oxidase studied by electrochemistry, IR spectroscopy, and theoretical calculation. **The Journal of Physical Chemistry. B** v.114, p. 12754 – 12764, 2010.

WILSON, L. *et al.* Cross-linked aggregates of multimeric enzymes: a simple and efficient methodology to stabilize their quaternary structure. **Biomacromolecules**, v. 5, n. 3, p. 814-7, 2004.

WILSON, L. *et al.* Co-Aggregation of Penicillin G Acylase and Polyionic Polymers: An Easy Methodology To Prepare Enzyme Biocatalysts Stable in Organic Media. **Biomacromolecules**, v. 5, n. 3, p. 852 - 857, 2004.

WITT, S. *et al.* Conserved arginine-516 of Penicillium amagasakiense glucose oxidase is essential for the efficient binding of β-D glucose. **Journal of Biochemistry**, v. 347, n. 2, p. 553–559. 2000.

WONG, C.; WONG, K.; CHEN, X. Glucose oxidase: natural occurrence, function, properties and industrial applications. **Applied Microbiology and Biotechnology**, v. 78, n. 6, p. 927-938, 2008.

XUE, R.; WOODLEY, J. Process technology for multi-enzymatic reaction systems. **Bioresource Technology**, v. 115, p. 183-195, 2012.

5.7 Supplementary data

Table 5.1S– Immobilization yield (Y) and recovered activity after the stability assay (RA) of glucose oxidase (GOD) immobilized by the CLEA technique: influence of the glutaraldehyde concentration (GLU) and BSA/enzymes ratios (w/w).

Assay	BSA(mg_{prot}/mL)	BSA/enzymes ratio (w/w)	[GLU] mM	μmol GLU/$mg_{protein}$	Y(%)	Activity (U/mL)	RA (%)	Activity recovered (U/mL)
1	0	0	25	4.55	29.7 ± 2.7	355.5 ± 32.0	8.03 ± 0.88	96.1 ± 10.6
2	0	0	50	9.09	42.3 ± 3.8	505.9 ± 45.5	15.96 ± 1.76	190.9 ± 21.0
3	0	0	100	18.18	41.2 ± 3.7	492.2 ± 44.3	10.84 ± 1.19	129.6 ± 14.3
4	0	0	200	36.36	24.2 ± 2.2	289.9 ± 26.1	5.19 ± 0.57	62.1 ± 6.8
5	15	1.36	25	1.92	59.4 ± 5.4	711.0 ± 64.0	39.63 ± 4.36	474.0 ± 52.1
6	15	1.36	50	3.85	55.8 ± 5.0	667.2 ± 60.0	41.84 ± 4.60	500.4 ± 55.0
7	15	1.36	100	7.69	50.8 ± 4.6	607.1 ± 54.6	30.45 ± 3.35	364.2 ± 40.1
8	15	1.36	200	15.38	45.7 ± 4.1	546.9 ± 49.2	34.30 ± 3.77	410.2 ± 45.1
9	30	2.73	25	1.22	59.4 ± 5.4	711.0 ± 64.0	54.59 ± 6.00	652.8 ± 71.8
10	30	2.73	50	2.44	32.0 ± 2.9	382.8 ± 34.5	24.01 ± 2.64	287.1 ± 31.6
11	30	2.73	100	4.88	32.0 ± 2.9	382.8 ± 34.5	25.33 ± 2.79	303.0 ± 33.3
12	30	2.73	200	9.76	19.2 ± 1.7	229.7 ± 20.7	19.21 ± 2.11	229.7 ± 25.3
13	60	5.45	25	0.70	70.9 ± 6.4	847.7 ± 76.3	53.16 ± 5.85	635.8 ± 69.9
14	60	5.45	50	1.41	42.8 ± 3.8	511.3 ± 46.0	34.20 ± 3.76	409.1 ± 45.0
15	60	5.45	100	2.82	44.4 ± 4.0	530.5 ± 47.7	43.42 ± 4.78	519.3 ± 57.1
16	60	5.45	200	5.63	34.5 ± 3.1	412.3 ± 37.1	32.24 ± 3.55	385.6 ± 42.4

Table 5.2S – Immobilization yield (Y) and recovered activity after the stability assay (RA) of catalase (CAT) immobilized by the Combi-CLEA technique: influence of the glutaraldehyde concentration (GLU) and BSA/enzymes ratios (w/w).

Assay	BSA(mg_{prot}/mL)	BSA/enzymes ratio (w/w)	[GLU] mM	μmol GLU/$mg_{protein}$	Y(%)	Activity (U/mL)	RA (%)	Activity recovered (U/mL)
1	0	0	25	4.55	42.19 ± 4.64	3320.6 ± 365.3	42.19 ± 5.02	3320.6 ± 395.2
2	0	0	50	9.09	48.37 ± 4.35	3806.6 ± 342.4	39.55 ± 5.76	3112.6 ± 453.0
3	0	0	100	18.18	10.33 ± 1.14	813.1 ± 89.4	10.33 ± 1.23	813.1 ± 96.8
4	0	0	200	36.36	6.77 ± 0.74	533.1 ± 58.6	6.77 ± 0.81	533.1 ± 63.4
5	15	1.36	25	1.92	48.45 ± 5.05	3812.6 ± 397.8	45.95 ± 5.76	3616.3 ± 453.7
6	15	1.36	50	3.85	50.20 ± 4.20	3950.6 ± 330.9	38.22 ± 5.97	3008.2 ± 470.1
7	15	1.36	100	7.69	51.50 ± 5.66	4053.1 ± 445.8	51.50 ± 6.13	4053.1 ± 482.3
8	15	1.36	200	15.38	40.56 ± 2.91	3192.2 ± 229.4	26.50 ± 4.83	2085.6 ± 379.9
9	30	2.73	25	1.22	62.69 ± 4.14	4933.7 ± 326.5	37.71 ± 7.46	2967.8 ± 587.1
10	30	2.73	50	2.44	46.70 ± 3.62	3675.2 ± 284.6	32.87 ± 5.56	2586.9 ± 437.3
11	30	2.73	100	4.88	27.01 ± 2.69	2125.4 ± 211.4	24.42 ± 3.21	1921.5 ± 252.9
12	30	2.73	200	9.76	24.74 ± 2.10	1947.0 ± 165.5	19.11 ± 2.94	1504.3 ± 231.7
13	60	5.45	25	0.70	80.42 ± 6.96	6329.2 ± 547.5	63.25 ± 9.57	4977.6 ± 753.2
14	60	5.45	50	1.41	78.86 ± 6.20	6206.5 ± 487.7	56.33 ± 9.38	4433.3 ± 738.6
15	60	5.45	100	2.82	68.93 ± 5.38	5424.6 ± 423.3	48.90 ± 8.20	3848.5 ± 645.5
16	60	5.45	200	5.63	39.14 ± 3.77	3080.3 ± 296.3	34.23 ± 4.66	2694.1 ± 366.6

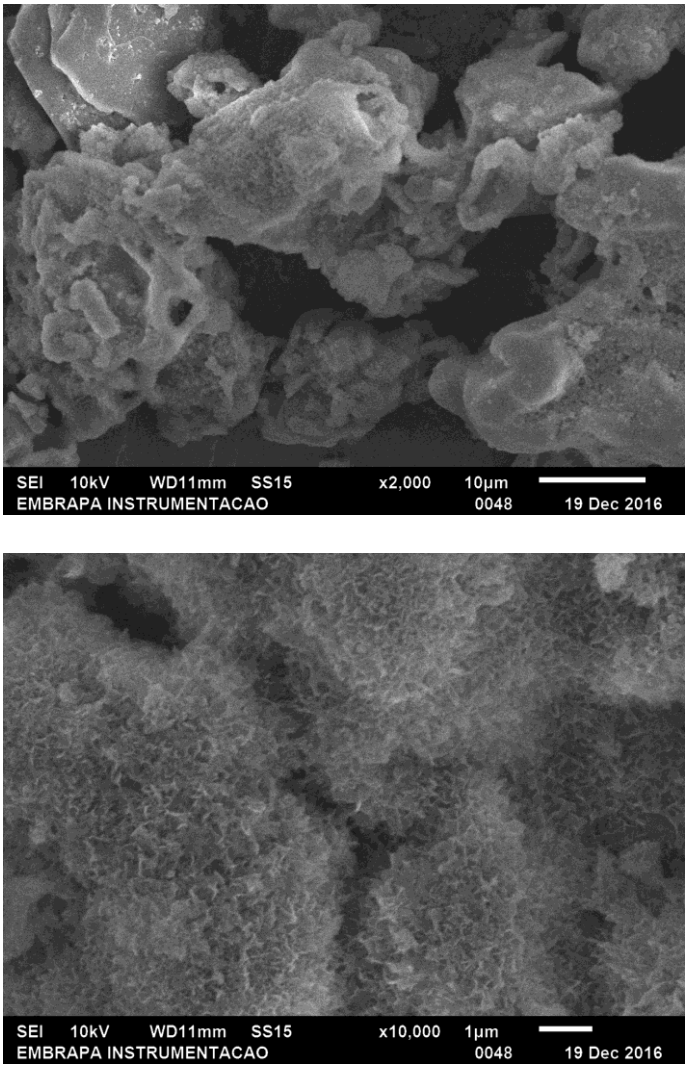
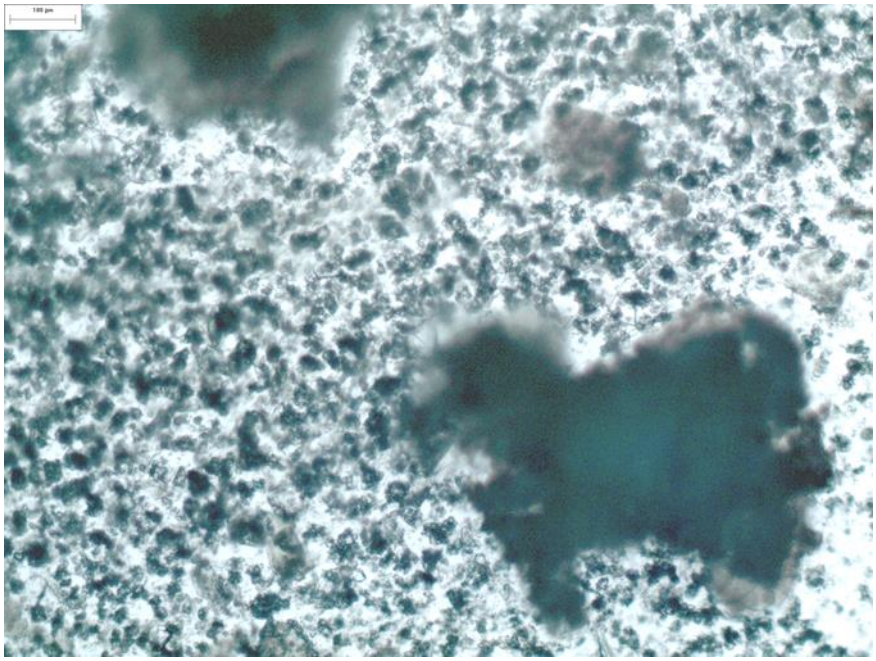


Fig. 5.1S - SEM images of *Aspergillus niger* glucose-oxidase and bovine liver catalase in Combi-CLEAs co-precipitated with BSA.



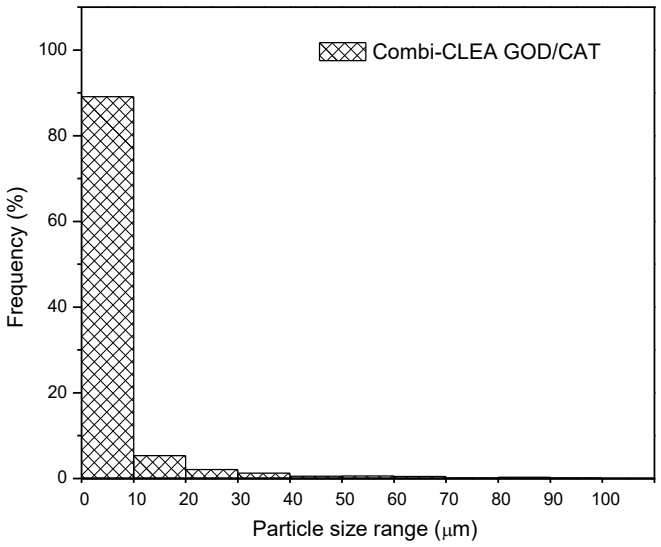


Fig. 5.2S – Particle size distribution for Combi-CLEAs GOD/CAT. The particle diameter was obtained from microscopy images of Combi-CLEAs using the ImageJ software, where the frequency count was performed using a step size of 10 μm.

6. Concluding remarks and future perspectives

The objective of this thesis was to produce an immobilized multi-enzymatic complex for the conversion of sucrose into gluconic acid and fructose syrup in a pneumatic bioreactor. In each step of the project the objective was to enhance the understanding of the CLEAs and Combi-CLEAs from the Chemical Engineer's point of view.

In the Chapter 2 – Literature Review - the countless possibilities for performing this technique were studied, still regarded as evolving and unknown by the research group. Based on literature papers which introduced evaluation and elaboration criteria the first experimental part of this Thesis was performed (Chapter 3 - *Diffusion effects of bovine serum albumin on cross-linked aggregates of catalase*). The main objective was to acquire information on the technique and validation from papers already published in the literature. With this paper, it was possible to experimentally verify that the use of inert proteins can admittedly decrease intraparticle diffusion problems.

The second experimental part of this Thesis was Chapter 4 - *Enzymatic production of inverted sugar using SOY-CLEAs of invertase*, which allows making progress in two main frontiers: the use of inert protein from soybean in CLEAs and the use of the window of economic viability to estimate the minimum reuse number for the catalytic converter to become feasible for the process and for the decision to conduct the process.

The last step of this Thesis comprised Chapter 5 - *Combi-CLEAs of glucose-oxidase and catalase for multiconversion of glucose to gluconic acid process*

in a pneumatic reactor. In this chapter the knowledge learned in the other two chapters can be put into practice. It was possible to propose a Combi-CLEA which operated for 50 hours in the pneumatic batch reactor, not yet described in the literature for gluconic acid production. The experimental data of the chapter herein can certainly be used for designs of more economically viable processes. Considering that the product concentration (determining factor for the economic feasibility window studied in this paper) is still low.

It is suggested to use the operational window methodology for the design of other biochemical processes as a tool for choosing the bio catalyst.

The CLEAs and Combi-CLEAs prepared in this Thesis had little optimization regarding the methodology, namely: agitation of the reaction, immobilization time and precipitation temperature. It is suggested that these optimizations be performed in future studies. Presumably, the effect of agitation can become the most relevant factor on the final activity of the biocatalyst.

Another issue that was tested in a preliminary manner was the CLEAs escalation in Taylor vortex reactors. These tests showed that keeping the proportions of the precipitant, crosslinker and enzymes, the agitation must be carefully chosen (and in laminar flow regime). This is because in the optimized small scale of CLEAs preparation (2mL), the Eppendorf tubes are kept lying down in the shaker under slow stirring. Where the height of solid precipitates of CLEAs and the liquid phase in which the crosslinking agent and the precipitating agent are, must perform a mass exchange as uniform as possible (for CLEAs with similar size dispersions) and possibly this will occur in the laminar flow regime.

7. List of publications

MAFRA, A. C. O.; KOPP, W. ; RAMOS, M. D. ; BELTRAME, M. B. ; RIBEIRO, M. P. A. ; BADINO, A. C ; TARDIOLI, P. W. Cross-linked enzyme aggregates of catalase from bovine liver. In: XX Congresso Brasileiro de Engenharia Química, 2015, Florianópolis. Anais do XX Congresso Brasileiro de Engenharia Química. São Paulo: Editora Edgard Blücher. v. 1. p. 1714-1721, 2014.

BELTRAME, M. B.; MAFRA, A. C. O.; RIBEIRO, M. P. A.; BADINO, A. C.; TARDIOLI, P. W. Avaliação de proteína de soja como agente espaçador na produção CLEAS de invertase. In: Congresso Brasileiro de Engenharia Química em Iniciação Científica, 2015, Campinas, SP. XI Congresso Brasileiro de Engenharia Química em Iniciação Científica- XI COBEQ IC, 2015.

MAFRA, A. C. O.; BELTRAME, M. B.; GIORDANO, R. L. C. ; RIBEIRO, M. P. A.; TARDIOLI, P. W. Síntese de agregados reticulados de invertase (CLEAS) como biocatalisador para a produção de açúcar invertido. In: Simpósio Nacional de Bioprocessos (SINAFERM) e Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2015, Fortaleza-CE. XX Simpósio Nacional de Bioprocessos (SINAFERM) e o XI Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2015.

MAFRA, A. C. O.; KOPP, W.; BELTRAME, M. B.; GIORDANO, R. L. C.; TARDIOLI, P. W; RIBEIRO, M. P. A. Modelagem da cinética de inativação de CLEAs de catalase de fígado bovino coimobilizados com albumina de soro bovino. In: Simpósio Nacional de Bioprocessos (SINAFERM) e Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2015, Fortaleza-CE. XX Simpósio Nacional de Bioprocessos (SINAFERM) e o XI Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2015.

SILVA, R. A.; MAFRA, A. C. O.; GIORDANO, R. L. C.; TARDIOLI, P. W. Imobilização de β-amilase de cevada por agregação/reticulação (CLEA). In: Simpósio Nacional de Bioprocessos (SINAFERM) e Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2015, Fortaleza-CE. XX Simpósio Nacional de

Bioprocessos (SINAFERM) e o XI Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2015.

MAFRA, A. C. O.; BELTRAME, M. B.; LOPES, L. A.; DIAZ, M.; SILVA, R. A.; LIMA, L. N., BADINO, A. C.; TARDIOLI, P. W. Cross-linked enzyme aggregates as biocatalysts for bioconversions of industrial potential. In: European Congress of Chemical Engineering, 2015, Nice, France. 10th European Congress of Chemical Engineering. 2015.

MAFRA, A. C. O.; BELTRAME, M. B.; GIORDANO, R. L. C.; RIBEIRO, M. P. A.; TARDIOLI, P. W. Produção de açúcar invertido a partir de sacarose catalisada por agregados reticulados de invertase de S. cerevisiae. In: XXI Congresso Brasileiro de Engenharia Química, 2016, Fortaleza. Anais do XXI Congresso Brasileiro de Engenharia Química, 2016.

MAFRA, A. C. O.; BELTRAME, M. B.; GIORDANO, R. L. C.; RIBEIRO, M. P. A.; TARDIOLI, P. W. Efeito de BSA na transferência de massa de agregados reticulados (CLEAs) de catalase de fígado. In: XXI Congresso Brasileiro de Engenharia Química, 2016, Fortaleza. Anais do XXI Congresso Brasileiro de Engenharia Química, 2016.

SCHULTZ, G.; ULRICH, L. G.; MAFRA, A. C. O.; GIORDANO, R. C.; RIBEIRO, M. P. A. Estabilidade de β-galactosidase de K. lactis. In: XXI Congresso Brasileiro de Engenharia Química, 2016, Fortaleza. Anais do XXI Congresso Brasileiro de Engenharia Química, 2016.

MAFRA, A. C. O.; KOOP, W.; BELTRAME, M. B.; GIORDANO, R. L. C.; RIBEIRO, M. P. A.; TARDIOLI, P. W. Diffusion effects of bovine serum albumin on cross-linked aggregates of catalase. Journal of Molecular Catalysis. B, Enzymatic (Print), v. 133, p. 107-116, 2016.

MAFRA, A. C. O.; ULRICH, L. G.; RIBEIRO, M. P. A.; TARDIOLI, P. W. Production of gluconic acid from glucose catalyzed by cross-linked aggregates of glucose oxidase and catalase in a bubble column reactor. In: Simpósio Nacional de Bioprocessos (SINAFERM) e Simpósio de Hidrólise Enzimática de Biomassas

(SHEB), 2017, Aracaju-SE. XXI Simpósio Nacional de Bioprocessos (SINAFERM) e o XII Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2017.

ULRICH, L. G.; MAFRA, A. C. O.; TARDIOLI, P. W.; RIBEIRO, M. P. A. Cross-linked enzyme aggregates (CLEAs) of β-galactosidase. In: Simpósio Nacional de Bioprocessos (SINAFERM) e Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2017, Aracaju-SE. XXI Simpósio Nacional de Bioprocessos (SINAFERM) e o XII Simpósio de Hidrólise Enzimática de Biomassas (SHEB), 2017.

ULRICH, L. G.; MAFRA, A. C. O.; TARDIOLI, P. W.; RIBEIRO, M. P. A. Imobilização de beta-galactosidase utilizando a técnica de CLEA (cross-linked enzyme aggregates). In: XXII Congresso Brasileiro de Engenharia Química em Iniciação Científica, 2017, São Carlos. Anais do XXII Congresso Brasileiro de Engenharia Química em Iniciação Científica, 2017.