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DESENVOLVIMENTO DE BIOCATALISADORES COMPOSTOS POR PECTINASES E CELULASES IMOBILIZADAS PARA A CLARIFICAÇÃO DE SUCOS DE FRUTAS

LUCAS DAL MAGRO

PORTO ALEGRE

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Tese submetida ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos como requisito parcial para obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Rafael Costa Rodrigues

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TESE

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RESUMO

O consumo de sucos de frutas vem apresentando grande crescimento, acompanhando a tendência mundial de bebidas saudáveis. Entretanto, sua composição rica em polissacarídeos pode influenciar o rendimento produtivo, bem como sua qualidade. Desta forma, o presente trabalho buscou, por meio do desenvolvimento de biocatalisadores compostos por pectinases e celulases imobilizadas, melhorar a eficiência do processo produtivo de clarificação e a qualidade dos sucos de frutas. Primeiramente, no intuito de melhorar a separação e a reutilização das enzimas imobilizadas dos sedimentos dos sucos, duas metodologias de imobilização com partículas magnéticas foram testadas, CLEAs-magnéticos (CLEA-MP*) e a imobilização na superfície da partícula magnética ativada com glutaraldeído (Enz-Glu-MP*). No entanto, uma queda quase linear no rendimento durante os 8 ciclos de clarificação do suco de uva foi verificado em ambos biocatalisadores, podendo estar relacionado a menor atração magnética do CLEA-MP* que dificultou a separação deste do suco, ou então, a menor estabilidade do Enz-Glu-MP*. Em seguida, buscando preparar biocatalisadores com alta estabilidade, aliada a boas propriedades magnéticas, estudou-se diferentes metodologias de recobrimento das partículas magnéticas com quitosana, alcançando 3 diferentes biocatalisadores com distintos tamanhos: Nano (Nano-CMag), Micro (Micro-CMag) e Macro (Macro-CMag). O tamanho do biocatalisador influenciou diretamente nas características das enzimas imobilizadas, onde o Macro-CMag obteve as maiores estabilidades, apresentando as maiores atividades residuais após 25 ciclos de clarificação dos sucos de uva, maçã e laranja. A seguir, procurou-se entender como cada enzima reage as diferentes condições de pH, visto que muitos protocolos de imobilização exigem condições alcalinas, nas quais as enzimas apresentam baixa estabilidade. Desta forma, diferentes agentes estabilizantes foram testados, onde verificou-se que 20 % de polietilenoglicol (PEG) apresentou os melhores resultados de estabilização para todas as enzimas do preparado em pH 10 a 25 °C, mantendo atividades próximas a 100 %. Posteriormente, a imobilização de duas das principais enzimas para a hidrólise da pectina, pectina liase (PL) e poligalacturonase (PG), foram estudadas variando a condição de imobilização nos suportes glioxil-agarose, vinilsulfona-agarose, MANAE e MANAE-glutaraldeído. Para ambas as enzimas o melhor suporte foi MANAE-glutaraldeído. Os diferentes pHs estudados para a imobilização apresentaram efeitos na recuperação da atividade enzimática, estabilidade térmica e operacional, sugerindo que os diferentes pHs testados permitiram obter enzimas imobilizadas com distintas orientações. Além disso, altas atividades foram alcançadas em amostras que apresentaram uma condição de imobilização mais lenta (alta força iônica). Já os biocatalisadores que foram incubados a pH 8 na parte final da imobilização, apresentaram as maiores estabilidades térmicas e operacionais, devido a formação de ligações multipontuais enzima-suporte. Por fim, visto as melhores estabilidades e a fácil separação da reação apresentada pelas macroesferas de quitosana, estas foram preparadas e aplicadas em reatores de leito empacotado e leito fluidizado para a clarificação contínua do suco de laranja. Devido ao seu maior tamanho, as partículas magnéticas não foram necessárias para a separação do suporte e uma maior concentração de enzima pôde ser imobilizada. Os melhores resultados foram alcançados utilizando o reator de leito fluidizado, provavelmente devido à melhor difusão de massa desse sistema, enquanto que zonas mortas e caminhos preferenciais foram observados no reator de leito empacotado. A aplicação de enzimas imobilizadas em reatores contínuos apresentou-se como uma tecnologia promissora para a clarificação de sucos de frutas industriais, trazendo contribuições para a eficiência do processo produtivo e para o aumento da qualidade dos sucos de frutas.

Palavras-chave: Complexo enzimático, partícula magnética; quitosana, agarose, imobilização enzimática; biorreatores enzimáticos.

ABSTRACT

The consumption of fruit juice has been growing, following the worldwide trend of consumption of healthy drinks. However, their rich composition in polysaccharides can influence the production yield, as well as their quality. Thus, this work sought to improve the efficiency of the clarification process and the fruit juices quality, through the development of biocatalysts composed of pectinases and cellulases immobilized. First, in order to improve the separation and reuse of the immobilized enzymes of the juice sediments, two immobilization methodologies with magnetic particles were tested, CLEAs-magnetic (CLEA-MP*) and immobilization on the magnetic particle surface activated with glutaraldehyde (Enz-Glu-MP*). However, a linear drop in yield during the 8 cycles of grape juice clarification was observed in both biocatalysts, which may be related to the lower magnetic attraction of the CLEA-MP * that made it difficult to separate from the juice, or then, the lower stability of the Enz-Glu-MP *. Then, seeking to prepare biocatalysts with high stability combined to good magnetic properties, different methodologies for covering the magnetic particles with chitosan were studied, obtaining 3 biocatalysts with different sizes: Nano (Nano-CMag), Micro (Micro-CMag) and Macro (Macro-CMag). The biocatalyst size influenced the immobilized enzymes characteristics, where the Macro-CMag achieved the greatest stability, presenting the higher residual activities after 25 cycles of grape, apple and orange juices clarification. Next, we sought to understand how each enzyme reacts to different pH conditions, since many immobilization protocols require alkaline conditions, in which the enzymes have low stability. In this way, different stabilizing agents were tested, where it was found that 20 % of polyethylene glycol (PEG) presented the best stabilization at pH 10 to 25 ° C, keeping activities close to 100 % for all enzymes. Subsequently, the immobilization of two of the main enzymes for the pectic polysaccharides hydrolysis, Pectin lyase (PL) and Polygalacturonase (PG), were studied, varying the immobilization condition on glyoxyl-agarose, vinylsulfone-agarose, MANAE and MANAE-glutaraldehyde supports. For both enzymes, the best support was MANAE-glutaraldehyde. The different pHs studied for the immobilization had effects on the enzyme recovered activity, thermal and operational stability, suggesting that the different pHs tested allowed to obtain immobilized enzymes with different orientations. In addition, high activities were achieved in samples that showed a slower immobilization condition (high ionic strength). Already the biocatalysts that were incubated at pH 8 in the final part of the immobilization, presented the higher thermal and operational stability, due to the formation of multipoint bonds between enzyme-support. Finally, in view the best stability and the easy separation of the reaction medium presented by the chitosan macrospheres, these were prepared and applied in packed-bed and fluidized-bed reactors for the continuous clarification of orange juice. Due to their larger size, the magnetic particles were not necessary for the biocatalyst separation of the reaction, so a higher enzyme concentration can be immobilized. The best results achieved by the fluidized-bed reactor may be related to the better mass diffusion of this system, whereas dead zones and preferential paths were observed in the packed-bed reactor. Immobilized enzymes application in continuous reactors is a promising technology for the industrial clarification of fruit juices, bringing contributions to the production process efficiency and to the improvement of the fruit juices quality.

Keywords: enzyme complex, magnetic particle, chitosan, agarose, enzymatic immobilization, enzymatic bioreactors.

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INTRODUÇÃO

A fruticultura é um dos setores de maior destaque do agronegócio brasileiro. Através de uma grande variedade de culturas, produzidas em todo o país e em diversos climas, a fruticultura conquista resultados expressivos e gera oportunidades para os pequenos negócios brasileiros (Carvalho et al., 2005; Monteiro, 2006).

A evolução da industrialização de frutas no Brasil e no mundo aponta o caminho da agregação de valor. As frutas são processadas por várias razões: para preservar e estender o prazo de validade, aumentar a digestibilidade, aumentar a disponibilidade de alguns nutrientes, melhorar a palatabilidade e a textura, preparar alimentos prontos para o consumo, eliminar microrganismos, inativar toxinas, remover partes não comestíveis, criar novos tipos de alimentos, entre outros (Shils, 2003). A fim de atender nichos de mercados diferenciados, o segmento das frutas processadas entrou na era da diversificação, no qual estas foram incorporadas à rotina das pessoas de diversas formas, com destaque para os sucos.

Os sucos de frutas são consumidos e apreciados em todo o mundo, não só pelo seu sabor, mas, também, por serem fontes naturais de carboidratos, carotenóides, vitaminas, polifenóis, minerais e outros componentes importantes (Carmo et al., 2014). Uma mudança apropriada na dieta em relação à inclusão desses componentes encontrados em frutas e suco de frutas pode ser importante na prevenção de doenças e para uma vida mais saudável (Blenford, 1996; Pinheiro et al., 2006).

Sucos de fruta prontos para beber são considerados bebidas refrescantes, capazes de saciar a sede, ao mesmo tempo em que respondem ao apelo por produtos naturais e com vantagens nutricionais, o que contribui para sua grande aceitação (Ferreira and Alcântara, 2013). O mercado brasileiro de sucos prontos para beber está em franca expansão, estando entre as bebidas que mais crescem no setor das não alcoólicas (Carmo et al., 2014). Este

crescimento é motivado pela falta de tempo da população em preparar o suco a partir das frutas *in natura*, pela praticidade oferecida pelos produtos, pela substituição do consumo de bebidas carbonatadas devido ao seu valor nutritivo e pela preocupação no consumo de alimentos mais saudáveis (Matsuura and Rolim, 2002). Por exemplo, a comercialização do suco de uva brasileiro vem apresentando grande crescimento nos últimos anos. Em 2018 foram comercializados 162.365.854 litros de suco de uva, um aumento de 23,16 % em relação a 2017 (UVIBRA, 2019).

Entretanto, com o crescimento do consumo de sucos de frutas, o mercado fica mais competitivo, o que leva o consumidor a exigir cada vez mais características de qualidade. Além da sua composição benéfica à saúde, os aspectos sensoriais são muito importantes para a aceitação do produto. Sucos com aroma e sabor pronunciados, boa aparência e coloração, são características fundamentais de qualidade. Porém, algumas propriedades dos sucos, como a sua composição rica em polissacarídeos, podem influenciar o rendimento produtivo, bem como sua qualidade, podendo este fator estar diretamente ligado com a aceitação do produto.

Os sucos de frutas recém-prensados são turvos devido à dispersão coloidal de pectina e outros polissacarídeos, tais como celulose e hemicelulose, presente na parede celular das células vegetais. A eliminação desses polissacarídeos é um dos maiores obstáculos para obtenção de sucos de frutas límpidos e transparentes (Vaillant et al., 2001; Tapre and Jain, 2014). A fim de evitar a turvação indesejável, bem como melhorar a limpidez, viscosidade, estabilidade de armazenamento e a qualidade dos sucos de frutas, preparados enzimáticos comerciais vêm sendo utilizados na produção. Estes são constituídos de um complexo de enzimas pectinolíticas, celulolíticas e hemicelulolíticas que atuam de modo combinado para a hidrólise dos polissacarídeos (Wilkins et al., 2007; Laaksonen et al., 2012; Jiménez-Sánchez et al., 2017). Além disso, na indústria de sucos de frutas, as enzimas são utilizadas para aumentar a produtividade, melhorar a dissolução, clarificação, filtração, maceração e extração

dos tecidos vegetais (Bhat, 2000; Uenojo and Pastore, 2007).

No entanto, a aplicação industrial de enzimas é quase sempre prejudicada devido a alguns fatores, tais como baixa estabilidade em condições operacionais, difícil recuperação e reutilização (Sheldon et al., 2005). Diante deste cenário, onde as condições do meio de reação irão afetar diretamente a atividade das enzimas, a imobilização surge como uma ferramenta importante para obtenção de biocatalisadores adequados para uma determinada aplicação de interesse. Os avanços científicos no campo de imobilização enzimática possibilitam a obtenção de biocatalisadores para aplicações industriais, melhorando as propriedades catalíticas frente às condições adversas de reação, facilitando a separação das enzimas do meio reacional e sua reutilização, tornando-as economicamente viáveis (Mateo et al., 2007).

Além disso, a imobilização de mais de uma enzima no mesmo suporte também confere um efeito de atividade em cascata ao biocatalisador, já que a proximidade das enzimas facilita que o produto de uma reação torne-se substrato para outra (Talekar et al., 2013). Esta abordagem de um sistema multi-enzimático confere algumas vantagens, tais como menor tempo reação, maior produtividade e transferência eficiente de substrato em reações enzimáticas sequenciais (Pinelo et al., 2010; Netto et al., 2013; Talekar et al., 2013).

Um suporte compatível e técnicas adequadas para a imobilização de enzimas são os fatores chave para o desenvolvimento de biocatalisadores com elevada atividade enzimática e poucas perdas durante a reação (Bickerstaff, 1997). A seleção do método de imobilização deve ser baseada em parâmetros como atividade global do biocatalisador, características de regeneração e inativação, custo do procedimento de imobilização, toxicidade dos reagentes, estabilidade operacional, propriedades hidrodinâmicas e características finais desejadas para a enzima imobilizada (Dalla-Vecchia et al., 2004; Sheldon, 2007; Mendes et al., 2011; Illanes et al., 2012). Embora não haja um suporte universal, existem características primordiais a serem observadas para a escolha de um suporte, tais como: área superficial, afinidade, grupos

reativos, permeabilidade, insolubilidade, capacidade de regeneração e reutilização, morfologia, composição, resistência ao ataque microbiano, resistência mecânica, custos, entre outras (Lei and Bi, 2007; Mateo et al., 2007).

Por fim, uma operação enzimática contínua pode ser alcançada por meio da construção de reatores com os biocatalisadores obtidos. Diversos fatores devem ser considerados para a construção de um biorreator enzimático eficiente. Além da sua configuração e modo de operação, características como, o formato e o tamanho do biocatalisador, propriedades do substrato, transferência de massa, condições operacionais, geometria, hidrodinâmica de fluido, inibidores e custo operacional, devem ser considerados (Castro et al., 2008; Poppe et al., 2015).

Neste contexto, o presente trabalho tem como objetivo melhorar a tecnologia enzimática da produção dos sucos de frutas, buscando, por meio da utilização de uma combinação de pectinases e celulases imobilizadas, preparar biocatalisadores eficientes para a clarificação contínua de sucos através da construção de reatores enzimáticos, gerando assim, uma nova tecnologia capaz de melhorar o processamento industrial, bem como as características organolépticas dos sucos de frutas.

A presente Tese de Doutorado foi desenvolvida principalmente no Laboratório de Biocatálise e Tecnologia Enzimática do Instituto de Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul, sendo uma parte realizada no Laboratório de Ingeniería de Biocatalizadores y Biotransformaciones, do Instituto de Catálisis y Petroleoquímica do Consejo Superior de Investigaciones Científicas em Madri na Espanha, durante um estágio de doutorado de oito meses. Além disso, o trabalho contou com apoio do Laboratório de Sólidos e Superfícies do Instituto de Química da UFRGS, do Laboratório de Magnetismo do Instituto de Física da UFRGS e de outros laboratórios do Instituto de Ciência e Tecnologia de Alimentos da UFRGS.

Este trabalho será apresentado na forma de artigos científicos de acordo com as normas estabelecidas pelo Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. No Capítulo 1 está apresentada a revisão bibliográfica abordando os principais pontos do tema proposto. O Capítulo 2 apresenta a descrição dos materiais e métodos utilizados nos experimentos. Os Capítulos 3, 4, 5, 6, 7 e 8 apresentam os resultados obtidos, na forma como foram submetidos à publicação em periódicos internacionais. Por fim, no Capítulo 9 é apresentado a discussão geral e, em seguida, as principais conclusões obtidas e as perspectivas para os trabalhos futuros.

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OBJETIVO

Este trabalho teve como **objetivo principal** desenvolver biocatalisadores compostos por pectinases e celulases imobilizadas para a clarificação de sucos de frutas.

Os objetivos específicos foram:

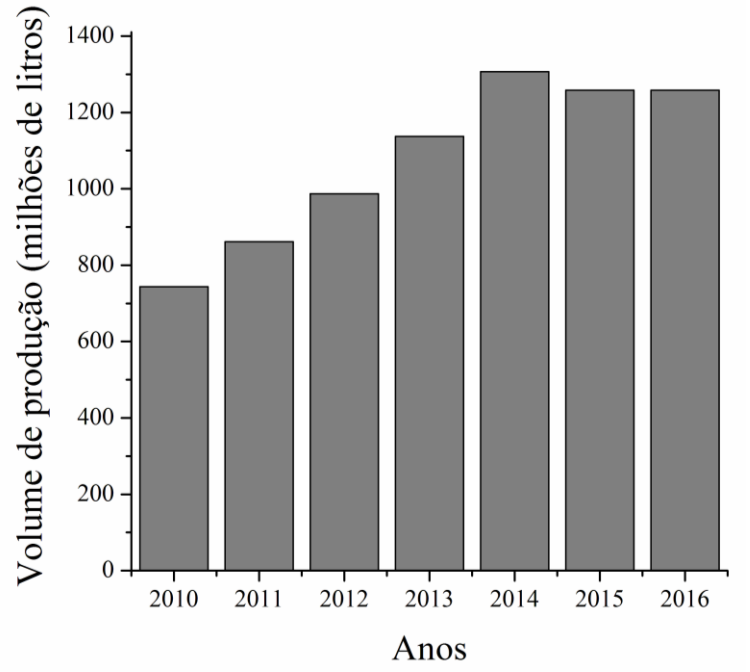
- Preparar diferentes biocatalisadores, utilizando partículas magnéticas;
- Estudar metodologias de recobrimento das partículas magnéticas com quitosana para obtenção de biocatalisadores eficientes;
- Avaliar a estabilidade das enzimas, utilizando diferentes agentes estabilizantes, sob condições extremas de pH e temperatura;
- Estudar diferentes condições de imobilização da pectina liase (PL) em MANAE-agarose;
- Avaliar diferentes condições de imobilização da poligalacturonase (PL) em agarose ativada com diferentes grupos funcionais;
- Desenvolver diferentes biorreatores enzimáticos, contendo os biocatalisadores preparados, para a clarificação contínua de sucos de frutas.

CAPÍTULO 1 – REVISÃO BIBLIOGRÁFICA

1.1. SUCOS DE FRUTAS

O mercado brasileiro de suco de fruta pronto pra beber vem crescendo nos últimos anos, seguindo a tendência mundial de consumo de bebidas que oferecem conveniência, sabor, inovação, praticidade e prazer, além da conscientização da população para a escolha de produtos saudáveis, buscando uma melhoria na qualidade de vida (Carmo et al., 2014). De 2010 para 2014, a produção de sucos de frutas e néctares expandiu-se em torno de 70 %, com um aumento de aproximadamente 15 % a cada ano (Figura 1), seguido por uma estabilização nos últimos anos. Ao mesmo tempo em que se observa uma redução no consumo de refrigerantes, segundo a Associação Brasileira das Indústrias de Refrigerantes e de Bebidas Não Alcoólicas (ABIR, 2017).

Figura 1. Volume de produção do mercado brasileiro de sucos de frutas e néctares entre 2010 e 2016.



Fonte: ABIR, 2017.

Esse cenário favorável é, em grande parte, devido aos compostos bioativos presentes nas frutas. As frutas são fontes de diversos compostos que proporcionam benefícios à saúde, como por exemplo, os antioxidantes naturais que combatem os radicais livres (Tripoli et al., 2007; Gattuso et al., 2007), fibras que auxiliam no controle dos níveis de colesterol (Stark and Madar, 1994; Camargo et al., 2007), carotenoides que atuam na manutenção da saúde dos olhos e ajudam no combate ao câncer (Fraser and Bramley, 2004; Rivera and Canela-Garayoa, 2012), minerais como o cálcio e o potássio, que garantem a saúde dos ossos e dentes (Pirillo and Sabio, 2009), além de vitaminas, como a vitamina C que auxilia no combate a infecções e na absorção do ferro (Block, 1993).

Pesquisas apontam que os sucos mais vendidos no Brasil são o de laranja, seguido pelos sucos de maçã e uva (Silva et al., 2005; Rosa et al., 2006; Ferrarezi et al., 2010). Os sucos devem atender à legislação específica, estando de acordo com definição, classificação, registro, padronização e requisitos de qualidade, devendo também atender à legislação sobre rotulagem, conforme definido no Decreto nº 6.871, de 4 de julho de 2009, que regulamenta a Lei nº 8.918, de 14 de julho de 1994, do MAPA, sendo o suco ou sumo definido como: a bebida não fermentada, não concentrada e não diluída, destinada ao consumo, obtida da fruta sã e madura, ou parte do vegetal de origem, por processo tecnológico adequado, submetida a tratamento que assegure a sua apresentação e conservação até o consumo.

1.2. APLICAÇÃO DE ENZIMAS EM SUCOS

Preparados enzimáticos comerciais, utilizados na produção de sucos, são constituídos, principalmente, de um complexo de enzimas pectinolíticas e celulolíticas que atuam de modo combinado para a hidrólise dos polissacarídeos das frutas (Sankaran et al., 2015). Essas enzimas foram as primeiras a serem utilizadas comercialmente nas preparações de vinhos e sucos de frutas em torno de 1930, mas somente a partir de 1960, quando os estudos sobre a

natureza química de tecidos vegetais se tornaram mais aparentes, é que os cientistas começaram a utilizar as enzimas mais eficientemente (Uenojo and Pastore, 2007).

As pectinases hidrolisam polissacarídeos complexos dos tecidos vegetais em moléculas mais simples, como os ácidos galacturônicos. Dependendo de seu mecanismo de ação, as enzimas pécticas podem ser divididas em, despolimerizantes, que agem catalisando o rompimento das ligações α-1,4 da cadeia principal do polissacarídeo péctico, e desmetoxilantes (pectina metil esterase, E.C. 3.1.1.11), que agem desesterificando a pectina a partir do ácido péctico por remoção dos grupos metílicos (Kashyap et al., 2001; Jayani et al., 2005). As enzimas despolimerizantes envolvem as hidrolases (poligalacturonase, E.C. 3.2.1.15) que catalisam a hidrólise das ligações glicosídicas e as liases (pectina liase, E.C. 4.2.2.10) que catalisam a β-eliminação das ligações glicosídicas (Alkorta et al., 1998).

Já as celulases são enzimas responsáveis pela degradação da celulose, promovendo a hidrólise das ligações químicas existentes entre as unidades de glicose. Estas enzimas são biocatalisadores altamente específicos que atuam em sinergia para a liberação de açúcares (Lynd et al., 2002; Castro and Pereira Jr, 2010). As endoglucanases (E.C. 3.2.1.4) promovem o rompimento das ligações β-1,4-D-glicosídicas em oligossacarídeos solúveis com grau de polimerização menor que 6 unidades. Esses oligossacarídeos solúveis são rapidamente hidrolisados pelas exoglucanases (EC. 3.2.1.91), formando em sua maioria celobiose, que pela ação das β-glicosidases (E.C. 3.2.1.21) são convertidas em glicose (Arantes and Saddler, 2010).

A combinação de pectinases e celulases, chamadas coletivamente de enzimas de maceração, são usadas na extração e clarificação de sucos de frutas e vegetais. As pectinases são usadas para reduzir a viscosidade, consistência e turbidez, melhorando o rendimento do suco, facilitando a clarificação e a concentração e aumentando a velocidade de prensagem e a liberação de componentes da casca. As celulases em combinação com as pectinases

favorecem a liberação de açúcares, sendo importantes no processo de liquefação do tecido vegetal, facilitando a separação líquido/sólido. Além disso, exercem um efeito favorável na melhoria da viscosidade e da filtrabilidade dos sucos (Kaur et al., 2004; Uenojo and Pastore, 2007).

A adição de preparados enzimáticos durante a maceração, antes da prensagem, é um pré-requisito para a obtenção de maiores rendimentos de suco, que são alcançados através de uma seleção adequada de enzimas (Alkorta et al., 1998; Lei and Bi, 2007). Segundo Mieszczakowska-Frac et al. (2012), o aumento da proporção de pectina liase e pectina metil esterase na maceração influenciam positivamente o rendimento do suco. Além disso, a degradação catalisada por enzimas das paredes celulares ajuda a liberar compostos bioativos, aumentando assim a recuperação de fenóis e antocianinas, que de outro modo seriam perdidos com os resíduos da prensagem (Sowbhagya and Chitra, 2010). Estes compostos são normalmente encontrados ligados aos polissacarídeos, exigindo, portanto, uma etapa de maceração efetiva para que ocorra a degradação desses polissacarídeos, permitindo que o conteúdo seja extraído (Bautista-Ortín et al., 2005).

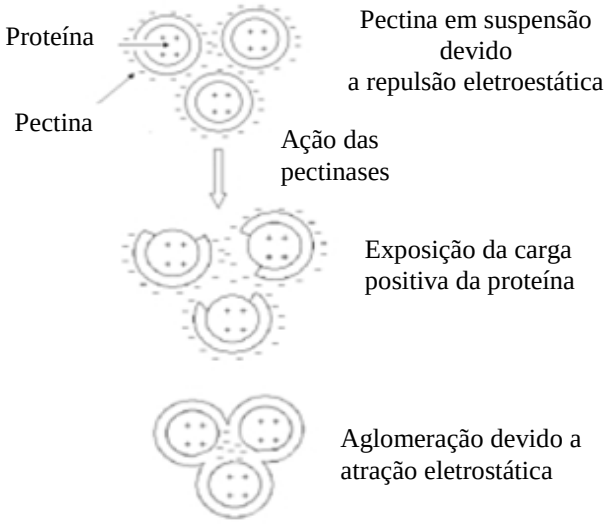
A etapa de prensagem das frutas provoca um rompimento das paredes celulares do mesocarpo levando à liberação do mosto (Lea, 1995). Esta etapa pode gerar também alguns problemas como excesso de viscosidade e turbidez por meio da formação de partículas insolúveis, as quais podem dificultar o processamento (Kashyap et al., 2001). Essa turbidez pode ser encontrada nos sucos de frutas em diferentes graus, especialmente devido à presença dos polissacarídeos (pectina, celulose, hemicelulose, lignina e amido) (Whitaker, 1984; Vaillant et al., 2001).

Os polissacarídeos são responsáveis pela consistência, turbidez e aparência dos sucos de frutas, e sua presença causa um aumento considerável da viscosidade, dificultando a filtração e a concentração dos sucos. As enzimas de maceração desempenham um papel vital

no processamento, reduzindo a viscosidade da polpa, o que por sua vez ajuda na filtração e clarificação de sucos (Panda et al., 1999). O processo de clarificação, requerido para facilitar a filtração e remover a turbidez do suco, representa a mais antiga aplicação destas enzimas (Pilnik and Rombouts, 1981).

Em grande parte, a turbidez dos sucos está relacionada a substâncias coloidais que tem por característica um núcleo proteico com carga positiva, revestido por moléculas de pectina com carga negativa. Essa carga negativa na superfície faz com que as moléculas sejam repelidas uma das outras (Pilnik and Voragen, 1993). Assim, através da degradação da pectina pelas pectinases, o núcleo proteico carregado positivamente é exposto, reduzindo a repulsão eletrostática e proporcionando a agregação em partículas maiores (complexo pectina-proteína) que irão sedimentar (Figura 2) (Pilnik and Voragen, 1993; Lea, 1995). Para melhorar o processo de sedimentação, podem ser utilizados agentes floculantes, como gelatina, tanino ou bentonite. Posteriormente, os processos de centrifugação e de filtração podem ser necessários para dar ao suco uma aparência límpida e clara que muitos consumidores preferem (Kashyap et al., 2001).

Figura 2. Mecanismo de formação do complexo pectina-proteína.



Fonte: Adaptado de Pilnik e Voragen (1993).

A presença desses polissacarídeos também pode causar problemas durante a concentração dos sucos, dificultando a retirada da água, causando o entupimento de filtros e diminuindo a velocidade do processamento. A adição das enzimas resulta em uma rápida redução da viscosidade, bem como na floculação destas micelas presentes (Alkorta et al., 1998), permitindo que estas partículas possam ser separadas por sedimentação ou filtração (Soares et al., 2001).

Algumas enzimas também têm a capacidade de extrair compostos glicosilados, como as β-glicosidases, que possuem capacidade de liberar compostos aromáticos durante a fabricação do vinho, como os terpenos, e alguns compostos fenólicos com capacidade antioxidante, propriedades nutracêuticas e flavorizantes (Daroit et al., 2007). A utilização de β-glicosidases em conjunto com pectinases faz com que aromas e características voláteis de frutas e vegetais aumentem, o que é de grande importância para a indústria de alimentos (Bhat, 2000).

Algumas enzimas utilizadas na indústria de alimentos e bebidas, com suas respectivas funções, são apresentadas na Tabela 1. Na indústria de sucos de frutas, estas enzimas são utilizadas para melhorar a qualidade sensorial da bebida, proporcionando uma maior extração e clarificação dos sucos, as quais contribuem para a eficiência do processamento, facilitando os processos de prensagem, filtração, clarificação e estabilização (Pinelo et al., 2010; Khandare et al., 2011; Sandri et al., 2011; Romero-Cascales et al., 2012).

Tabela 1. Funções e aplicações de enzimas na indústria de alimentos e de vinhos.

ENZIMA	FUNÇÃO	APLICAÇÃO
Enzimas de maceração (pectinases, celulases e hemicelulases)	Hidrólise de pectina solúvel e de componentes de parede celular (polissacarídeos), diminuição de viscosidade e manutenção de textura de sucos de frutas	Melhoramento na extração de sucos de frutas, pigmentos da casca, óleo de oliva, liberação de aromas, enzimas, proteínas, polissacarídeos, amido e ágar, auxilia a estabilidade, filtração e clarificação dos vinhos
Pectinase ácida e termo-estável como poligalacturonase, pectina esterase e pectina transeliminase	Rápida diminuição de viscosidade e hidrólise dos tecidos vegetais	Melhora o rompimento da fruta e aumenta a extração de pigmentos de cor
Poligalacturonase com alta atividade de pró-pectinase e baixa atividade celulolítica	Hidrólise parcial de pró-pectina	Produção de purês com alta viscosidade
Poligalacturonase e pectina trans-eliminase com baixa atividade de pectina esterase e hemicelulase	Hidrólise parcial de pró-pectina e de pectina solúvel em fragmentos de tamanho médio, formação de precipitado e remoção de hidrocoloides de celulose	Produção de sucos vegetais não clarificados de baixa viscosidade
Poligalacturonase, pectina trans-eliminase e hemicelulase	Hidrólise completa de pectina e de polissacarídeos ramificados	Clarificação de sucos de frutas
Pectinase e β-glicosidade	Infusão de pectinase e glicosidase para facilitar o descascamento e melhorar a firmeza de frutas	Alteração das propriedades sensoriais de frutas e vegetais
Pectina esterase com atividade de poligalacturonase e de pectina liase	Processamento de frutas	Produção de *ketchup* de alta qualidade e de polpa de frutas
Pectina esterase	Desesterificação e gelificação de pectina	Melhoramento na clarificação de sidra

Fonte: Uenojo e Pastore (2007).

1.3. IMOBILIZAÇÃO DE ENZIMAS

Atualmente, os avanços científicos nas áreas da biotecnologia e engenharia de proteínas vêm viabilizando a produção de diversas enzimas para uso comercial, com custo aceitável e propriedades convenientes, como especificidade, atividade, seletividade e

estabilidade (Sheldon and Van Pelt, 2013). Entretanto, as enzimas são moléculas complexas, altamente sensíveis, com estruturas tridimensionais que são essenciais para as suas atividades. A exposição a determinadas condições do meio de reação, tais como temperatura elevada ou pH extremo, pode levar à desnaturação e a perda da sua atividade. Além disso, as enzimas são geralmente usadas na forma de soluções, dificultando a sua recuperação e reutilização, podendo também gerar uma contaminação do produto (Sheldon, 2011). Assim, muitos trabalhos vêm estudando ferramentas que possam superar estas desvantagens e melhorar o desempenho da aplicação enzimática industrial, como é o caso da imobilização de enzimas.

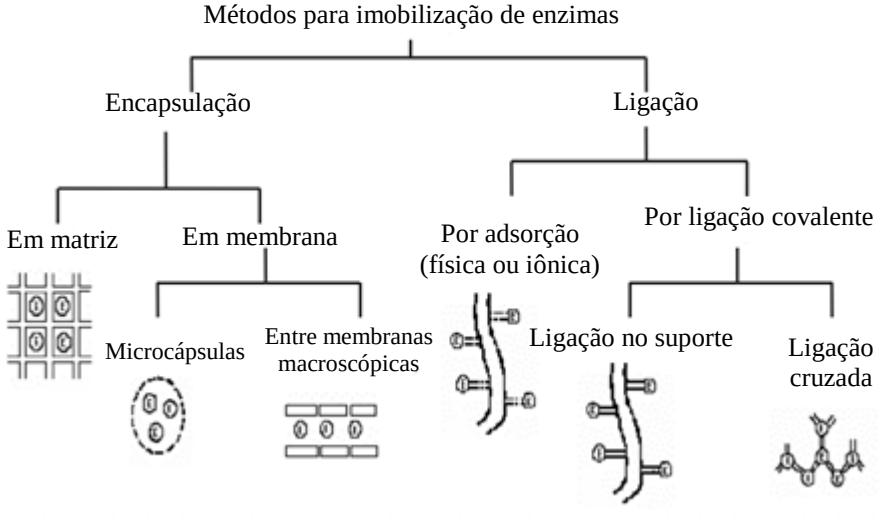
Enzimas imobilizadas podem ser definidas como aquelas fisicamente retidas, normalmente em uma matriz sólida, com manutenção da sua atividade catalítica, podendo ser utilizadas continuamente (Garcia-Galan et al., 2011). Em geral, a imobilização oferece uma série de vantagens, incluindo: possibilidade de uso da enzima por um maior período de tempo, possibilidade de operação em modo contínuo, maior controle do processo, facilidade de separação do produto final e de interrupção da reação pela simples remoção da enzima, caso o processo seja em batelada, ou ajuste do tempo de residência em reatores contínuos. Além disso, a imobilização, na maioria dos casos, produz ligeiras distorções na estrutura das enzimas, o que pode alterar as propriedades catalíticas, conferindo maior estabilidade frente às condições adversas da reação, como faixas extremas de pH e temperatura. Por outro lado, essas distorções estruturais também podem estar ligadas a perda da capacidade catalítica da enzima, relatada em muitos trabalhos de imobilização (Guisan, 2006; Hanefeld et al., 2009; Klein et al., 2013; Liese and Hilterhaus, 2013; Asgher et al., 2014).

O tipo de imobilização empregada confere características químicas e propriedades cinéticas particulares para as enzimas. A seleção do método de imobilização deve ser baseada em parâmetros como atividade global do biocatalisador, características de regeneração e inativação, custo do procedimento de imobilização, toxicidade dos reagentes, estabilidade

operacional, propriedades hidrodinâmicas e características finais desejadas para a enzima imobilizada (Dalla-Vecchia et al., 2004; Sheldon, 2007; Mendes et al., 2011; Illanes et al., 2012).

Várias metodologias podem ser aplicadas para imobilizar enzimas, como por exemplo, a encapsulação, a adsorção em materiais insolúveis, o entrecruzamento enzimático ou a ligação covalente a uma matriz (Figura 3) (Dalla-Vecchia et al., 2004; Guisan, 2006; Romaškevič et al., 2006; Hanefeld et al., 2009).

Figura 3. Métodos de imobilização de enzimas.



Fonte: Dalla-Vecchia et al. (2004).

O aumento da estabilidade e a recuperação das enzimas são as principais razões que torna a imobilização enzimática uma técnica atrativa do ponto de vista econômico (López-Gallego et al., 2005; Mendes et al., 2011).

1.3.1. Imobilização por ligação covalente em suporte sólido

A retenção da enzima ao suporte por ligações covalentes é um dos métodos de imobilização mais utilizados (Dalla-Vecchia et al., 2004). Esta ligação baseia-se na ativação de suportes com a inserção de grupos reativos que reagem com os grupos nucleofílicos da

enzima, sendo necessário certo conhecimento de sua estrutura e dos grupamentos ativos do suporte, tais como -OH, -NH_2, -COOH, -SH. Ligações covalentes entre enzimas e suportes ocorrem por interação da cadeia lateral de aminoácidos como lisina, arginina, ácido aspártico, histidina, com base no grau de reatividade de diferentes grupos funcionais como imidazol, indol, fenólico, hidroxil (Cowan and Fernandez-Lafuente, 2011).

Normalmente, a imobilização covalente enzimática é iniciada com a modificação da superfície do suporte por meio de reações de ativação, na qual os grupos funcionais são modificados para produzir intermediários reativos (Fernández-Fernández et al., 2013). O glutaraldeído é o reagente bifuncional geralmente utilizado na ativação de suportes e/ou como braço espaçador, devido à simplicidade dos seus métodos e pela obtenção de biocatalisadores enzimáticos ativos e estáveis (Mateo et al., 2007; Chaubey et al., 2009; Barbosa et al., 2012). Nesse caso, as extremidades do glutaraldeído se ligam ao suporte e à enzima, formando uma ligação covalente através de grupos amino (α-NH_2 da cadeia terminal, ε-NH_2 da lisina e/ou NH_2 proveniente de aminação química), que se ligam aos grupos aldeídos do suporte, formando as bases de Schiff (Mateo et al., 2007; Brady and Jordaan, 2009; Barbosa et al., 2012).

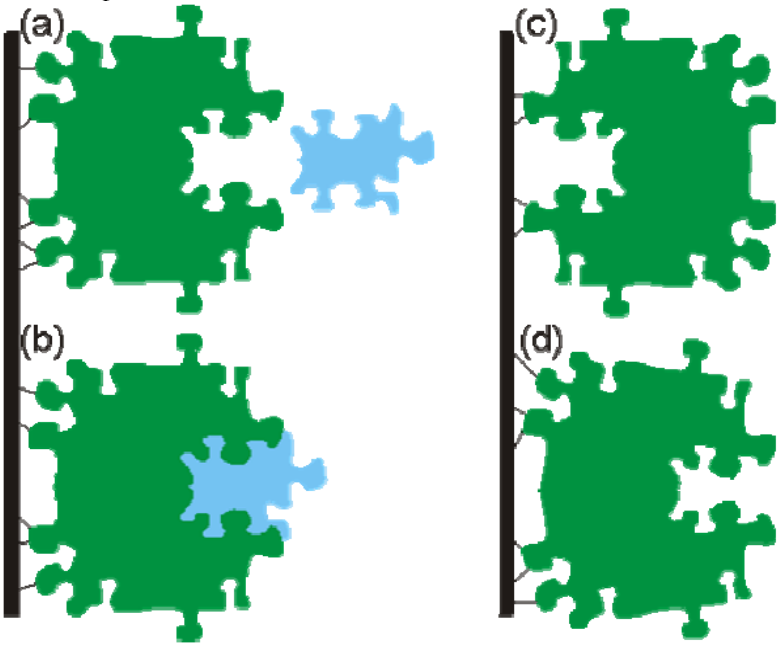
A próxima etapa de imobilização covalente ocorre pela adsorção das enzimas na superfície do material, seguida pela formação de ligações covalentes entre os resíduos de aminoácidos e os grupos reativos na superfície do suporte. Essa ligação ocorre dependendo do nível de reatividade do aminoácido; normalmente, o aminoácido presente na superfície das enzimas é a lisina, que pode ser influenciada pelo pH do meio. Essa reatividade pode ser muito baixa a pH neutro, devido a esses grupos estarem na forma de eletrófilos (protonados). Dessa forma, a ligação covalente pode levar um maior período de tempo, necessitando, muitas vezes, o uso de temperaturas moderadamente elevadas, promovendo uma menor rigidez na molécula da enzima que favorece a reatividade para formação das ligações (Mateo et al.,

2000). Assim, a imobilização de enzimas por ligações covalentes pode necessitar de condições que favoreçam o processo de imobilização, dependendo da enzima, como variações de tempo, temperatura, pH e força iônica, a fim de melhorar a reatividade dos resíduos de aminoácidos para a ligação ao suporte.

A ligação covalente pode envolver vários grupamentos da enzima, proporcionando uma maior rigidez a sua estrutura, a qual confere uma maior resistência perante aos agentes desnaturantes, como calor, solventes orgânicos, pH extremos e outros (Mateo et al., 2007; Macario et al., 2009; Miletić et al., 2009). Entre os métodos de imobilização disponíveis, a ligação covalente é o mais efetivo em termos de estabilização térmica e operacional das enzimas, sendo uma das suas principais vantagens a forte ligação da enzima com o suporte sólido, diminuindo a possibilidade de dessorção (Van de Velde et al., 2002; Rodrigues et al., 2008; Mendes et al., 2011).

Entretanto, ao imobilizar uma enzima a um suporte inerte pode-se esperar algum tipo de efeito sobre sua estrutura e, consequentemente, sobre sua atividade catalítica (Macario et al., 2009). A parcial desativação ou redução da atividade catalítica da enzima devido às restrições na sua conformação, impostas pelas ligações entre as enzimas e os grupos reativos do suporte, é um dos inconvenientes geralmente relatados na imobilização por ligação covalente (Erdemir and Yilmaz, 2009). Durante o processo de interação da enzima com o suporte, a região do sítio ativo pode se tornar menos acessível ao substrato, ocasionando um impedimento estérico (Figura 4) (Mateo et al., 2007).

Figura 4. Imobilização da enzima por ligação covalente em suporte sólido. (a) e (b) Enzima imobilizada com o sítio ativo inalterado e disponível para aceitar a molécula de substrato; (c) Enzima ligada de modo não produtivo devido à inacessibilidade do substrato ao sítio ativo; (d) Distorção da conformação da enzima impedindo a entrada do substrato.



Fonte: Rodrigues (2009).

1.3.2. Agregados enzimáticos entrecruzados

Agregados enzimáticos entrecruzados (CLEAs, do inglês *cross-linked enzyme aggregates*) ganharam considerável atenção industrial como uma alternativa aos métodos convencionais de imobilização, destacando-se por ser um processo simples, rápido e econômico, sem a necessidade de um suporte sólido (Cao et al., 2003; Sheldon, 2011). A preparação de CLEAs apresenta vantagens como, alta retenção de atividade, estabilidade mecânica, estabilidade frente às condições de reação, estabilidade de armazenamento, baixos custos de produção, excelente capacidade de recuperação, além de não haver necessidade de purificação prévia das enzimas (Wilson et al., 2004; Sheldon, 2011; Bhattacharya and Pletschke, 2014; Nadar et al., 2016). Essa metodologia de imobilização combina as operações de purificação e imobilização em um único procedimento, uma vez que a precipitação enzimática é um processo utilizado para purificação (Sheldon and Van Pelt, 2013).

Inicialmente, os CLEAs são preparados através da precipitação da enzima com adição de agentes precipitantes específicos como solventes orgânicos, polímeros não-iônicos e sais inorgânicos, seguido por ligação cruzada com um agente de entrecruzamento bifuncional (Cao et al., 2003; Garcia-Galan et al., 2011; Cruz et al., 2012). Vários agentes precipitantes podem ser utilizados para preparação de CLEAs, entretanto, visto as variações bioquímicas e estruturais das enzimas, cada agente precipitante pode ter um comportamento diferente sobre cada uma delas. Dessa forma, uma etapa prévia de seleção do melhor agente precipitante e sua concentração são essenciais para alcançar um biocatalisador eficiente (Talekar et al., 2013). Maiores recuperações de atividades são observadas quando se utilizam altas concentrações, pois a agregação e precipitação ocorrem de forma mais rápida, evitando a desnaturação da enzima. Entretanto, deve-se ter cuidado com altas concentrações do agente precipitante em conjunto com longos tempos de precipitação, o que pode causar agregados enzimáticos rígidos e com baixa atividade catalítica (Zhao et al., 2008; Yang et al., 2012).

Os agentes de precipitação proporcionam uma agregação física das moléculas de enzima em grandes estruturas moleculares, sem perturbação da estrutura tridimensional original da proteína. Estes agregados sólidos são mantidos por ligação não covalente e facilmente se redissolvem, quando dispersos em meio aquoso. Assim, uma etapa de entrecruzamento por ligações covalentes é necessária para estabilizar os agregados. As moléculas de enzimas são ligadas quimicamente umas às outras por um reagente bifuncional, através da reação dos grupamentos amino presentes na sua superfície, tornando os agregados permanentemente insolúveis (Sheldon, 2007; Kartal et al., 2011; Sheldon, 2011).

Por esse motivo, a seleção do agente precipitante deve ser sempre seguida da etapa de entrecruzamento, pois a enzima precipitada pode apresentar uma conformação inativa, mas que ao ser ressuspendida retoma sua atividade normal. Entretanto, com a etapa de

entrecruzamento as enzimas apresentarão a mesma conformação inativa da enzima precipitada, exibindo baixa atividade catalítica (Talekar et al., 2013).

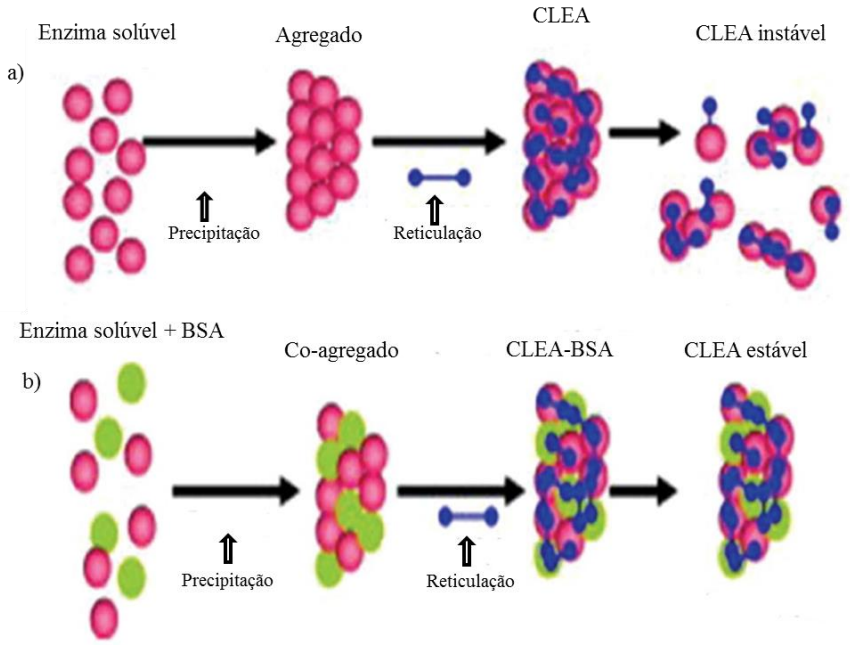
O agente de entrecruzamento, bem como a sua concentração, tem papel muito importante para a atividade, estabilidade e tamanho dos CLEAs. Estudos têm mostrado que a atividade do CLEA aumenta com o aumento da concentração do agente de entrecruzamento até um ponto ótimo, seguindo pela diminuição da atividade com o aumento da concentração (Talekar et al., 2013). Em baixas concentrações do agente de entrecruzamento, as ligações entre as enzimas são insuficientes para formação dos agregados, deixando muita enzima livre em solução. Já para altas concentrações, fortes ligações entre as enzimas são formadas, tornando o CLEA um agregado rígido, impedindo o acesso do substrato ao sítio ativo das enzimas imobilizadas no interior do agregado (Yu et al., 2006; Dal Magro et al., 2016b).

Entre os agentes entrecruzantes, o glutaraldeído também tem sido amplamente utilizado para a preparação dos CLEAs devido a sua capacidade de formar ligações covalentes com os resíduos de aminoácidos reativos na superfície das enzimas (Barbosa et al., 2014), como apresentado anteriormente. Porém, a alta reatividade e o tamanho extremamente pequeno do glutaraldeído permitem que ele penetre no sítio ativo das enzimas, reagindo com grupos amino que são cruciais para a atividade catalítica (Mateo et al., 2004).

Para que a reação de entrecruzamento seja efetiva, também é necessário que a superfície da enzima apresente uma quantidade razoável de resíduos de aminoácidos livres para ligação. Enzimas com baixo conteúdo de resíduos de aminoácidos livres podem resultar em CLEAs frágeis com baixa estabilidade mecânica e com baixa atividade, devido à lixiviação de enzimas para o meio durante a reação (Talekar et al., 2013a). Como uma opção para resolver esses problemas, Lopez-Gallego et al., (2005) e Shah et al., (2006) propuseram a adição de um polímero e albumina de soro bovino (BSA), contendo vários grupos amino livres na preparação de CLEAs altamente estáveis (Figura 5). Embora as enzimas estudadas

tivessem um número reduzido de grupos amino na superfície, os grupamentos amino do polímero e da BSA proporcionaram um adequado entrecruzamento entre estes aditivos e as moléculas de enzimas resultando em elevadas atividades recuperadas. Entretanto, quando adicionado em quantidade excessiva o coadjuvante proteico pode prejudicar a recuperação da atividade, por dificultar a mobilidade entre enzima e substrato e pela diluição da atividade catalítica (Dal Magro et al., 2016a).

Figura 5. Produção de CLEAs com enzimas deficientes em grupamentos amino (a) e com adição de BSA (b).



Fonte: Adaptado de Talekar et al. (2013a).

Qualquer fator que pode alterar a precipitação da proteína ou o entrecruzamento do agregado pode afetar o tamanho de partícula, a estabilidade e a recuperação da atividade (Yu et al., 2006). O tamanho de partícula é uma propriedade importante no contexto de aplicações industriais, uma vez que afeta diretamente a transferência de massa e a separação do meio reacional sob as condições operacionais (Talekar et al., 2013a). Um tamanho típico das partículas dos CLEAs é de 5 μm a 50 μm. Igualmente como acontece com muitos

catalisadores sólidos, pode-se esperar perda de atividade devido a limitações difusionais. No entanto, os CLEAs são estruturas porosas, o que facilita o acesso do substrato ao sítio ativo das enzimas no interior dos CLEAs (Sheldon, 2011).

Alguns estudos também têm demonstrado que os CLEAs podem catalisar uma sequência de reações envolvendo mais de uma enzima, sendo chamados de combi-CLEAs. Nos combi-CLEAs, populações heterogêneas de enzimas podem ser simultaneamente confinadas no mesmo agregado, podendo desempenhar a bioconversão em cascata ou não, melhorando desse modo as interações sinérgicas entre as diferentes enzimas na catálise (Taboada-Puig et al., 2011; Jung et al., 2013; Bhattacharya and Pletschke, 2015). Processos catalíticos em cascata têm grande potencial no contexto da produção industrial, envolvendo menos operações unitárias, reatores menores, maiores rendimentos volumétricos e de espaço/tempo, tempos de ciclo mais curtos e que geram menos resíduos em comparação com os processos convencionais de múltiplas etapas (Sheldon, 2011). Essa possibilidade da preparação dos combi-CLEAs com diferentes enzimas no mesmo agregado é uma característica interessante para a aplicação em sucos, visto que são várias enzimas atuando em conjunto na degradação dos polissacarídeos.

1.3.3. Suportes para imobilização de enzimas

A utilização de um suporte adequado para a imobilização de enzimas é um fator-chave para o desenvolvimento de biocatalisadores eficientes (Bickerstaff, 1997). Embora não haja um suporte universal, existem características primordiais a serem observadas para a escolha do mesmo, tais como: área superficial, afinidade, grupos reativos, permeabilidade, insolubilidade, capacidade de regeneração e reutilização, morfologia, composição, resistência ao ataque microbiano, resistência mecânica, custos, entre outras (Lei and Bi, 2007; Mateo et al., 2007).

A escolha também deve avaliar as características do processo, como o tipo de reator utilizado (batelada, tanque agitado, coluna e fluxo pistonado), características do meio reacional (aquoso, solvente orgânico ou sistema bifásico), o sistema de reação (pasta, líquido-líquido, as condições do líquido-sólido ou sólido para sólido) e as condições do processo (pH, temperatura e pressão) (Cao et al., 2003).

A natureza física dos suportes pode variar desde materiais gelatinosos até superfícies rígidas recobertas com substâncias capazes de interagir com a enzima. Sua escolha também dependerá das características peculiares da enzima e das condições de uso da biomolécula imobilizada (Miletić et al., 2009). Assim, para alcançar uma imobilização eficiente é indispensável conhecer previamente as características da enzima, do suporte e das possíveis interações entre eles, as quais irão conferir propriedades químicas, bioquímicas, mecânicas e cinéticas específicas para o biocatalisador (Tischer and Kasche, 1999). Um suporte adequado pode aumentar o tempo de meia-vida da enzima imobilizada, porém a escolha incorreta pode afetar não só a estabilidade, mas o desempenho global do sistema (Mendes et al., 2011).

Os suportes podem ser classificados de acordo com sua composição e morfologia, como mostra a Tabela 2.

Tabela 2. Classificação dos suportes de acordo com a composição.

SUPORTES				
Orgânicos			**Inorgânicos**	
Naturais		**Sintéticos**	**Minerais**	**Fabricados**
Polissacarídeos	Proteínas	Poliestireno	Areia	Vidro
Celulose	Colágeno	Poliacrilatos	Bentonita	Cerâmica
Agarose	Albumina	Polivinilos	Homeblenda	Sílica
Ágar	Gelatina	Nylon	Pedra-pome	Aluminossilicato
Quitosana	Seda	Poliamidas		Óxido de Ferro
Amido		Vinil		Óxido de Níquel
		Policrilamidas		

Fonte: Villeneuve et al. (2000).

Os suportes podem ser ainda classificados como porosos ou não-porosos. Os suportes porosos têm grande área superficial interna disponível para a imobilização, que protege a enzima contra turbulências externas. Contudo, é importante que o diâmetro dos poros do suporte seja suficientemente grande para acomodar a enzima e permitir o acesso do substrato e a difusão dos produtos (Dalla-Vecchia et al., 2004). Já os não-porosos tem a desvantagem de não possuir grande área para a imobilização, mas diminuem o problema de transferência de massa interna (Galvão, 2004).

Nos últimos anos, além dos suportes tradicionais, as partículas magnéticas vêm ganhando destaque em várias áreas de aplicação, em especial, na imobilização de enzimas, devido à sua baixa toxicidade, boas propriedades mecânicas, estabilidade térmica, elevada área superficial, resistência a solventes orgânicos e ao ataque microbiano, e, principalmente, devido à rápida separação do meio reacional pela simples aplicação de um campo magnético externo (Tischer and Kasche, 1999). Estudos de imobilização enzimática mostraram que esse material pode ser uma alternativa capaz de manter eficientemente a atividade catalítica (Liao et al., 2010; Altun et al., 2015; Feng et al., 2016; Xie and Zang, 2016).

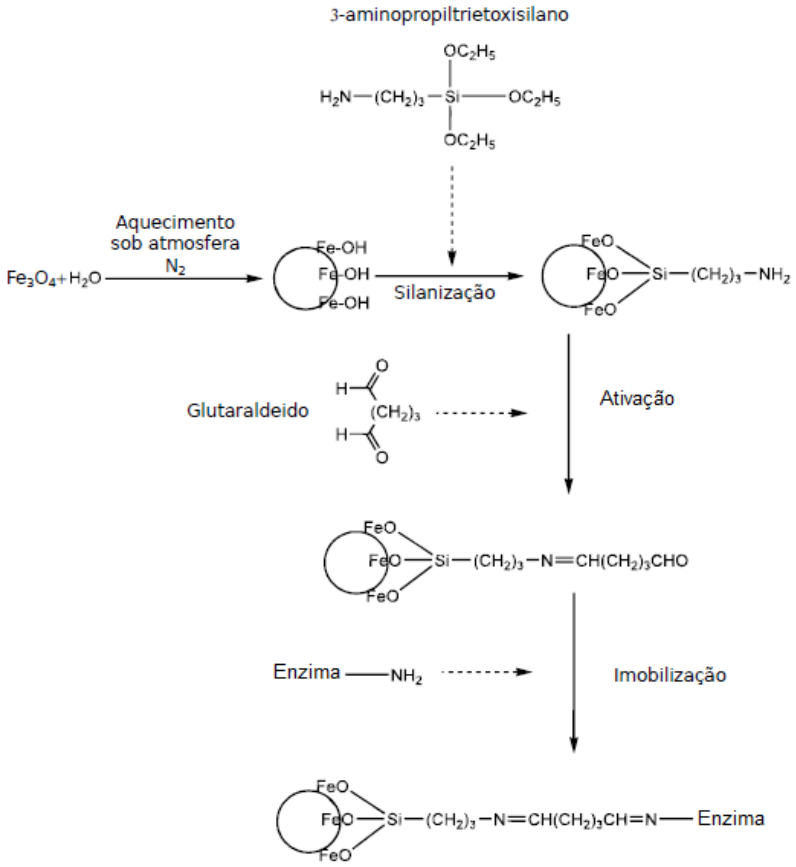
A magnetização surge como uma possível solução para materiais que são de difícil remoção do meio de reação (Barbosa et al., 2012). O uso de suportes com núcleo magnético diminui a necessidade de centrifugação, indesejável diluição da amostra e perda do suporte durante as lavagens, fatores que muitas vezes complicam o uso em reatores de enzimas não magnéticas (Tüzmen et al., 2012). As separações magnéticas são relativamente rápidas, fáceis e requerem aparelhagem simples. Portanto, técnicas de separação magnética têm atualmente encontrado muitas aplicações em áreas diferentes das ciências biológicas, incluindo aplicações biomédicas e ambientais (Jordan et al., 2011). Além disso, enzimas imobilizadas em partículas magnéticas vêm sendo aplicada em reatores revestidos por um campo magnético, o qual promove uma boa dispersão das enzimas no seu interior, melhorando a

transferência de massa do reator (Al-Qodah et al., 2017).

A imobilização de enzimas ou biomoléculas em partículas magnéticas normalmente é alcançada pela interação com grupos reativos da sua superfície. Grupos funcionais podem ser produzidos de diversas formas, porém geralmente envolve um revestimento da partícula magnética com um polímero ou biomacromoléculas (Neri et al. 2008). Como são muito reativas, as partículas magnéticas são facilmente oxidadas e sua estrutura pode ser alterada quando expostas ao meio reacional, resultando em perda do magnetismo. Portanto, para muitas aplicações é crucial que as partículas sejam revestidas com surfactantes, polímeros ou material inorgânico, para prevenir sua possível oxidação e aglomeração. Além disso, o material de revestimento pode fornecer grupos funcionais para ligação de outras moléculas, como enzimas e fármacos (Lu et al., 2007).

A presença de grupos hidroxila sobre a superfície da partícula magnética pode permitir a ligação de compostos funcionais. Além disso, a modificação da superfície, por exemplo, com aminopropiltrietoxisilano (APTES) ou 3-aminopropiltrimetoxisilano (APTMS), conduz a um revestimento que ajuda a estabilizar e proteger as partículas magnéticas contra a oxidação e proporciona pontos de ligações (grupos amina) para diferentes moléculas (Xu et al., 1997; Netto et al., 2009). Em geral, a modificação da superfície das partículas magnéticas é necessária para o processo de imobilização. Na Figura 6 é demonstrado um esquema da funcionalização da partícula magnética com APTMS, seguida da reticulação com glutaraldeído e imobilização da enzima.

Figura 6. Modificação da superfície das partículas magnéticas, seguido da ativação com glutaraldeído e imobilização da enzima.



Fonte: Adaptado de Tang et al. (2011).

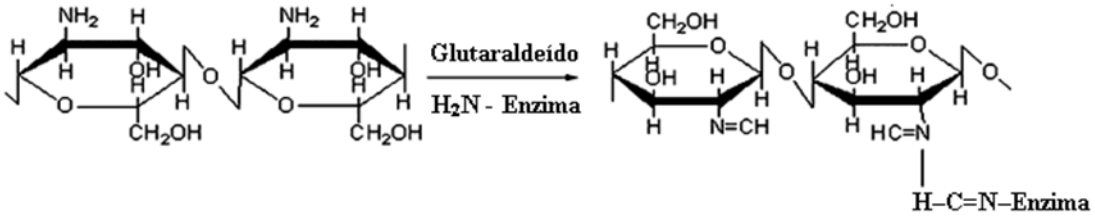
O uso de quitosana como suporte ou material de revestimento tem demostrado bons resultados, modificando as propriedades superficiais das partículas magnéticas e melhorando a interação com as moléculas de interesse (Grenha, 2012). A quitosana possui grupos amino e hidroxila livres que permitem a ligação a uma diversidade de grupos e íons químicos, sendo utilizada em uma série de aplicações, como a adsorção de proteínas e metais, a administração de medicamentos, ressonância magnética, engenharia de tecidos e imobilização enzimática (Linlin et al., 2007; Sasaki et al., 2008; Fang et al., 2009; Liu et al., 2009; Ruge et al., 2011; Kuo et al., 2012; Xie and Wang, 2012).

A quitosana já tem demostrado bons resultados para a imobilização de enzimas, apresentando características como elevada afinidade com proteínas, alta recuperação da atividade enzimática, disponibilidade de grupos funcionais reativos para reações diretas com

enzimas, hidrofilicidade, biocompatibilidade, reduzida adsorção não específica, resistência à degradação química, propriedades antibacterianas e facilidade de preparação em diferentes formatos físicos (Lei and Bi, 2007; Adriano et al., 2008). Além disso, a quitosana apresenta um custo relativamente baixo, já que é derivado da quitina, o segundo polímero natural mais abundante depois da celulose (Krajewska, 2004; Biró et al., 2008).

A imobilização de enzimas em suporte de quitosana é facilmente realizada utilizando um reagente bifuncional, como o glutaraldeído, uma vez que os seus grupos funcionais (-CHO) reagem simultaneamente com os sítios de ligação da quitosana ($-NH_2$) e o grupo amino da enzima (Figura 7) (Krajewska, 2004). Além disso, pectinases imobilizada em quitosana exibem uma maior resistência ao tratamento térmico e a diferentes pHs (Lei and Bi, 2007).

Figura 7. Possível mecanismo de imobilização de enzimas na partícula de quitosana revestida.



Fonte: Lei e Bi (2007).

A agarose é outro polissacarídeo natural que vem se destacando como suporte na imobilização de enzimas. Geralmente extraída de algumas algas vermelhas pertencentes à classe *Rhodophyceae*, a agarose é um dos principais componentes do ágar, composta por unidades repetitivas de agarobiose, que é um dissacarídeo composto de β-D-galactose e 3,6-anidro-α-L-galactose, ligados por ligações glicosídicas β (1–4) (Zucca et al., 2016).

As esferas à base de agarose são altamente porosas, resistentes mecanicamente, quimicamente e fisicamente inertes, e hidrofílicas, tornando-as particularmente adequadas

para imobilização enzimática, através de uma ampla gama de métodos de modificação química das hidroxilas do polímero (Zucca et al., 2016). Além disso, as características das esferas de agarose podem ser moduladas por reticulação covalente, conferindo elevada resistência física, química, mecânica e térmica, sendo encontrada em uma grande variedade de tamanhos e de diâmetro de poros (Mateo et al., 2006; Zucca et al., 2016).

O caráter hidrofílico da agarose é responsável por sua reatividade, onde suas hidroxilas podem ser ativadas com uma grande variedade de reagentes. Dessa maneira, vários grupamentos químicos podem ser adicionados ao longo das cadeias poliméricas, como amina, carboxil, sulfonato, ciano, diclorotriazinil, entre outros (Zucca et al., 2016). A preparação do suporte glioxil-agarose tem sido descrito como uma ferramenta muito adequada para obter uma ligação covalente multipontual entre enzima e suporte (Mateo et al., 2006). O suporte é preparado por eterificação dos grupos hidroxilas do suporte com glicidol, que são posteriormente oxidados com periodato de sódio para obter o grupo glioxil (López-Gallego et al., 2013).

A partir desse suporte, é possível introduzir grupos aminos na superfície do mesmo, por meio da incubação em uma solução concentrada de etilenodiamina, preparando o suporte MANAE, o qual pode ser facilmente ativado com glutaraldeído. Para a ligação covalente de enzimas em MANAE, é possível usar suportes pré-ativados ou adicionar glutaraldeído após troca iônica da enzima no MANAE (López-Gallego et al., 2005).

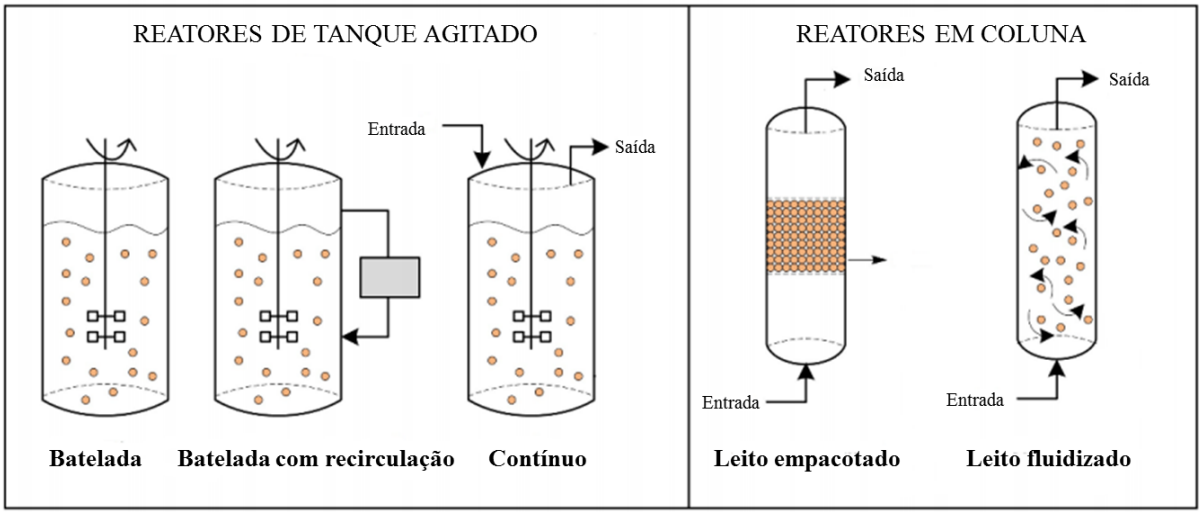
Além disso, a agarose ativada com grupos vinilsulfona também revela boas propriedade para obter uma intensa ligação covalente multipontual (dos Santos et al., 2015a). Este grupo reage com diferentes aminoácidos das proteínas, como histidina, lisina, cisteína, tirosina, entre outros, e sua reatividade é alta em pH alcalino. Uma das principais características desse suporte é o braço espaçador relativamente grande, que reduz a rigidez induzida pela ligação covalente (dos Santos et al., 2015a; dos Santos et al., 2015b).

Dessa forma, a partir dos diferentes métodos de ativação da agarose é possível direcionar a imobilização enzimática pela área mais rica em grupos reativos da proteína (Zucca et al., 2016).

1.4. REATORES ENZIMÁTICOS

Reações catalisadas por enzimas imobilizadas, em geral, podem ser feitas em reatores com diferentes configurações. Os reatores enzimáticos mais comuns são os de tanques agitados e os reatores de leito empacotado ou leito fluidizado, que podem ser operados de forma contínua, descontínua (batelada) ou descontínua alimentada (Figura 8).

Figura 8. Representação esquemática dos principais tipos de reatores.



Fonte: Poppe et al. (2015).

Nos reatores de tanque agitado, as enzimas imobilizadas são misturadas ao meio reacional, no qual a agitação pode ser feita por agitadores orbitais ou mecânicos. A agitação promove a mistura do meio reacional, evitando gradientes de temperatura e concentração. Este tipo de reator tem sido o mais utilizado em escala laboratorial, devido à simplicidade e pequena quantidade de reagentes necessários para utilização nessa escala. No entanto, a utilização deste processo em maior escala não é muito indicada, visto a baixa produtividade, necessidade frequente de esgotamento, limpeza e abastecimento a cada reação (Balcão et al.,

1996; Zanin and Moraes, 2004; Illanes, 2008). Além disso, a perda gradual da atividade enzimática, devido às reutilizações, faz com que o tempo de reação seja aumentado para manter a taxa de conversão constante. Com o passar do tempo, há uma inevitável diminuição de produção e neste ponto a enzima deve ser substituída (Nielsen et al., 2008).

Em reatores de leito empacotado, as enzimas imobilizadas encontram-se empacotadas, imóveis, enquanto a solução é bombeada através da coluna. Estes reatores são os mais indicados para uso com biocatalisadores em escala piloto e industrial, devido à sua maior eficiência, possibilidade de reutilização da enzima sem a necessidade de separação do meio reacional, minimização dos danos ao biocatalisador devido à menor tensão de cisalhamento, maior área superficial por unidade de volume reacional, possibilidade de remoção de substâncias que causam inibição e, consequentemente, menor custo operacional. No entanto, algumas desvantagens deste sistema são relatadas, como a possibilidade de compactação do leito, a formação de caminhos preferenciais e a limitações de transferência de massa e calor (Watanabe et al., 2001; Laudani et al., 2007; Illanes, 2008).

Já nos reatores de leito fluidizado, o biocatalisador imobilizado é mantido em suspensão através da recirculação da solução de substrato ou pela injeção de ar no sistema, proporcionando a livre circulação das partículas por todo o leito e evitando a decantação das enzimas no fundo do reator (Kosseva et al., 2009). Além disso, não há ocorrência de caminhos preferencias e a transferência de massa é facilitada (Ray, 2012). Biocatalisadores grandes geralmente são necessárias, devido à elevada viscosidade dos líquidos (Feng et al., 2013). A fim de operar de forma eficiente, os reatores de leito fluidizado requerem menores quantidades de enzima por unidade de volume, reduzindo a eficiência global do reator. A maior desvantagem do desenvolvimento desses reatores é a dificuldade de aumento de escala (Kosseva et al., 2009).

Diano et al. (2008) obtiveram bons resultados de clarificação para o suco de maçã em

reatores de leito empacotado e fluidizado, utilizando pectinases imobilizadas. O reator de leito fluidizado reduziu em 0,25 vezes o tempo de despectinização quando comparado ao reator de leito empacotado.

Além da configuração e do modo de operação, outros fatores devem ser considerados para a construção de um biorreator enzimático eficiente, como formato e tamanho do suporte de imobilização, natureza do substrato, transferência de massa, condições operacionais, geometria, hidrodinâmica de fluido, inibidores e custo operacional (Castro et al., 2008; Poppe et al., 2015).

CAPÍTULO 2 – MATERIAIS E MÉTODOS

2.1. MATERIAIS

Os sucos de frutas, laranja, maçã e uva foram fornecidos por uma empresa do setor de bebidas do RS, e coletados logo após a etapa de prensagem. Os preparados enzimáticos comerciais utilizados foram o Rohapect® 10L (AB *Enzymes*, Alemanha) e o Novozym® 33095 (*Novozymes*, Espanha), sendo declarados pelos seus fornecedores como complexos de pectinases com atividade de 70000 $ADJU.mL^{-1}$ e 10000 $PECTU.mL^{-1}$, produzidos por *Aspergillus niger* e *Aspergillus aculeatus*. Os preparados Rohapect® 10L e Novozym® 33095 foram selecionados dentre 8 preparados enzimáticos comerciais para aplicação em sucos de frutas, visto suas maiores eficiências na clarificação do suco de uva (Dal Magro et al., 2016a).

Pectina (75 % grau de esterificação), ácido poligalacturônico, ácido galacturônico, quitosana (de casca de camarão, 75 % desacetilada), 3-aminopropiltrimetoxisilano (APTMS), periodato de sódio, borohidreto de sódio, glutaraldeído a 25 %, divinilsulfona (DVS), etilenodiamina, glicidol, dodecilsulfato de sódio (SDS), glicerina e dextrano foram adquiridos da Sigma Aldrich Co. Ltd. (St. Louis, MO, EUA). Glutaraldeído 50 % foi adquirido da Dinâmica Química Contemporânea Ltda. (São Paulo, Brasil). Agarose 4 % BLC foi adquirida da Agarose Bead Technologies (Madrid, Espanha). O polietilenoglicol (600 $g.mol^{-1}$) foi adquirido da Merck (Hohenbrunn, Alemanha). Os reagentes de eletroforese foram obtidos da Bio-Rad (Hercules, CA, EUA). Todos os demais reagentes, solventes e produtos químicos foram de grau analítico.

2.2. DETERMINAÇÕES ANALÍTICAS

2.2.1. Determinação da atividade de pectinase total (PE)

A atividade de pectinase total foi determinada pela liberação de substâncias redutoras pela hidrólise da solução de pectina, quantificadas pelo método de ácido dinitrosalicílico (DNS) (Miller, 1959). Em um tubo de ensaio foram adicionados 0,1 mL da solução enzimática diluída e 0,9 mL de substrato (1 $g.L^{-1}$ de pectina em tampão citrato de sódio a 50 mM e pH 4,8), seguindo para incubação a 37 °C por 1 min, sob agitação. Após reação, foi adicionado 1 mL de DNS e incubado a 100°C por 5 min, sendo imediatamente resfriado em banho de gelo. Por fim, a amostra foi lida em espectrofotômetro, a 540 nm, contra uma amostra controle preparada sem enzima e submetida ao mesmo procedimento.

Os valores de absorbância encontrados em cada amostra foram convertidos em concentração através da curva de calibração construída com soluções de ácido galacturônico, entre 0,2 $mg.L^{-1}$ a 1,2 $mg.L^{-1}$. Uma unidade de pectinase total foi definida como a quantidade de enzima necessária para liberar 1 μmol de grupos redutores por minuto, nas condições da reação e expressa em unidades por mL ($U.mL^{-1}$). A atividade de pectinase total foi calculada pela Equação 1.

$$U/ml = \frac{C * v_f * f_{dil}}{t * v_e} \quad (1)$$

Onde:

C = concentração de grupos redutores liberados ($mg.mL^{-1}$)

v_f = volume final da reação (mL)

f_{dil} = fator de diluição

t = tempo de reação (min)

v_e = volume de enzima (mL)

2.2.2. Determinação da atividade de poligalacturonase (PG)

A atividade de poligalacturonase foi determinada pela hidrólise de ácido poligalacturônico, liberando ácidos galacturônicos que foram quantificadas pelo método de DNS (Miller, 1959). Em um tubo de ensaio foram adicionados 0,1 mL da solução enzimática diluída e 0,9 mL de substrato (1 $g.L^{-1}$ de ácido poligalacturônico em tampão citrato de sódio a 50 mM e pH 4,8), seguindo para incubação a 37 °C por 2 min, sob agitação. Após reação, foi adicionado 1 mL de DNS e incubado a 100 °C por 5 min, sendo imediatamente resfriado em banho de gelo. Por fim, a amostra foi lida em espectrofotômetro, a 540 nm, contra uma amostra controle preparada sem enzima e submetida ao mesmo procedimento.

Os valores de absorbância encontrados em cada amostra foram convertidos em concentração de ácido galacturônico através da curva de calibração construída conforme citado anteriormente (Item 2.2.1). Uma unidade de PG foi definida como a quantidade de enzima necessária para liberar 1 μmol de ácido galacturônico por minuto, nas condições da reação e expressa em unidades por mL ($U.mL^{-1}$). A atividade de PG foi calculada pela Equação 1.

2.2.3. Determinação da atividade de pectina liase (PL)

A atividade de pectina liase foi determinada pelo aumento da absorbância em 235 nm devido a formação de produtos insaturados, conforme descrito por Albersheim e Killias (1962), com modificações. Em um tubo de ensaio foram adicionados 0,05 mL da solução enzimática diluída e 0,95 mL de substrato (4 $g.L^{-1}$ de pectina em tampão de citrato de sódio a 50 mM e pH 4,8), seguindo para incubação a 37 °C por 1 min, sob agitação. Após a reação, foi adicionado 3 mL de tampão de parada (0,5 M de ácido clorídrico). Por fim, a amostra foi lida em espectrofotômetro, a 235 nm, contra uma amostra controle preparada sem enzima e submetida ao mesmo procedimento.

Os valores de absorbância encontrados em cada amostra foram convertidos em concentração utilizando o coeficiente de extinção molar do uronídeo insaturado (ϵ = 5500 $M^{-1}.cm^{-1}$) (Equação 2). Uma unidade de PL foi definida como a quantidade de enzima que produz 1 nmol de uronídeo insaturado por min, nas condições da reação e expressa em unidades por mL ($U.mL^{-1}$).

$$U/ml = \frac{Abs * v_f * f_{dil}}{\epsilon_m * L * t * v_e} \quad (2)$$

Onde:

Abs = absorbância das amostras

v_f = volume final da reação (mL)

f_{dil} = fator de diluição

ϵ_m = coeficiente de extinção molar

L = comprimento que a luz atravessa na amostra (cm)

t = tempo de reação (min)

v_e = volume de enzima (mL)

2.2.4. Determinação da atividade de pectina metil esterase (PME)

A atividade de pectina metil esterase foi determinada por titulação de grupos carboxílicos liberados através da desesterificação da pectina, de acordo com o método descrito por Rouse e Atkins (1952), com modificações. Em um tubo de ensaio foram adicionados 0,1 mL da solução enzimática diluída e 9,9 mL de substrato (5 $g.L^{-1}$ de pectina em solução de cloreto de sódio a 150 mM e pH 4,5), seguindo para incubação a 30 °C por 10 min, sob agitação. Após a reação, a amostra foi titulada com uma solução de 20 mM de hidróxido de sódio até o pH inicial de 4,5.

Os valores de NaOH gastos em cada amostra foram convertidos em concentração utilizando a Equação 3. Uma unidade de PME foi definida como a quantidade de enzima que libera 1 mEq de grupos carboxílicos por mL por minuto, nas condições da reação e expressa em unidades por mL ($U.mL^{-1}$).

$$U/ml = \frac{mL\ NaOH * M\ NaOH * f_{dil} * 1000}{t * v_e} \quad (3)$$

Onde:

mL NaOH = volume gasto de NaOH (mL)

M NaOH = molaridade da solução de NaOH

f_{dil} = fator de diluição

t = tempo de reação (min)

v_e = volume de enzima (mL)

2.2.5. Determinação de atividade de celulase total (CE)

A atividade de celulase total foi determinada utilizando papel filtro Whatman n°1 como substrato, seguindo o método proposto por Ghose (1987), com modificações. Em um tubo de ensaio foram adicionados 0,5 mL da solução enzimática diluída, 50 mg de papel filtro e 0,5 mL de tampão de citrato de sódio (50 mM e pH 4,8), seguindo para incubação a 50 °C por 5 min, sob agitação. Após reação, foi adicionado 1 mL de DNS e incubado a 100 °C por 5 min, sendo imediatamente resfriado em banho de gelo. Por fim, a amostra foi lida em espectrofotômetro, a 540 nm, contra uma amostra controle preparada sem enzima e submetida ao mesmo procedimento.

Os valores de absorbância encontrados em cada amostra foram convertidos em concentração através da curva de calibração construída com soluções de glicose, entre 0,2 $mg.L^{-1}$ a 1,2 $mg.L^{-1}$. Uma unidade de CE foi definida como a quantidade de enzima necessária

para liberar 1 μmol de grupos redutores por min, nas condições da reação e expressa em unidades por mL ($U.mL^{-1}$). A atividade de celulase total foi calculada pela Equação 1.

2.2.6. Quantificação de proteínas

O teor de proteínas total foi determinado pelo método de Bradford, que utiliza o corante de *Coomassie brilliant blue* BG-250. Em um tubo de ensaio foram adicionados 20 μL da amostra diluída e 1 mL do reagente de Bradford. Após 5 min para estabilização da cor, foi realizada a leitura em espectrofotômetro, a 595 nm, contra um controle preparado com água destilada, submetido ao mesmo procedimento.

Os valores de absorbância encontrados em cada amostra foram convertidos em concentração utilizando uma curva de calibração construída com soluções de BSA (padrão de proteína, albumina do soro bovino), entre 0,1 $mg.mL^{-1}$ a 1,0 $mg.mL^{-1}$. Os resultados foram expressos em $mg.mL^{-1}$ de albumina.

2.2.7. Determinação da turbidez

A turbidez dos sucos foi medida através da luz dispersa por espectrofotometria, como proposto por Anderson (2005), com modificações. As amostras foram centrifugadas por 2 min a 5000 × g e suas absorbâncias foram detectadas em espectrofotômetro a 860 nm. A porcentagem (%) de clarificação foi determinada através da diferença entre a turbidez inicial e final das amostras, conforme a equação 4.

$$\text{Porcetagem de clarificação (\%)} = \frac{\text{turbidez inicial} - \text{turbidez final}}{\text{turbidez inicial}} \text{ x } 100 \qquad (4)$$

2.2.8. Gel de eletroforese SDS-PAGE

O gel eletroforese de SDS-poliacrilamida foi realizado de acordo com Laemmli (1970), usando a Miniprotean tetra-cell (Bio-Rad). Um gel de corrida a 12 % de poliacrilamida foi adicionada em uma zona de separação de 9 cm × 6 cm, seguido pela adição de poliacrilamida a 5 %. As amostras de enzimas foram suspensas em tampão de ruptura (SDS a 2 % e mercaptoetanol a 10 %) e fervidas por 5 min. Após, 14 μL do sobrenadante foram coletados e utilizados nos experimentos. Os géis foram corados com azul brilhante de Coomassie e marcadores de baixo peso molecular da GE Healthcare (14.000 a 97.000 Da) foram utilizados.

2.2.9. Magnetismo

As características magnéticas das amostras foram avaliadas utilizando um magnetômetro de amostra vibrante (VSM) modelo EZ9 da MicroSense à temperatura ambiente com um campo magnético circulado entre -22 kOe e +22 kOe.

2.2.10. Características morfológicas

O diâmetro e distribuição de poros foram caracterizados por isotermas de adsorção e dessorção de N_2, obtidas na temperatura de ebulição do nitrogênio líquido, no Equipamento Micromeritics Tristar Kr 3020. As amostras foram previamente desgaseificadas a 50 ºC, sob vácuo, durante 24 h. As áreas de superfície específicas foram estimadas pela técnica de multiponto BET (Brunauer, Emmett e Teller) e o volume de poros foi obtido usando o método BJH (Barret, Joyner e Halenda) (Gregg and Sing, 1982).

2.2.11. Microscopia electrónica de varredura

As imagens da microscopia electrónica de varredura (MEV) foram obtidas utilizando um modelo de microscópio eletrônico de varredura EVO MA10 (Zeiss). Os materiais foram dispersos em uma fita condutora de dupla face em um suporte de alumínio e revestidos com uma fina película de carbono usando um aparelho Sputter Coater modelo SCD 050 (Baltec). As imagens foram obtidas com tensão de aceleração de 5 kV e 15 kV.

2.3. SÍNTESE E FUNCIONALIZAÇÃO DAS PARTÍCULAS MAGNÉTICAS

A síntese e funcionalização das partículas magnéticas foram realizadas conforme descrito por Schätz et al., (2009). Para a síntese das partículas magnéticas, 4,32 g de $FeCl_3$ foram dissolvidos em 80 mL de etilenoglicol sob agitação magnética. Subsequentemente, 7,2 g de acetato de sódio anidro foram adicionados e deixados sob agitação por 1 h. Após a formação de uma mistura homogênea, esta foi transferida para autoclave e aquecida a 200 °C por 10 h. Posteriormente, as partículas magnéticas foram separadas com auxílio de um imã, lavadas com etanol e secas à vácuo a 50 °C por 4 h.

Para a funcionalização, em um balão de 3 bocas de 250 mL, 1 g de Fe_3O_4 foi disperso em uma solução de tolueno (50 mL) e 3-aminopropiltrimetoxisilano (APTMS) (0,174 mL, 1 mmol). A reação foi feita sob agitação mecânica, em atmosfera de argônio, por 12 h a 80 °C. O material magnético foi separado com auxílio de um imã, lavado com tolueno, etanol, água e etanol novamente, e posteriormente, seco em linha de vácuo a 50 °C por 2 h.

2.4. RECOBRIMENTO DAS PARTÍCULAS MAGNÉTICAS COM QUITOSANA

2.4.1. Nanopartículas magnéticas recobertas com quitosana

As nanopartículas magnéticas de quitosana foram preparadas pelo método de gelificação ionotrópica utilizando sulfato de sódio como agente de gelificação, como descrito

por Klein et al. (2012), com modificações. 25 mg de quitosana foi misturado com 25 mg de partículas magnéticas funcionalizadas em 9,5 mL de ácido acético (0,35 M) contendo 1 % de Tween 80. Em seguida, foram gotejados 0,5 mL de sulfato de sódio (1,4 M) em banho de ultrassom. Então, esta mistura foi agitada magneticamente por 2 h. As nanopartículas assim obtidas foram separadas magneticamente e lavadas com água destilada.

2.4.2. Micropartículas magnéticas recobertas com quitosana

As micropartículas magnéticas de quitosana foram preparadas usando a técnica de reticulação em emulsão, de acordo com Denkbas et al. (2002), com modificações. 200 mg de quitosana foram misturados com 200 mg de partículas magnéticas funcionalizadas em 10 mL de ácido acético (0,35 M). Esta solução foi vertida em um meio de dispersão, composto por 25 mL de óleo mineral e 35 mL de éter de petróleo com 0,5 % de Tween 80, sob agitação. Após 10 min do início do processo de emulsão, foram adicionados 0,3 mL de glutaraldeído. Transcorrido 1 h de reação, mais 0,3 mL de glutaraldeído foram adicionados, totalizando 1 % na mistura final, seguindo por mais 2 h de agitação. Ao final da reação, as micropartículas magnéticas com quitosana foram separadas da fase oleosa por centrifugação (30 minutos a $3000 \times g$), seguido por sucessivas lavagens com acetona, etanol e água destilada.

2.4.3. Macropartículas magnéticas recobertas com quitosana

As macropartículas magnéticas de quitosana foram preparadas pelo método de precipitação, de acordo com Klein et al. (2012), com modificações. 400 mg de quitosana foram misturados com 400 mg de partículas magnéticas em 20 mL de ácido acético (0,35 M). A mistura foi sonicada por 2 h para a remoção das bolhas de ar, seguido pelo gotejamento da solução em 250 mL de hidróxido de sódio (1 M), sob agitação lenta. As macropartículas magnéticas de quitosana formadas foram separadas magneticamente e lavadas com água

destilada até atingir a neutralidade.

2.5. PREPARAÇÃO DOS SUPORTES DE AGAROSE

2.5.1. Suporte glioxil-agarose

A preparação do suporte de glioxil-agarose foi realizada de acordo com o procedimento descrito por Mateo et al. (2006). 50 g de esferas de agarose (4 % BCL) foram suspendidas em 85 mL de água destilada. Em seguida, foram adicionados 23,8 mL de NaOH (1,7 N), contendo 1,6 g de borohidreto de sódio, à solução de agarose. Esta suspensão foi mantida em um banho de gelo sob agitação mecânica, enquanto 17,2 mL de glicidol foram adicionados gota a gota. A suspensão foi agitada suavemente durante a noite (18 h) à temperatura ambiente. Após esse período, a agarose ativada foi exaustivamente lavada com água destilada e filtrada. Em seguida, para cada grama de glioxil-agarose foram adicionados 10 mL de água destilada, contendo 60 μmol de $NaIO_4$ para cada grama úmida de suporte. A reação de oxidação foi realizada por 3 h sob agitação à temperatura ambiente. Em seguida, o suporte foi lavado com água destilada, filtrado e armazenado a 4 ° C.

2.5.2. Suporte vinilsulfona-agarose

A preparação do suporte de vinilsulfona-agarose foi realizada conforme descrito por dos Santos et al. (2015). Uma solução de divinilsulfona (DVS) foi preparada misturando 7,5 mL de DVS com 200 mL de tampão de carbonato de sódio (333 mM) a pH 12,5, até a solução se tornar homogênea. Depois disso, 10 g de agarose (4 % BCL) foram adicionados à solução sob agitação suave por 35 min. Transcorrido esse tempo, o suporte foi lavado exaustivamente lavado com água destilada, filtrado e armazenado a 4 ° C.

2.5.3. Suporte MANAE e MANAE-glutaraldeído

O suporte de monoaminoetil-N-aminoetil (MANAE) foi preparado a partir de um suporte glioxil-agarose, como descrito no item 2.4.1, e modificado com etilenodiamina. Para cada grama do suporte glioxil-agarose foi adicionado 4 mL de etilenodiamina (2 M, pH 10), a suspensão ficou sob agitação suave por 24 h. Transcorrido esse tempo, 10 $mg.mL^{-1}$ de borohidreto de sódio foram adicionados para reduzir o suporte, o qual ficou por mais 2 h sob agitação. Em seguida, o MANAE obtido foi lavado com tampão carbonato (100 mM, pH 9), com tampão acetato (100 mM, pH 4) e, finalmente, com água destilada, seguido pela filtração e armazenamento a 4 °C (Zaak et al., 2017).

A partir desse suporte também foi preparado o MANAE-glutaraldeído, onde 10 g de MANAE foi adicionado a 50 mL de uma solução de glutaraldeído a 10 % (v/v) preparado em tampão fosfato de sódio (200 mM, pH 7), sob agitação suave durante 15 h a temperatura ambiente (Zaak et al., 2017). Este protocolo permitiu a introdução de duas moléculas de glutaraldeído por grupo amino primário do suporte (Barbosa et al., 2014). Em seguida, o MANAE-glutaraldeído obtido foi lavado exaustivamente com água destilada para remover todo o excesso de glutaraldeido e encaminhado para a imobilização.

2.6. IMOBILIZAÇÃO DE ENZIMAS

2.6.1. Considerações gerais

Para as metodologias de imobilização descritas abaixo, as atividades recuperadas (AR), os rendimentos de imobilização (RI) e as eficiências de imobilização (EI) foram calculadas de acordo com Sheldon e Van Pelt (2013), seguindo as equações 5, 6 e 7.

$$\text{AR}\ (\%) = \frac{\text{Atividade do biocatalisador (U)}}{\text{Atividade inicial (U)}} \text{ x } 100 \qquad (5)$$

$$\text{RI}\ (\%) = \frac{\text{Atividade imobilizada}\ (\text{U})}{\text{Atividade inicial}\ \ (\text{U})}\ \text{x}\ 100 \qquad (6)$$

$$\text{EI}\ (\%) = \frac{\text{Atividade do biocatalisador}\ \ (\text{U})}{\text{Atividade imobilizada}\ \ (\text{U})}\ \text{x}\ 100 \qquad (7)$$

2.6.2. Agregados enzimáticos entrecruzados magnéticos (CLEAs-Mag)

A preparação dos CLEAs-magnéticos foi realizada através do procedimento proposto por Schoevaart et al., (2004), com modificações. Para isso, 1 mg da partícula magnética funcionalizada foi adicionado à solução enzimática (0,4 $mg.mL^{-1}$), seguindo para a precipitação. A fim de escolher o melhor agente precipitante para a preparação dos CLEAs magnéticos, quatro solventes orgânicos foram avaliados: acetona, etanol, iso-propanol e terc-butanol em diferentes concentrações. Após a precipitação das proteínas, 50 mM de glutaraldeído foi adicionado à solução, dando início a reação de entrecruzamento que foi realizada durante 1 a 7 h à temperatura ambiente em um homogeneizador horizontal de rolos. Transcorrido o tempo de reação, os CLEAs magnéticos foram separados pela aproximação de um campo magnético (íman) e lavado 3 vezes com tampão de citrato de sódio (50 mM, pH 4,8) para remover o agente precipitante e as enzimas fracamente adsorvidas. Por fim, os CLEAs magnéticos foram suspensos em tampão de citrato de sódio (50 mM, pH 4,8) e armazenados a 4 ºC até a sua utilização.

2.6.3. Imobilização covalente em partículas magnéticas

A imobilização covalente das enzimas em partículas magnéticas foi baseada na metodologia proposta por Bayramoglu et al., (2017), com modificações. Inicialmente, 1 mg de partículas magnéticas funcionalizadas foram ativadas com diferentes concentrações de glutaraldeído (2,5 mM a 250 mM) durante 3 h. Após ativação, as partículas magnéticas foram

separadas magneticamente e lavadas 3 vezes com tampão citrato de sódio (50 mM, pH 4,8) para remover o excesso de glutaraldeído. Posteriormente, as partículas magnéticas ativadas foram incubadas durante a noite com diferentes concentrações de enzimas (0,1 mg a 0,8 mg de proteína) em tampão citrato de sódio (50 mM, pH 4,8) à temperatura ambiente em um homogeneizador de rolos horizontal. Ao final da imobilização, o biocatalisador foi separado pela aproximação de um campo magnético (íman) e lavado com tampão citrato de sódio (50 mM, pH 4,8) por 3 vezes para a remoção das enzimas fracamente adsorvidas. Por fim, o biocatalisador foi suspenso em tampão citrato de sódio (50 mM, pH 4,8) e armazenado a 4 °C até a sua utilização.

2.6.4. Imobilização covalente em partículas magnéticas com quitosana

Primeiramente, para a imobilização covalente das enzimas em partículas magnéticas com quitosana, foram realizados experimentos variando o tempo de ativação. Assim, 50 $mg.mL^{-1}$ do suporte foi incubado com 1 % de glutaraldeído em tampão fosfato de potássio (0,1 M, pH 7,0) a 37 °C sob agitação. Após ativação, os suportes foram separados magneticamente e lavados 3 vezes com tampão citrato de sódio (50 mM, pH 4,8) para remover o excesso de glutaraldeído. Posteriormente, os suportes ativados foram incubados com diferentes concentrações de enzimas (0,2 mg a 1,6 mg de proteína) em diferentes tempos de imobilização (5 h a 30 h), à temperatura ambiente em um homogeneizador de rolos horizontal. No final da imobilização, os biocatalisadores foram separados magneticamente e lavados 3 vezes com tampão citrato de sódio (50 mM, pH 4,8) para remover as enzimas fracamente adsorvidas. Finalmente, os biocatalisadores foram suspensos em tampão citrato de sódio (50 mM, pH 4,8) e armazenados a 4 °C até a sua utilização.

2.6.5. Imobilização em suportes de agarose

A imobilização das enzimas foi realizada em glioxil-agarose, vinilsulfona-agarose, MANAE e MANAE-glutaraldeído. Para isso, 1 g de cada suporte foi adicionado a uma solução contendo 1 mL da preparação enzimática (5,4 mg de proteína) e 9 mL de tampão (5 mM, contendo 20 % de polietilenoglicol para a estabilização enzimática). Dependendo do pH de imobilização diferentes tampões foram utilizados, citrato de sódio 5 mM para pH 5, fosfato de sódio 5 mM para pHs entre 6 e 8, ou carbonato de sódio 5 mM para pH 9 e 10. As imobilizações foram realizadas à temperatura ambiente em um agitador de rolos horizontal. Durante o período de imobilização, as atividades enzimáticas do sobrenadante, da suspensão e da referência (enzima livre em condições idênticas às da suspensão de imobilização, utilizando agarose inerte) foram monitoradas.

A imobilização em glioxil-agarose foi realizada em pH 10, com e sem 20 % de polietilenoglicol. Após 5 h de imobilização, foi adicionado 1 mg de borohidreto de sódio por mL de suspensão de imobilização. Transcorrido 30 min, os biocatalisadores obtidos foram exaustivamente lavados com água destilada e com tampão citrato de sódio (50 mM, pH 4,8), filtrados a vácuo, usando um filtro de vidro sinterizado, e armazenados a 4 °C até a sua utilização.

A imobilização em vinilsulfona-agarose foi realizada em pH 8 e pH 10, contendo 20 % de polietilenoglicol. Após 5 h de imobilização, os biocatalisadores foram incubados em uma solução de etilenodiamina (1 M, pH 8) durante 15 h, para bloquear os demais grupos ativos do suporte. Transcorrido esse tempo, os biocatalisadores obtidos foram exaustivamente lavados com água destilada e com tampão citrato de sódio (50 mM, pH 4,8), filtrados a vácuo, usando um filtro de vidro sinterizado, e armazenados a 4 °C até a sua utilização.

A imobilização em MANAE foi realizada em pHs 5, 7, 8 e 9, usando 20 % de polietilenoglicol como estabilizador. Após a troca iônica da enzima com o suporte, o pH foi

ajustado para 7 e 1 % de glutaraldeído foi adicionado à suspensão de imobilização, para promover a ligação covalente da enzima com o suporte. Transcorrido 1 h de reação, os biocatalisadores foram lavados com água destilada para eliminar o excesso de glutaraldeído e incubados a pH 8 por um período de 24 h, permitindo um aumento do número de ligações da enzima com suporte. Finalizada essa etapa, os biocatalisadores obtidos foram exaustivamente lavados com água destilada e com tampão citrato de sódio (50 mM, pH 4,8), filtrados a vácuo, usando um filtro de vidro sinterizado, e armazenados a 4 °C até a sua utilização.

Por fim, a imobilização em suportes MANAE pré-ativados com glutaraldeído (MANAE-glutaraldeído) foi realizada em pH 5, pH 6,5 e pH 8. Após 3 h de imobilização, as amostras foram incubadas em pH 8 por um período de 24 h, permitindo um aumento do número de ligações da enzima com suporte. Uma amostra foi mantida em pH 5 até o final da imobilização (24 h). Além disso, outra amostra foi imobilizada em pH 8 com 300 mM de NaCl. Finalizado as imobilizações, os biocatalisadores obtidos foram exaustivamente lavados com água destilada e com tampão citrato de sódio (50 mM, pH 4,8), filtrados a vácuo, usando um filtro de vidro sinterizado, e armazenados a 4 °C até a sua utilização.

2.6.6. Imobilização em esferas de quitosana

Após a preparação das esferas de quitosana, como descrito por Klein et al. (2012), estas foram ativadas através da incubação em uma solução de glutaraldeído a 5 %, preparada em tampão fosfato 0,1 M (pH 7,0), por 3 h sob agitação suave. Posteriormente, as esferas foram lavadas abundantemente com água destilada para remover o excesso de glutaraldeído. Para a imobilização enzimática, as esferas ativadas foram incubadas com diferentes concentrações de enzima, variando de 12,5 mg a 300 mg de proteína por grama de suporte seco, preparadas em tampão citrato de sódio (50 mM, pH 4,8). A imobilização foi realizada por 15 h em temperatura ambiente e sob agitação suave em um homogeneizador de rolos

horizontal. Após este tempo, as enzimas imobilizadas foram separadas da solução e lavadas com tampão citrato de sódio (50 mM, pH 4,8) para remover as enzimas fracamente adsorvidas. Por fim, os biocatalisadores foram suspensos em tampão citrato de sódio (50 mM, pH 4,8) e armazenados a 4 °C até a sua utilização.

2.7. CARACTERIZAÇÃO DOS BIOCATALISADORES

2.7.1. Parâmetros cinéticos

Os parâmetros cinéticos, velocidade máxima de reação (V_{max}), constante de Michaelis (K_M) e eficiência catalítica (V_{max}/K_M), foram determinados utilizando diferentes concentrações de pectina (1,39 μmol.mL^{-1} e 55,5 μmol.mL^{-1}) em tampão citrato de sódio (50 mM, pH 4,8) a 37 °C. O K_M e V_{max} foram calculados por regressão não linear das taxas iniciais de reação correspondentes a diferentes concentrações de substrato usando o programa Solver (Microsoft Office Excel 2016).

2.7.2. Determinação da temperatura ótima

A temperatura ótima para atividade da enzima livre e dos biocatalisadores imobilizados foi determinada pelas análises das atividades enzimáticas, descritas no item 2.2, em diferentes temperaturas, variando de 30 a 90 °C, com pH constante de 4,8.

2.7.3. Determinação do pH ótimo

O pH ótimo para atividade da enzima livre e dos biocatalisadores imobilizados foi determinado através pelas análises das atividades enzimáticas, descritas no item 2.2, em diferentes pHs, variando de pH 3 a 10, em temperatura ambiente ou de 60 °C.

2.7.4. Avaliação da inativação térmica

A inativação térmica foi determinada através da incubação da enzima livre e dos biocatalisadores imobilizados em distintas condições de temperatura e pH. Diferentes combinações de temperatura e pH foram selecionadas para ter cursos de inativação confiáveis. Assim, foram utilizadas temperaturas, variando de 25 a 60 ºC, e pHs, variando de 4 a 10, usando tampões citrato de sódio (50 mM), fosfato de sódio (50 mM) ou carbonato de sódio (50 mM), dependendo do pH. Periodicamente, as amostras foram coletadas para determinação das atividades enzimáticas, descritas no item 2.2.

2.7.5. Avaliação da estabilidade térmica

As estabilidades térmicas da enzima livre e imobilizadas foram avaliadas conforme descrito por Lorenzoni (2014), com modificações. As enzimas foram incubadas a 60 °C em tampão de citrato de sódio (50 mM, pH 4,8). Periodicamente, alíquotas foram retiradas para determinação da atividade de pectinase total (Item 2.2.1). A inativação térmica foi descrita por uma equação de primeira ordem:

$$\frac{A}{A_0} = \exp(-kt) \qquad (8)$$

Onde: A é a atividade enzimática no tempo t, A_0 é atividade enzimática inicial, t é o tempo de tratamento e k é a taxa de inativação constante na temperatura estudada. Os tempos de meia-vida das enzimas ($t_{1/2}$) representam os tempos necessários para que as enzimas percam 50 % da sua atividade inicial, sendo calculados a partir dos valores de k, com a seguinte equação:

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

2.7.6. Avaliação da estabilidade ao armazenamento

A estabilidade ao armazenamento foi avaliada através da incubação das enzimas livre e imobilizadas no pH 3,5 em tampão citrato de sódio (50 mM) a 4 ºC. Periodicamente, amostras foram retiradas e atividade enzimática foi medida, conforme descrito no item 2.2.

2.7.7. Estabilidade operacional em batelada

A estabilidade operacional em batelada dos biocatalisadores imobilizados foi avaliada durante vários ciclos de clarificação de sucos. Para isso, foram adicionados 5 U de atividade total de pectinase por mL de suco fresco e incubados a 40 ºC durante 2 h, para cada biocatalisador. Entre cada ciclo de reutilização, as enzimas imobilizadas foram recuperadas magneticamente e lavadas 3 vezes com tampão citrato de sódio (50 mM, pH 4,8), seguido da adição de um novo suco para iniciar um novo ciclo de clarificação. A diminuição da turbidez do suco foi determinada para cada ciclo, considerando o valor do primeiro ciclo como 100 %. A turbidez foi determinada conforme descrito no item 2.2.7.

2.7.8. Estabilidade operacional em reatores de fluxo continuo

O reatores de leito empacotado e leito fluidizado são formados por um tubo de vidro com 1 cm de diâmetro interno e 18 cm e 39 cm de comprimento, respectivamente, envolvidos por uma camisa externa para recirculação da água com temperatura controlada de 40 ºC. Os volumes internos dos reatores foram de 14 mL e 31 mL, respectivamente para o reator de leito empacotado e leito fluidizado, os quais foram preenchidos com 650 esferas (0,22 g de massa seca com atividade de 1340 $U.g^{-1}$). Para o reator de leito fluidizado, uma bomba peristáltica, com alta vazão, foi utilizada para recirculação do suco e suspensão das esferas.

A clarificação contínua do suco de laranja foi avaliada em diferentes vazões, variando de 0,5 a 3 $mL.min^{-1}$. Após 2 h de operação, as amostras foram coletadas e a porcentagem de clarificação foi determinada. Em seguida, a estabilidade operacional em ambos os reatores foi avaliada através da clarificação contínua do suco de laranja com uma vazão fixa de 0,5 $mL.min^{-1}$. Periodicamente, amostras foram coletadas na saída dos reatores e a porcentagem de clarificação do suco de laranja foi determinada conforme descrito no item 2.2.7.

CAPÍTULO 3 – Magnetic biocatalysts of pectinase and cellulase: Synthesis and characterization of two preparations for application in grape juice clarification

No intuito de melhorar a separação e a reutilização das enzimas imobilizadas dos sedimentos dos sucos, partículas magnéticas foram utilizadas em diferentes protocolos de imobilização, buscando superar esse problema. Assim, neste trabalho foi avaliada a preparação de dois biocatalisadores magnéticos de pectinase e celulase: CLEAs-magnéticos (CLEA-MP*), preparados através do entrecruzamento das enzimas com as partículas magnéticas, e a imobilização das enzimas na superfície da partícula magnética ativada com glutaraldeído (Enz-Glu-MP*). Os biocatalisadores foram comparados em relação as suas propriedades magnéticas, texturais, morfológicas, parâmetros cinéticos, estabilidades e na clarificação do suco de uva. Os resultados estão apresentados no manuscrito a seguir, publicado na revista *International Journal of Biological Macromolecules*, v. 115, p. 35-44, 2018.

CAPÍTULO 3 - Magnetic biocatalysts of pectinase and cellulase: Synthesis and characterization of two preparations for application in grape juice clarification

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Abstract

In the present study, we prepared two different magnetic biocatalysts of pectinase and cellulase: carrier-free magnetic CLEAs (CLEA-MP*) and immobilization on glutaraldehyde-activated magnetite (Enz-Glu-MP*). The biocatalysts were compared to their magnetic properties, immobilization parameters, stability and grape juice clarification. Enz-Glu-MP* presented highermagnetic properties than CLEA-MP*, whereas this presented higher surface area and pore volume. The KM of the enzyme immobilized on Enz-Glu-MP* was 25.65 mM, lower in comparison to the CLEA-MP* (33.83 mM). On the other hand, CLEA-MP* was the most active and stable biocatalyst, presenting higher recovered activity (33.4 % of cellulase), higher thermal stability (2.39 stabilization factor) and improved reusability (8 cycles). The integration of magnetic technology with enzymatic immobilization emerges as a possibility to increase the recover and reuse of biocatalysts for application in juice technology.

Keywords: magnetic particle; enzyme immobilization; CLEA; pectinase; cellulase; juice clarification.

1. Introduction

In the processing of grape juices, the pressing step leads to a disruption of the vegetable cell wall, releasing the internal juice. Then, due to the colloidal dispersion of pectin, the freshly pressed juices are turbid and cloudy in appearance, which is one of the major hurdles in the process [1,2].Additionally, polysaccharides such as cellulose and hemicellulose tend to settle during storage, resulting in a clear, flavorless and poor quality fruit juice [3]. In order to overcome these problems, commercial enzyme preparations, containing mainly pectinases, cellulases and hemicellulases, are extensively used in fruit juice industries in their soluble forms, since they promote the hydrolysis of such compounds, improving filtration, clarification and stabilization of the final product [4–7].

Immobilization technology emerges as a viable strategy to build heterogeneous biocatalysts and improve enzyme recovery and reusability [8–10]. Enzyme immobilization enzyme stability against adverse reaction conditions, facilitates enzyme separation from reaction systems and allows the reuse of the biocatalyst during multiple cycles or mainly in continuous process, making it commercially and industrially attractive [9,11–17]. Some studies have demonstrated that the enzyme immobilization could be applied for the clarification of apple, grape, pineapple, orange and carrot juices [18–23]. Nevertheless, due to the low costs involving juice technology and the, sometimes, high costs for enzyme immobilization, none immobilized preparation was industrially used for this purpose, open the field for new research efforts.

From several methods for enzyme immobilization, the covalent immobilization is usually preferred since it may prevent enzymes desorption from the support during the reaction [11,24–27]. In addition, the type of support is an important factor in the enzyme immobilization process, since it can influence the amount of enzyme loading, as well as their catalytic activity and operational stability [12,28–30].

In general, small size biocatalysts are very attractive because of their high specific surface area available to enzyme attachment. However, the separation step from reaction medium can be hard, because the juice clarification generates insoluble sediments, and the separation of immobilized enzymes and sediments is difficult by traditional methods, such as centrifugation and filtration. In this sense, magnetic particles can be considered as a potential support for enzyme immobilization in order to overcome these limitations, since their magnetic core allows easy, quick and efficient separation of enzyme from the reaction mixture by using external magnetic field; moreover its size can be tailored to provide high surface and, subsequently, high enzyme activity [31,32]. Despite all these advantages, the magnetic particles are highly sensitive to acidic and oxidative conditions. Therefore, their functionalization is extremely important to maintain their stability [33].

Another strategy to enzyme immobilization is the preparation of carrier-free biocatalysts. Cross-linked enzyme aggregates (CLEAs) are generally prepared by directly cross-linking different enzyme preparations [34]. This approach offers clear advantages, for instance, highly concentrated enzyme activity in the catalyst, high stability and the low production cost due to the exclusion of an additional carrier [29].

Based on these aspects, the objective of the present work was to prepare two different magnetic biocatalysts for application in juice clarification aiming to help biocatalyst separation. The magnetic particles were functionalized using aminopropyltrimethoxysilane (APTMS) and used to prepare the immobilized biocatalyst by two strategies: by covalent attachment to glutaraldehyde-activated magnetite (Enz-Glu-MP*) or by magnetic cross-linking enzyme aggregates (CLEA-MP*). These strategies were tested to verify biocatalysts properties in relation to their magnetic, morphological and textural characteristics, recovered enzymatic activities, kinetic parameters (Vmax and KM), optimal pH and temperature and

thermal stability. Finally, the immobilized enzymes were used for grape juice clarification in several batches of reuse.

2. Materials and methods

2.1. Materials

The freshly pressed grape juice without any treatment was kindly donated by Vitivinícola Jolimont (Canela, RS, Brazil). Rohapect 10 L was acquired from AB Enzymes (Darmstadt, Germany). Pectin from apple, polygalacturonic acid and galacturonic acid were from Sigma Aldrich (St. Louis, MO). All other reagents and solvents were of analytical grade.

2.2. Determination of enzyme activities

All enzymatic activities - total cellulase (CE), total pectinase (PE), polygalacturonase (PG), pectinlyase (PL) and pectin methyl esterase (PME) - were performed as described by Dal Magro et al. [35].

2.3. Synthesis of the magnetic particles (MP) biocatalyst

The magnetite particles (MP) were synthesized by the dissolution of 4.32 g of $FeCl_3$ in 80 mL of ethylene glycol under stirring. Subsequently, 7.2 g of anhydrous sodium acetate was added and mixed for 1 h. After formation of a homogeneous mixture, it was treated at 200 °C for 10 h in an autoclave. Then, the particles were magnetically separated, washed with ethanol (8×) and dried under vacuum at 50 °C for 4 h.

The magnetite particles (1 g) were functionalized by dispersing them into a solution of toluene (50 mL) and 3-aminopropyltrimethoxysilane (APTMS) (0.174 mL, 1 mM). The reaction was carried out under argon atmosphere and stirring for 12 h at 80 °C. Finally, the functionalized APTMS-magnetite particles, named as MP*, were magnetically separated,

washed with toluene (3×), ethanol (5×), water (5×), ethanol (2×) and dried under vacuum at 50 °C for 2 h.

2.4. Enzyme immobilization

2.4.1 Immobilization by covalent attachment to glutaraldehyde-activated MP*

For the covalent immobilization of Rohapect 10 L, the MP*were previously activated with different glutaraldehyde concentrations (2.5mM to 250 mM) for 3 h. After activation, the support was washed with distilled water and incubated overnight with the enzyme solution prepared with sodium citrate buffer (50 mM, pH 4.8) overnight at room temperature, under stirring in a roller mixer. Different enzyme concentrations (0.1 mg to 0.8 mg of protein per g of MP*) were tested in order to find the best recovered activity (RA %). At the end of immobilization, the covalent attached magnetic particle, named as Enz-Glu- MP*, biocatalyst was magnetically separated and washed 3-times with sodium citrate buffer (50 mM, pH 4.8) to remove the enzymes that did not bound. Finally, the Enz-Glu-MP* was suspended in sodium citrate buffer (50 mM, pH 4.8) and stored at 4 °C. The recovered activity was calculated according to the following equation:

$$\text{RA}\ (\%) = \frac{\text{Total pectinase activity in biocatalyst}\ (\text{U})}{\text{Initial pectinase activity}\ \ (\text{U})}\ \text{x}\ 100 \qquad (1)$$

2.4.2 Synthesis of the Magnetic CLEA

The magnetic CLEA was synthesized according to Schoevaart et al., [36], with some modifications. For this, the MP* (1 mg) were added to the enzyme solution (0.4 $mg.mL^{-1}$) and then precipitated with organic solvent. Different concentrations of four organic solvents (ethanol, acetone, isopropanol and tert-butanol) were tested in order to achieve high recovered activities (RA %). After protein precipitation, glutaraldehyde was added to a final

concentration of 50 mM, and the cross-linking reaction was allowed to proceed at room temperature during 1 h to 7 h. Afterwards, the magnetic CLEA, named as CLEA-MP*, was magnetically separated and washed 3-times with sodium citrate buffer (50 mM, pH 4.8) to remove the enzymes that did not bound. Finally, the CLEAMP* was suspended in sodium citrate buffer (50mM, pH 4.8) and stored at 4 °C. The recovered activity was calculated according to the Eq. (1).

2.5. Characterization of the materials

Themagnetic characteristics of the sampleswere evaluated using an EZ9 MicroSense vibrating sample magnetometer (VSM) at room temperature with a magnetic field cycled between −22 kOe and +22 kOe.

N_2 adsorption isotherms were determined at liquid nitrogen boiling point, using a Tristar II 3020 Kr Micrometrics equipment. The samples were previously degassed at 50 °C, under vacuum, for 24 h. The specific surface areas were determined by the BET (Brunauer, Emmett and Teller) multipoint technique and the pore volume was obtained by using the BJH (Barret, Joyner and Halenda) method [37].

Scanning Electron Microscopy (SEM) images were obtained using a scanning electron microscope model Zeiss EVO MA10. The materials were dispersed on a double-sided conducting tape on an aluminum support and coated with a thin film of carbon using a BaltedSCD 050 Sputter Coater apparatus. The images were obtained at accelerating voltage of 5 kV and 15 kV.

2.6. Kinetic parameters

Kinetic parameters (K_M and V_{max}) of the soluble enzyme and immobilized biocatalysts (Enz-Glu-MP* and CLEA-MP*) were determined by using different pectin concentrations,

varying between 1.39 μmol.mL^{-1} and 55.5 μmol.mL^{-1}, in sodium citrate buffer (50 mM, pH 4.8) at 37 °C. The Km and V_{max} were calculated by non-linear regression of the initial reaction rates corresponding to different substrate concentrations using the program Solver (Microsoft Office Excel 2016).

2.7. Optimal pH and temperature

Soluble enzyme and the immobilized biocatalysts (Enz-Glu-MP* and CLEA-MP*) were analyzed and compared with respect to their optimal pH and temperature of activities. The range of temperatures tested varied between 30 °C to 80 °C, at pH4.8whereas the range of pH tested varied between 3.0 and 6.0 at 60 °C.

2.8. Thermal and storage stability

The thermal stability of the soluble and immobilized enzymes (Enz- Glu-MP* and CLEA-MP*) were performed by incubating an aliquot of each sample in sodium citrate buffer (50 mM, pH 4.8) at 60 °C. The pH stability was performed by incubating the enzymes (soluble and immobilized) in sodium citrate buffer (50 mM, pH 3.5), at 4 °C. Periodically, samples were withdrawn and the total pectinase activity was measured. The thermal inactivation was described by a first order reaction:

$$\frac{A}{A_0} = \exp(-kt) \qquad (2)$$

where A is the enzyme activity at time t, A0 the initial enzyme activity, t is the treatment time, and k is the constant inactivation rate at the studied temperature. The half-life time ($t_{1/2}$) was calculated through the k values, according to the equation:

$$t_{1/2} = \frac{\ln(2)}{k} \qquad (3)$$

2.9. Operational stability

Operational stability of the Enz-Glu-MP* and CLEA-MP* biocatalysts was evaluated during repeated cycles of grape juice clarification. For this, Enz-Glu-MP* or CLEA-MP* biocatalyst (5 U of total pectinase) were added to 1.0 mL of fresh juice and incubated at 40 °C during 2 h under agitation. Between each cycle of reuse, Enz-Glu-MP* and CLEAMP* were magnetically separated from the reaction medium and washed with sodium citrate buffer (50 mM, pH 4.8). The decrease on grape juice turbidity was measured for each cycle, considering the turbidity reduction of the first cycle as 100 %. Turbidity was measured spectrophotometrically by detection of the scattered light at 860 nm, as proposed by Anderson [38].

3. Results and discussion

3.1. Preparation of magnetic CLEA

3.1.1. Screening of precipitant agents

An important step in the synthesis of the CLEA-MP* biocatalyst preparation is precipitation, which transforms free enzymes into their physically aggregated form. The nature and concentration of the precipitant can have great effect on the recovered activity, thus these parameters are relevant to be evaluated.

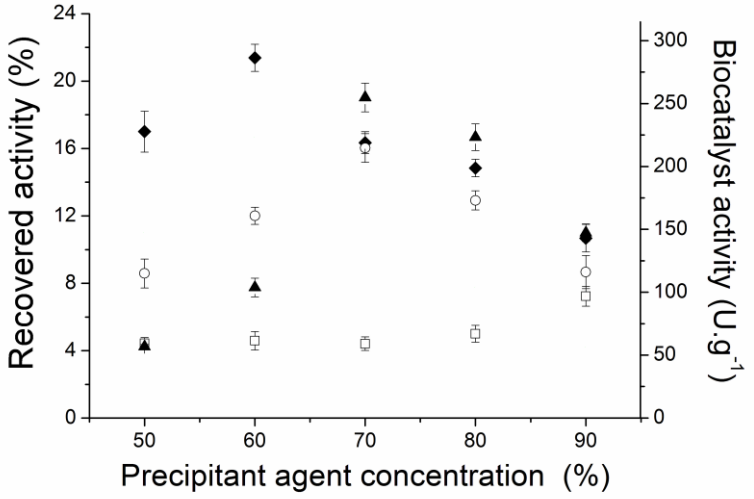
Some works deal with several precipitant agents for different enzymes, and activities even higher than 100 % have been found [36,39]. However, in many cases, just the recovered activity after enzyme precipitation and dissolution is considered. High activity on dissolution of the precipitate in buffer does not automatically mean that the aggregates will retain this high activity after cross-linking [34]. Therefore, in this work, the effect of precipitant solvents (acetone, ethanol, isopropanol and tert-butanol) on the recovered activity, at different

concentrations, was analyzed after cross-linking with glutaraldehyde (50 mM), and the results are shown in Fig. 1.

For ethanol, isopropanol and tert-butanol, small recovered activities were initially observed, increasing with solvent concentration until 70 % (v/v), 60 % (v/v) and 70 % (v/v), respectively. Higher solvent concentrations caused a decrease in the recovered activity. According to Talekar et al. [40] at low concentration the precipitation process is slow and the damage in the enzyme structure is marked, causing its denaturation. In higher concentration of precipitant, the precipitation process is faster and the enzyme molecule is able to find neighboring molecules to surround it. In this way, the enzyme can maintain its active tertiary structure. However, high concentrations of precipitant agent form too large aggregates, reducing the mass transfer of substrate to the internal side of CLEAs and the catalytic activity [41].When acetone was used as precipitant, the lowest recovered activities were observed, remaining always below 8 %.

The best precipitation condition was achieved with the use of isopropanol 60 % (v/v), and the recovered activity was 21.4 %. Often, the recovered enzyme activity from CLEAs is low due to conformational changes caused by removal of hydration water on the enzyme surface required to retain activity. Moreover, the penetration of solvent into the enzyme active sites can lead to damages in the protein structure. The rigidification and confinement of the enzymes in the aggregates can also hinder the access of substrates, thus reducing enzyme activity [42,43].

Figure 1. Effect of acetone (□), ethanol (▲), isopropanol (◆) and *tert*-butanol (○) in different concentrations, as precipitant solvents, on the CLEA-MP* preparation, under the conditions of 50 mM of glutaraldehyde, 2 h of reaction and 0.4 mg.mL^{-1} of enzyme concentration.

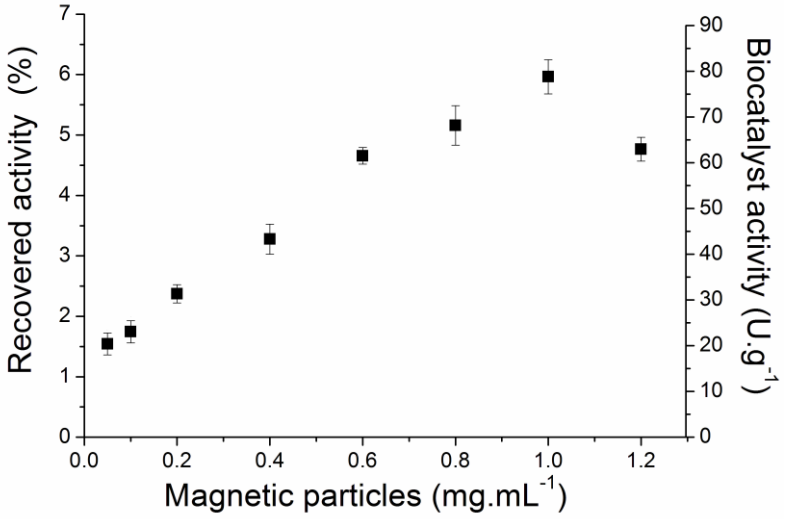


3.1.2. Effect of magnetite concentration on CLEA-MP* synthesis

After selection of the precipitant solvent, different concentrations of the MP* were tested for the synthesis of CLEA-MP* biocatalyst. The cross-linking was performed with glutaraldehyde (50 mM), during 2 h using an enzyme solution (0.4 mg.mL^{-1}).

As can be seen in Fig. 2, the increase in the magnetite concentration resulted in a positive effect on RA % up to 1.0 mg.mL^{-1}. On the other hand, higher concentrations resulted in a decrease in the RA %, probably due to the dilution effect on the enzyme with the addition of the MP*. Moreover, increasing the concentration of MP* the CLEA-MP* magnetization was improved. Nadar and Rathod [44] and Talekar et al. [45] also observed that the increase in magnetite concentration had a positive effect on the recovered activity from the biocatalyst to a certain extent, and that higher concentrations did not lead to the increase of the recovered activity.

Figure 2. Influence of magnetite concentration on the CLEA-MP* preparation, under the conditions of 50 mM of glutaraldehyde, 2 h of reaction and 0.4 mg.mL^{-1} of enzyme concentration.

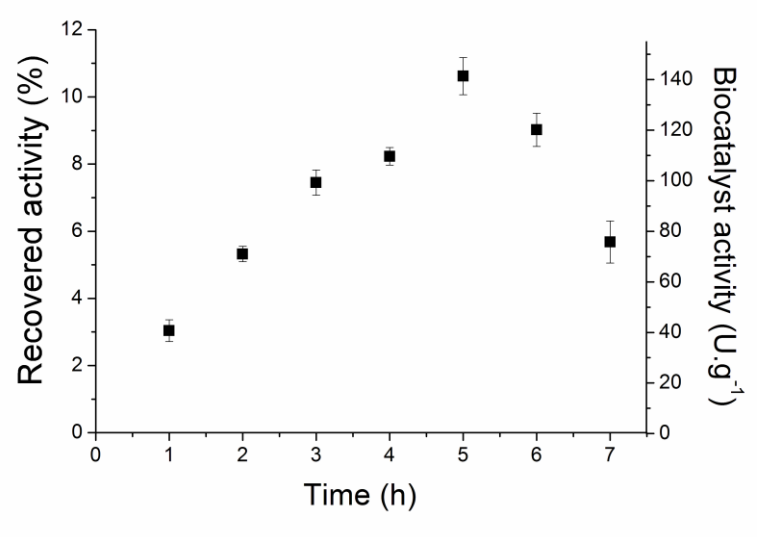


3.1.3. Effect of the cross-linking time

The reaction time of cross-linking for the synthesis of CLEA-MP* biocatalyst was tested using glutaraldehyde 50 mM, an enzyme solution (0.4 mg.mL^{-1} of protein) and MP* (1.0 mg.mL^{-1}).

Analyzing the results of Fig. 3, it can be seen that the RA % of the CLEA-MP* was improved up to 5 h of cross-linking. As observed by Dalal et al. [39], a short reaction time resulted in inadequate crosslinking, providing low recovered activity and low operational stability. However, the same authors have also observed that prolonged reaction time restricts the enzyme flexibility, inhibiting enzyme activity due to more intense cross-linking. Thus, optimal cross-linking time may involve a compromise among efficient cross-linking and enzyme activity and stability [40,46].

Figure 3. Influence of cross-linking time on the CLEA-MP* preparation, under the conditions of 50 mM of glutaraldehyde, 0.4 mg.mL^{-1} of enzyme concentration and 1.0 mg.mL^{-1} of MP*.



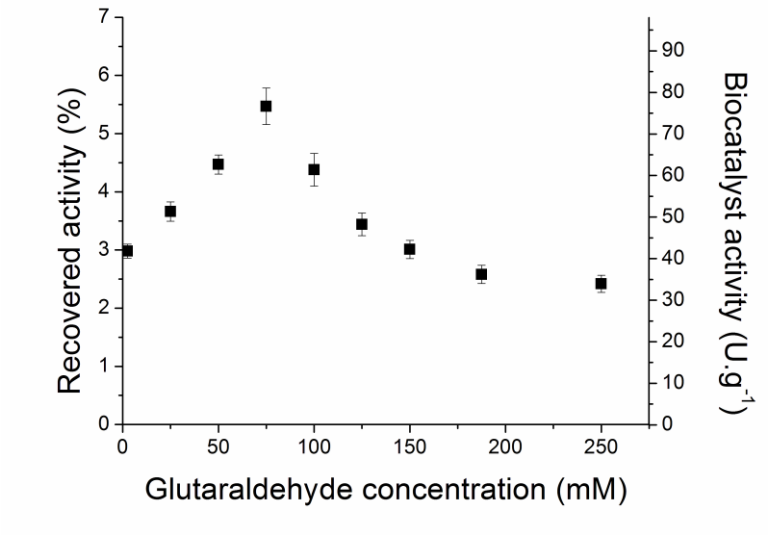
3.2. Immobilization by covalent attachment to magnetic particles

3.2.1. Activation of the magnetic particles

Firstly, the magnetic particles functionalized with APTMS (MP*), were activated with different concentrations of glutaraldehyde. Glutaraldehyde is a bi-functional cross-linking reagent that has been extensively used because of its low cost, ease of handling and ability to form covalent bonds, reacting with the amino groups of the modified support and of the enzymes, allowing the enzyme immobilization [47].

As shown in Fig. 4, recovered activity of the Enz-Glu-MP* increased with glutaraldehyde concentrations, up to 75 mM (5.5 % of RA). When low concentrations of glutaraldehyde are used, the enzyme molecules may not be bound to the support. However, increasing in glutaraldehyde concentration provided a higher amount of free aldehyde groups on the support surface, improving the interactions with enzymes and their retention, as well as decreasing leaching [48]. On the other hand, higher glutaraldehyde concentration leads to a decrease in recovered activity. This can be due to 3D conformation loss, due to extensive interaction of enzyme molecules with free aldehyde groups on the support surface [45,49].

Figure 4. Influence of the glutaraldehyde concentration in MP* activation on the Enz-Glu-MP* preparation, under the conditions 3 h of activation and 0.1 mg.mL^{-1} of enzyme concentration.



3.2.2. Protein concentration

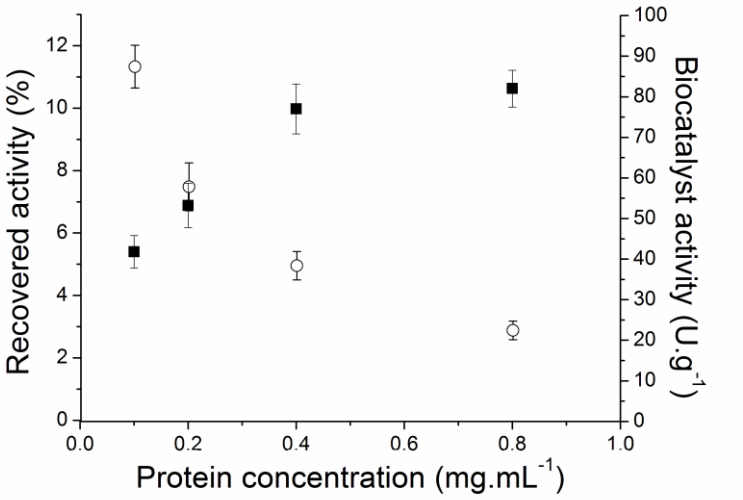
After studying MP* activation, different enzymes concentrations were tested, with the aim to obtain a Enz-Glu-MP* biocatalyst with higher activity. Enzyme concentrations varied from 0.1 mg.g^{-1} to 0.8 mg.g^{-1} of MP* and the results are shown in Fig. 5.

The highest recovered activity (11.33 %) was obtained when using the lowest concentration of enzyme. However, the activity of the Enz- Glu-MP* biocatalyst slowly increased with higher enzyme concentration.

The decrease in the recovered activity with the increase of the enzyme concentration may be related to the saturation and multilayering of the enzyme on the Enz-Glu-MP* biocatalyst surface that could cause intermolecular steric hindrance, hindering the substrate diffusion [50]. When the enzyme concentration is low, the enzyme is easily fixed on the surface of the support due to less steric hindrance and more active sites [51]. On contrary, when the enzyme concentration increases, the enzymatic aggregation on the surface of the support could block the active sites of the enzyme [52].

Despite that the recovered activity was higher at low enzyme concentration, such a small amount of enzyme loaded onto the support resulted in low activity of the biocatalyst. Thus, to combine high enzyme activity and satisfactory RA %, the enzyme concentration of 0.4 $mg.g^{-1}$ of support was chosen for the next experiments.

Figure 5. Influence of enzyme concentration on the recovered activity (○) and on the Enz-Glu-MP* biocatalyst activity (■), under the conditions of 1.0 $mg.mL^{-1}$ of MP* activated with 75 mM of glutaraldehyde for 3h.



3.3. Magnetic, textural and morphological properties

Fig. 6 shows the normalized magnetization curves for the APTMS-magnetite particles (MP*), magnetic CLEAs (CLEA-MP*) and glutaraldehyde-activated magnetic particles (Enz-Glu-MP*). It is observed magnetic saturation with the available maximum field, and similar curves shape. Thus, these magnetic materials can be easily and rapidly separated by applying the external magnetic field [53].

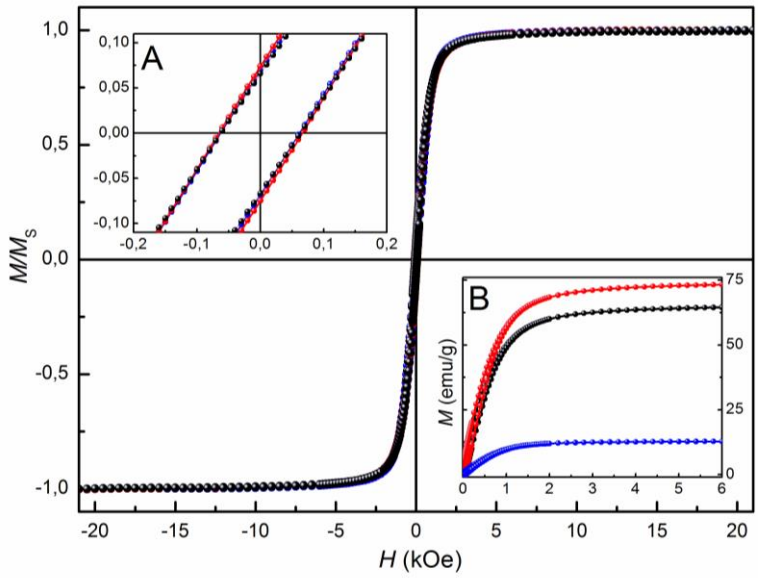
The inset (B) of Fig. 6 shows the non-normalized magnetization curves. The observed saturation magnetizations are M_S = 74.4 emu/g for MP, M_S = 65.6 emu/g for Enz-Glu-MP* and M_S = 13 emu/g for CLEA-MP*. Different saturation of magnetization can confirm the enzyme immobilization. The coating substance decreases the force exerted by an external

magnetic field on the nanoparticles owing to the difficult alignment of magnetic dominions in the material [54,55]. There is a decrease in the M_S after modifications that is strongly dependent on the particle size and amount of coatingmaterials [56]. The substantial decrease of the saturation magnetization after CLEA-MP* immobilization indicates a smaller magnetite amount per gram of biocatalyst compared to Enz-Glu-MP*, which implies in a large amount of enzyme immobilized on the magnetite surface, indicating the superior enzyme binding property of the procedure [57].

The inset (A) shows the same coercive field H_C = 63 ± 2 Oe and M_R/M_S = 0.07 that indicate comparable magnetic response of the samples. This suggests that the immobilization of enzymes in CLEA-MP* and Enz- Glu-MP* did not produce substantial changes in their intrinsic magnetic properties, *i.e*., there is no substantial modifications of surface magnetic anisotropy or interactions among the magnetic particles.

SEM images of the samples with different magnifications are presented in Fig. 7. In the MP* sample (Fig. 7A and B) is clearly observed a spherical shape, with a macroporous structure (interparticles space) formed by the magnetite agglomeration. The images 7C and 7D show the CLEA-MP* biocatalyst, where it is possible to see the spherical MP* surrounded by a heterogeneous mass of cross-linked enzymes. It can also be seen a fraction of macropores. Similar characteristics were observed in the images of the Enz-Glu-MP* sample (Fig. 7E and F), however, the MP* seems to be more aggregated.

Figure 6. Normalized magnetization curves for MP* (●), CLEA-MP* (●) and Enz-Glu-MP* (●) materials. Inset B: non-normalized magnetization curves and inset A: similar coercive fields and relative remanence.



The textural characteristics of the samples were investigated using N_2 adsorption isotherms. The surface area and pore volume are presented on Table 1, and the isotherms are shown in Fig. 8. It is possible to observe that MP* and CLEA-MP* samples presented higher values of surface area and pore volume than Enz-Glu-MP*. The isotherms show a profile of non-porous or macroporous material, i.e. porous with diameter larger than 50 nm. In fact, the isotherms of MP* samples and CLEA-MP* show a clear inflection at high relative pressure values. This trend means that the nitrogen pore saturation was not reached. This behavior occurs due to the presence of a significant fraction of macropores. It is important to emphasize that, for the Enz-Glu-MP* sample, this trend were not too marked, and also the surface area and pore volume were lower, indicating that, the immobilization of the enzyme on magnetic particles surface leads to a more compacted structure (less porous) when compared to CLEA-MP* sample, also according to SEM results discussion above.

Figure 7. SEM images of the samples at 10,000 and 30,000 × magnifications: (A,B) APTMS-magnetites, (C,D) CLEA-MP* and (E,F) Enz-Glu-MP*.

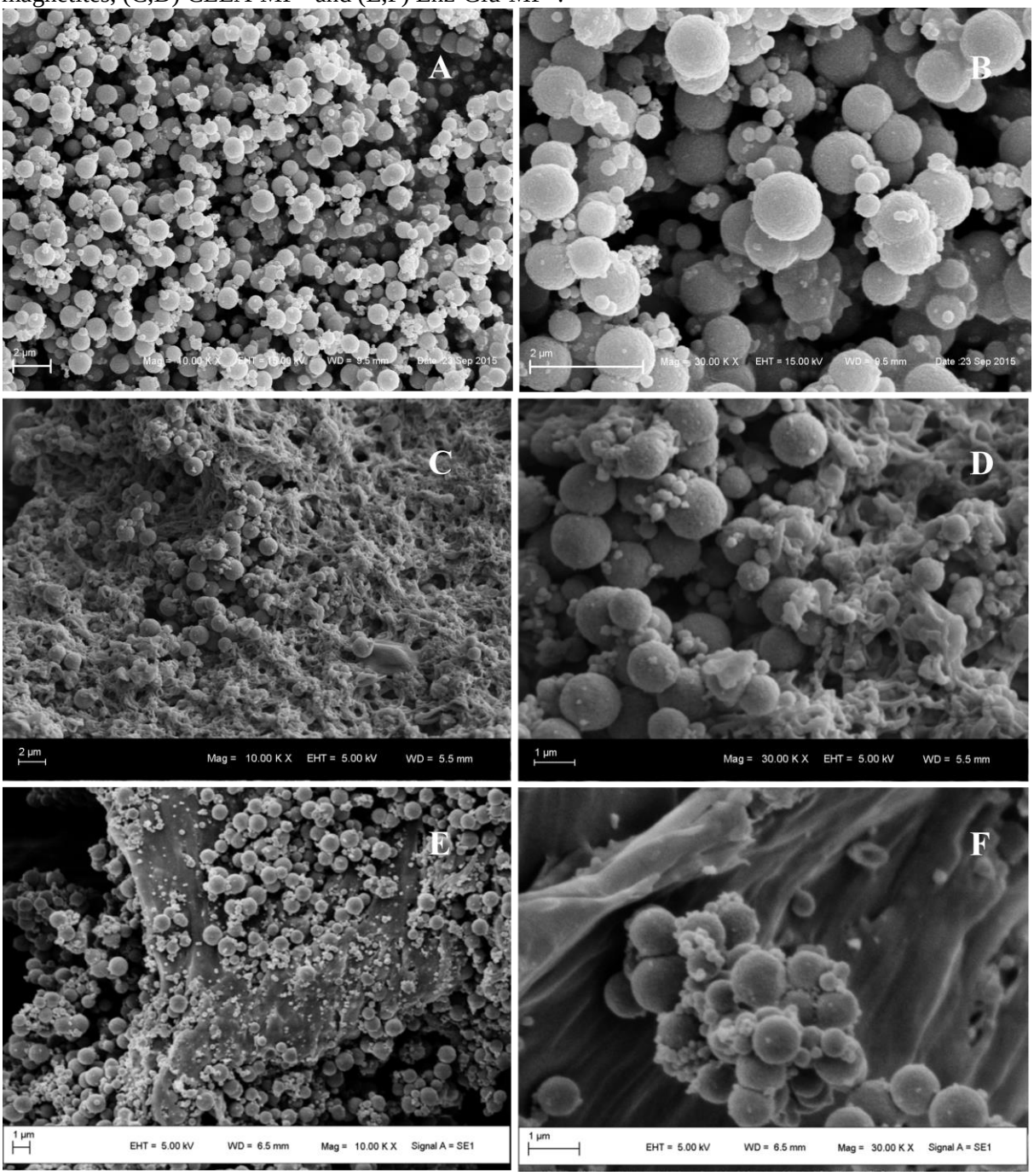


Figure 8. N_2 adsorption isotherms of MP* (■), CLEA-MP*(●) and Enz-Glu-MP* (▲).

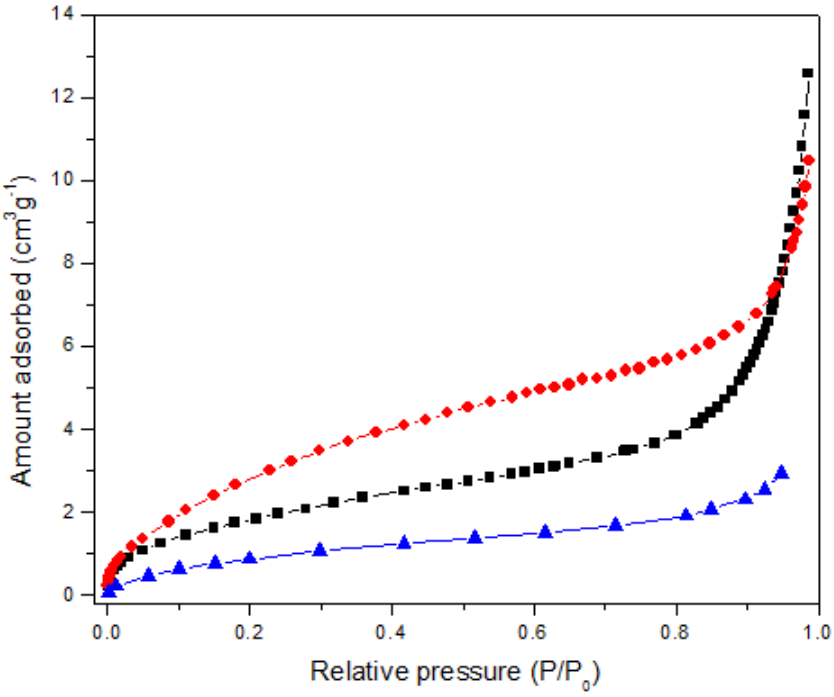


Table 1. Textural analysis of the materials

Sample	**BET surface area (m^2g^{-1})**	**Pore volume (cm^3g^{-1})**
MP*	7.0 ± 0.5	0.018 ± 0.001
CLEA-MP* biocatalyst	12.0 ± 1.0	0.014 ± 0.001
Enz-Glu-MP* biocatalyst	3.7 ± 0.5	0.003 ± 0.001

3.4. Kinetic parameters

Kinetic parameters (K_M and V_{max}) of soluble and immobilized enzymes (CLEA-MP* and Enz-Glu-MP* biocatalysts) were determined by measuring initial reaction rates, varying the amount of substrate.

Regarding K_M values, both immobilized enzymes showed higher values than the soluble enzyme (17.60 μmol.mL^{-1}), 2-fold and 1.5-fold for CLEA-MP* (33.83 μmol.mL^{-1}) and Enz-Glu-MP* (25.65 μmol.mL^{-1}), respectively. This could be probably due to the reduction of enzyme conformational flexibility after immobilization and also to the restricted

accessibility for the macromolecular substrate (pectin) [22,58]. In addition, K_M value of the CLEA-MP* was even higher than the K_M value of the Enz-Glu-MP*,which is possibly due to the structure assembled by glutaraldehyde in CLEA-MP*, in which the active sites of some enzymes were inside the CLEA structure, decreasing K_M [44], while in the Enz-Glu-MP* biocatalyst, due to the smaller pore sizes (as seen in Section 3.3), the enzymes were more immobilized on the MP* surface, facilitating the access to the substrate.

V_{max} values of both immobilized biocatalysts (26.87 μmol.min^{-1} and 26.66 μmol.min^{-1} for CLEA-MP* and Enz-Glu-MP*, respectively) were lower than the V_{max} values for the soluble enzyme (40.58 μmol.min^{-1}), indicating that the substrate hydrolysis rate was reduced after immobilization. The formation of a rigid and compact structure provided a steric hindrance, causing in mass transfer resistance and difficulty in the substrate diffusion to reach active site of the immobilized enzymes [22,42], resulting in lower catalytic efficiency after immobilization, which were 2.31, 0.79, 1.04 for soluble enzyme CLEA-MP* and Enz-Glu-MP*, respectively.

3.5. Enzyme activities of the soluble enzyme, CLEA-MP and Enz-Glu-MP* biocatalysts*

The CLEA-MP* and Enz-Glu-MP* biocatalysts were characterized according to their activity of total pectinase (PE), polygalacturonase (PG), pectinlyase (PL), pectin methyl esterase (PME) and cellulase (CE), and were compared to soluble enzyme activity. For all enzymatic activities analyzed, the CLEA-MP* presented higher values than Enz-Glu-MP* highlighting the CE activity, in which the recovered activity was 33.6 % for the CLEA-MP* while for the Enz-Glu-MP*, the recovered activity was only 5.6 % (Table 2).

The methodology for the CLEA-MP* biocatalyst preparation may have recovered a more heterogeneous portion of all enzymes, through the multipoint interactions among glutaraldehyde, enzymes and MP*, increasing the activity of this biocatalyst. On the other

hand, in the Enz-Glu-MP* preparation, the different enzymes of the commercial preparation may have presented different affinities and kinetics of binding immobilizations, favoring the pectinases binding and disfavoring the cellulases binding. The spatial arrangement and orientation of multiple enzymes in confined spaces are very critical in facilitating cooperative enzymatic activity in multi-enzyme co-localization [59]. The multi-enzyme complexes enable highly cooperative catalytic mechanisms in Nature, where the reactive intermediates can be transported rapidly from one active site to the next to avoid diffusion losses, as in cascade reactions [60]. In multiple enzymes immobilization, the order of each enzyme layer adsorbed on the support was found to affect the overall product conversion rate, in which co-localizing the enzymes on the same layer showed the highest catalytic kinetic performance [60–62].

Table 2. Enzymatic activities of the soluble enzyme, CLEA-MP* and Enz-Glu-MP* biocatalysts.

Treatments	**Recovered activities** (%)				
	PE	**PG**	**PL**	**PME**	**CE**
Soluble enzyme	100	100	100	100	100
CLEA-MP* biocatalyst	10.95	6.61	17.60	14.49	33.57
Enz-Glu-MP* biocatalyst	5.07	3.75	10.00	5.51	5.59

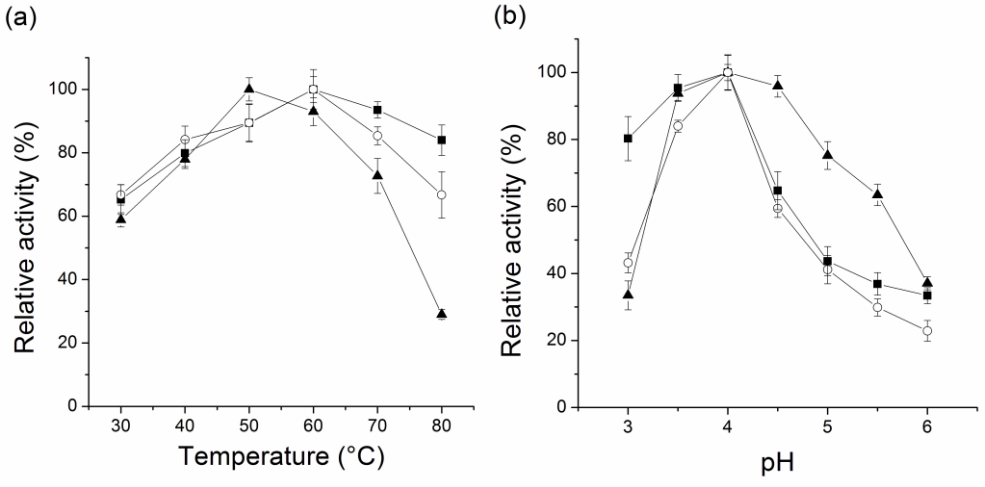
3.6. Optimal pH and temperature

The immobilization may change many enzyme characteristics, as the optimal pH and temperature conditions. As can be seen in Fig. 9(a), the soluble enzyme used in this research showed maximum activity near 50 °C, while the CLEA-MP* and Enz-Glu-MP* biocatalysts presented maximum activity at 60 °C. In addition, the immobilized biocatalysts presented a tendency to maintain higher activities at elevated temperatures compared to the soluble enzyme. At 80 °C, the CLEAMP* and Enz-Glu-MP* still presented 84 % and 67 % of their initial activities, while the soluble counterpart remained with 29 % of its initial activity. These

results may be explained by covalent binding between enzymes (in the case of CLEA-MP*) and between enzymes and support (in the case of Enz-Glu-MP*), that restrict the conformational mobility of the enzyme molecules and protected the enzyme active conformation from distortion or damage by heat exchange [48,63,64].

Variation in pH can result in damage of the enzyme structure, preventing the binding to the substrate, thus affecting its activity [63]. Changes in optimal pH were not observed for the immobilized biocatalysts compared to the soluble enzyme, as shown in Fig. 9(b). However, near to pH 3, Enz-Glu-MP* and CLEA-MP* were very active, especially the CLEA-MP*, that presented 80 % of its initial activity, proving the positive effect of immobilization on the stability of enzymes under adverse conditions. This effect might be occasioned by changes in the ionization state of the microenvironment around the active site due to the MP* and/or glutaraldehyde [45,64,65].

Figure 9. Relative activity of soluble enzyme (▲),CLEA-MP* (■) and Enz-Glu-MP* (○) under different conditions of temperature (a) and pH (b).



3.7. Thermal and storage stability

The thermal stability of soluble and immobilized biocatalysts was determined by incubating them in a thermostatic bath at 60 °C. The residual activities were determined after

different time intervals up to 30 min. Thermal constant inactivation rate (kd), half-life time ($t_{1/2}$) and stabilization factors were determined.

Enzyme immobilization can enhance stability under adverse conditions of pH and temperature [26]. A significant improvement in the thermal stability was observed for the CLEA-MP* and Enz-Glu-MP* biocatalysts, since *k* decreased (0.10 and 0.18 min^{-1}, respectively) and $t_{1/2}$ increased (6.73 and 3.83 min, respectively), when compared with *k* (0.25 min^{-1}) and $t_{1/2}$ (2.82 min) of the soluble enzyme, providing stabilization factors of 2.39 and 1.36 for the CLEA-MP* and Enz-Glu-MP* biocatalysts, respectively.

Chemical cross-linking restricts conformational changes of the enzyme induced by heat and stabilizes the quaternary structure of the enzyme [42,47]. Stabilization of the enzymes due to covalent bond might be responsible for the thermal stability observed, because the covalent bond between the terminal amino group of enzyme and the aldehyde group of glutaraldehyde linked to the MP* restricts the enzyme molecular flexibility, which is essential to catalytic activity [25,48] being necessary more energy to distort this active conformation [44]. When the enzymes are immobilized as magnetic cross-linked enzyme aggregates (CLEA-MP*), a large number of multiples interactions are formed between enzyme-enzyme and enzyme-MP*. These bonds provide a confinement effect and a more effective conformational stabilization of protein structure [23,58]. Some studies have reported that larger and more porous supports that confer a molecular confinement, are able to restrict the molecular movement, reducing the possibility of enzyme deactivation, increasing its thermal stability [66,67], and a similar effect can be expected in the porous structure of CLEA-MP*, where the enzymes in the internal side of the particle are less sensitive to thermal inactivation due to the neighborhood protection.

The storage stability of the enzymes was also evaluated in the pH of the grape juice (around 3.5). The soluble enzyme, CLEA-MP* and Enz-Glu-MP* biocatalysts were

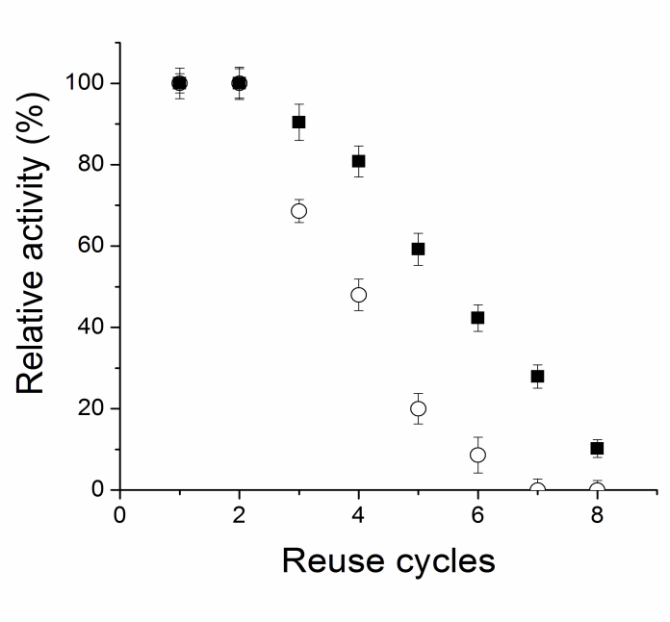
incubated in sodium citrate buffer (50 mM, pH 3.5, 4 °C) and analyzed during 30 days of incubation. For all enzyme form, the enzymatic activities remained constant along all time of incubation. Several studies point out the positive effects on the conservation and stabilization of enzymes by immobilization, which prevents possible distortion effects on the active sites of enzyme caused by storage in buffer solution [21,22,45].

3.8. Operational stability

Reusability of immobilized enzymes is important for its cost effective industrial application, being one of the main reasons for immobilization. Integration of magnetic technology with the enzyme immobilization can enhance recoverability and reusability of the biocatalysts [68]. To measure the operational stability of CLEA-MP* and Enz-Glu-MP* biocatalysts, the clarification of fresh grape juice was evaluated, and percentage of turbidity reduction was measured for each cycle.

The results presented in Fig. 10 showed that the magnetic biocatalysts could be reused for several cycles. However, the magnetic biocatalysts progressively decreased their enzymatic activities, in which the Enz-Glu-MP* lost all its activity in the seventh cycle, while CLEA-MP* still presented 10 % of its initial activity at the end of the 8 cycle. The loss of activity is related to the number of cycles and can have many reasons: leaching of enzymes during incubation, washing of the biocatalysts after use, protein denaturation due to temperature, hydrodynamic stress and mechanical damage [68].

Figure 10. Reusability of CLEA-MP* (■) and Enz-Glu-MP* (○) biocatalysts assayed in grape juice.



4. Conclusions

The results presented in this study demonstrate that magnetic biocatalysts of pectinase and cellulase can be a suitable strategy of immobilizing enzymes for application in the clarification of grape juice, as it facilitates the separation of the enzymes from the reaction systems and, subsequently, the reuse of multiple cycles. Comparing the both biocatalysts, the magnetic CLEAs presented the most suitable characteristics: thermal stability (2.39-times compared to soluble enzyme), high activities recovery of pectinases and cellulases compared to the Enz-Glu-MP*, and reusability (8 cycles against 6 cycles for Enz- Glu-MP*) in grape juice clarification. The results from this study induce future researches to improve this technology, such as the increase of the surface area to immobilize a higher enzyme load, recover of the magnetite with chitosan, and the use of alternative cross-linking agents to avoid the negative effect of glutaraldehyde on enzymatic activity, looking for a magnetic biocatalyst with high activity, stability and of easy separation. As stated before, there is none industrial application of immobilized enzymes for juice clarification. We believe that more research efforts are needed in this field to make this technology feasible.

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CAPÍTULO 4 – Immobilization of pectinase on chitosan-magnetic particles: Influence of particle preparation protocol on enzyme properties for fruit juice clarification

A partir dos resultados do trabalho anterior, o recobrimento das partículas magnéticas com quitosana surgiu como uma possibilidade de alcançar biocatalisadores que apresentassem alta atividade e estabilidade, aliado a boas propriedades magnéticas para a fácil separação e reutilização. Assim, neste trabalho estudou-se diferentes metodologias de recobrimento das partículas magnéticas com quitosana, preparando-se 3 diferentes biocatalisadores com distintos tamanhos. As nanopartículas magnéticas de quitosana (Nano-CMag) foram preparadas por gelificação ionotrópica, as micropartículas magnéticas de quitosana (Micro-CMag) foram preparadas usando a técnica de reticulação em emulsão e as macropartículas magnéticas de quitosana (Macro-CMag) foram preparadas pelo método de precipitação. Os efeitos do tempo de ativação com glutaraldeído, a concentração de enzimas e o tempo de imobilização foram estudados para os diferentes biocatalisadores. Além disso, as características morfológicas, texturais, magnéticas, bem como os parâmetros cinéticos, estabilidades e os reusos para a clarificação dos sucos de uva, maçã e laranja foram estudados. Os resultados estão apresentados no manuscrito a seguir, publicado na revista *Biotechnology Reports*, v. 24, e00373, 2019.

CAPÍTULO 4 – Immobilization of pectinase on chitosan-magnetic particles: Influence of particle preparation protocol on enzyme properties for fruit juice clarification

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Abstract

Magnetic-chitosan particles were prepared following three different protocols enabling the preparation of particles with different sizes – Nano (Nano-CMag), Micro (Micro-CMag) and Macro (Macro-CMag) – and used for pectinase immobilization and clarification of grape, apple and orange juices. The particle size had a great effect in the kinetic parameters, Nano-CMag biocatalyst presented the highest V_{max} value (78.95 $mg.min^{-1}$), followed by Micro-CMag and Macro-CMag, with V_{max} of 57.20 $mg.min^{-1}$ and 46.03 $mg.min^{-1}$, respectively. However, the highest thermal stability was achieved using Macro-CMag, that was 8 and 3-times more stable than Nano-CMag and Micro-CMag biocatalysts, respectively. Pectinase immobilized on Macro-CMag kept 85 % of its initial activity after 25 batch cycles in orange juice clarification. These results suggested that the chitosan magnetic biocatalysts presented great potential application as clarifying catalysts for the fruit juice industry and the great importance of the chitosan particles preparation on the final biocatalyst properties.

Keywords: chitosan, magnetic particle; pectin; pectinase; immobilization; juice clarification.

1. Introduction

There is a growing demand for more selective and clean processes every day, in which the enzymes have been attracting the attention of many researchers. Enzymes are biocatalysts with high potentiality in diverse industries thanks to their high activity under mild conditions, specificity and selectivity, [1,2]. Enzymes are one of the most important tools in modern food processing industry because many intermediate processes are simplified by their use [3].

Nevertheless, their industrial implementation are impaired because of some factors, such as their usual low stability under operational conditions, and the difficulties to their recovery and reuse [4–6]. In this situation, where the reaction conditions will directly define the enzyme activity, the immobilization appears as a way to achieve a suitable biocatalyst for each specific application [7,8]. The scientific advances in enzyme immobilization allow obtaining biocatalysts for industrial applications, improving catalytic properties even in adverse reaction conditions, facilitating the recovering of the enzymes from the reaction medium and their reuse, turning them economically feasible [9–13].

In the juice industry, the application of commercial enzyme preparations, composed by different enzyme, such as hemicellulases, cellulases and pectinases, is usual for obtaining stable and clarified fruit juices [14–17]. These enzymes are responsible for the breakdown of the structural polysaccharides of fruit pulp, which caused juice turbidity [18–20]. The addition of pectinases to the juice reduces its viscosity, improving the press ability of the pulp, disintegrating the jelly structure and increasing the fruit juice yields [3]. Some academic studies have utilized immobilized enzymes for the clarification of orange, apple, pineapple, grape, and carrot juices [21–28], but to the best of our knowledge there is not an industrial process using immobilized enzymes for juice clarification, which makes the development of stable and active biocatalysts useful for this task an attractive challenge.

Support nature and immobilization protocols suitable for enzyme immobilization are the key factors for the preparation of an enzyme biocatalysts with high enzymatic activity and operationally stable [29,30]. Chitin is an abundant natural polymer obtained from the shells of shellfish and wastes from the seafood processing industry, that by N-deacetylation produces chitosan [4,31]. The combination of chitosan and magnetic particles could produce an ideal support for the immobilization of enzymes, with high affinity for proteins, easy functionalization with reactive groups for reactions with enzymes, and at same time, providing magnetic separability, allowing easy reuse. Moreover, its hydrophilicity, non-toxicity, biocompatibility, high resistance to chemical degradation and antibacterial properties increase its interest [4,32,33]. Moreover, the magnetic core allows easy and efficient separation of enzyme from the reaction medium by using external magnetic fields [25,34,35], which is important if the product presents some solids in suspension (making filtering useless). Additionally, chitosan particles with different characteristics, especially particle size, can be produced by diverse synthesis methods, such as spray drying, emulsion cross-linking, ionotropic gelation, emulsion-droplet coalescence, reverse micellar and precipitation, methods, providing supports with diverse porosity and different surface/volume ratio for enzyme immobilization [31,33,36–39]. The differences in each method for preparation of chitosan particles may alter the final performance of the biocatalysts. Each synthetic protocol can alter the superficial density of amino groups or the rigidity of the chitosan chains, affecting the final biocatalyst features.

Based on these aspects, the objective of the present work was to prepare chitosan-magnetic biocatalysts with different characteristics by varying the method of synthesis of the support, assessing the effect of the support preparation protocol on the final particle size of the particles and on the immobilized enzyme properties. The biocatalysts were applied to the clarification of apple, orange, and grape juices. The supports were prepared by mixing

magnetic particles with chitosan by using three different methods: emulsion cross-linking, precipitation and ionotropic gelation [33,40]. For the immobilization of the enzymes, we used glutaraldehyde as coupling agent, which has been considered an efficient procedure to attain stable covalently attached enzymes [30,41–44]. All biocatalysts were characterized in relation to their magnetic, morphological and textural properties, kinetic parameters (V_{max} and K_M) and thermal stability. Finally, the operational stability of the biocatalysts was study in the clarification of several juices (apple, orange and grape).

2. Materials and methods

2.1. Materials

Freshly pressed apple, orange and grape juices without any treatment were kindly provided by Vitivinícola Jolimont (Canela, RS, Brazil). Rohapect 10 L was from Amazon group (Brazil). Pectin from apple, polygalacturonic acid, galacturonic acid and chitosan were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents were of analytical grade.

2.2. Enzymatic preparation

The enzymatic preparation used in this work was Rohapect 10 L. It is a cocktail of pectinase and cellulase enzymes. The protein concentration was 5.4 $mg.mL^{-1}$, and the main specific activities ($U.mg^{-1}$) present in the preparation were: total pectinase, 233.29; polygalacturonase, 537.57; pectin lyase, 13.53; pectin methyl esterase, 168.33; cellulase, 31.99 [45].

2.3. Determination of enzyme activities

Enzymatic activities were measured as previously described by Dal Magro et al. [46]. The polygalacturonase (PG) and total pectinase (PE) activities were determined by the hydrolysis of polygalacturonic acid and pectin (1 $g.L^{-1}$), respectively. The enzyme was added to substrate prepared in sodium citrate buffer (50 mM, pH 4.8), and incubated at 37 ºC, for 1 min for PE activity and 2 min for PG activity, under agitation. The amount of reducing groups was quantified by the 3,5-dinitrosalicyclic acid (DNS) method [47]. The pectin lyase (PL) activity was measured spectrophotometrically by determining the increase in absorbance at 235 nm, through the formation of unsaturated uronide (e = 5500 $M^{-1}.cm^{-1}$) [48]. Briefly, the enzyme was added to pectin solution (4 $g.L^{-1}$) prepared in sodium citrate buffer (50 mM, pH 4.8) and incubated at 37 ºC for 1 min, under agitation. The reaction was stopped by adding HCl, 0.5 M. The pectin methyl esterase (PME) activity was measured by titration of carboxylic groups released through the de-esterification of pectin [49]. The enzyme was added to pectin solution (5 $g.L^{-1}$) prepared in NaCl buffer (0.15 M, pH 4.5). The reaction was conducted at 30 ºC for 10 min, under agitation. Total cellulase (CE) activity was determined using Whatman no. 1 filter paper as substrate [50]. The enzyme was added to sodium citrate buffer (50 mM, pH 4.8) containing 50 mg. mL^{-1} of filter paper. The reaction was carried out at 50 ºC for 5 min, under agitation, and the amount of released reducing groups was quantified by the 3,5-dinitrosalicyclic acid (DNS) method.

2.4. Synthesis and functionalization of the magnetic particles (MP)

Synthesis of magnetic particles was carried out through solvothermal method [51], using iron (III) chloride as iron precursor and ethylene glycol as high boiling point solvent and reducing reagent. Briefly, $FeCl_3.6H_2O$ (16 mmol, 4.32 g) was dissolved in 100 mL of ethylene glycol, under stirring. Afterward, sodium acetate trihydrate (80 mmol, 10.89 g) was

added and the mixture was stirred at room temperature for 1 h, then transferred to a Teflon lined stainless steel autoclave and heated up to 180 ºC for 12 h, with heating rate of 3.0 $^{o}C.min^{-1}$. The system was cooled to room temperature and the precipitate was captured using an external magnet. The magnetic particles, named as MP, were washed thoroughly with ethanol and vacuum-dried for 2 h. In sequence, MP (1.00 g) were dispersed in toluene (50 mL) with 3-aminopropyltrimethoxysilane (APTMS) (1 mmol, 0.174 mL) and stirred under argon atmosphere for 12 h at 70 ºC. Subsequently, the functionalized MP with APTMS, named as MP-APTMS, was magnetically separated, washed several times with non-polar and polar solvents to extract any impurity or residue from the product. The sequence of used solvents starts with toluene, which is the solvent used in the reaction, ethanol, water, and finishing with ethanol that is easier to evaporate. Finally the solid was dried under vacuum at 50 ºC for 2 h.

2.5. Preparation and activation of chitosan magnetic particles

The chitosan magnetic particles were prepared by three different methodologies to obtain supports with different properties.

Method 1: Chitosan magnetic nano particles (Nano-CMag) were prepared by ionotropic gelation using sodium sulfate as the gelation agent [33]. For this, 0.5 mL of 1.4 $mol.L^{-1}$ sodium sulfate solution was dropped, under sonication, in 9.5 mL of 0.25 % w/v chitosan solution in 0.35 $mol.L^{-1}$ acetic acid containing 1 % v/v Tween 80 and 0.25 % w/v magnetic particles. Then, it was mechanically stirred for 2 h. The Nano-CMag obtained were magnetically separated and washed with distilled water.

Method 2: Chitosan magnetic micro particles (Micro-CMag) were prepared using the cross-linking emulsion technique [40]. 2 % w/v chitosan suspension and 2 % w/v magnetic particles in 0.35 $mol.L^{-1}$ acetic acid were mixed into a dispersion medium composed of

mineral oil:petroleum ether (25:35 v/v) with 0.5 % v/v Tween 80. 0.5 % (v/v) Glutaraldehyde was added twice (after 10 min and after 1 h), totaling 1 % (v/v) in the final mixture. After the addition of glutaraldehyde, the mixture was stirred for 2 h. Finally, the Micro-CMag particles were separated from the oil phase by centrifugation followed by successive washes with acetone, ethanol and distilled water.

Method 3: Chitosan magnetic macro particles (Macro-CMag) were prepared by the precipitation method [33]. A suspension of 2 % w/v chitosan and 2 % w/v magnetic particles in 0.35 mol.L^{-1} acetic acid was sonicated for 30 min to remove air bubbles. Then, the mixture was added dropwise in the coagulation solution (1 mol.L^{-1} NaOH) under slow stirring. The formed Macro-CMag particles were magnetically separated and washed with distilled water until neutrality was reached.

2.6. Enzyme immobilization

For immobilization of Rohapect 10 L, all supports were previously activated with glutaraldehyde [26,51]. The activation was performed by incubating 50 mg of each support in 1 mL of phosphate-potassium 0.1 mol.L^{-1} buffer (pH 7.0) with 1 % (v/v) glutaraldehyde at 37 ºC under stirring for different times (from 30 min to 5 h). After, the supports were magnetically separated and washed 3 times with 50 mmol.L^{-1} sodium citrate at pH 4.8 to remove the excess of glutaraldehyde.

Subsequently, 50 mg of the activated supports were added to 1 mL of the enzyme solution with different protein concentrations (0.2–1.6 mg.mL^{-1}) for different times (5–30 h), at room temperature under gently stirring in a roller mixer. Finally, the biocatalysts were magnetically separated and washed 3 times with 5 mL of 50 mmol sodium citrate at pH 4.8 to remove the non-bound enzymes. To store the biocatalysts, they were suspended in 50 mmol.L^{-1} sodium citrate buffer at pH 4.8 and 4 ºC. The immobilization yield (IY),

immobilization efficiency (IE) and recovered activity (RA) were determined by the following equations according to Sheldon and Van Pelt [6]:

$$\text{IY}\ (\%) = \frac{\text{Immobilized activity (U)}}{\text{Initial activity (U)}} \text{ x } 100 \qquad (1)$$

$$\text{IE}\ (\%) = \frac{\text{Biocatalyst activity (U)}}{\text{Immobilized activity (U)}} \text{ x } 100 \qquad (2)$$

$$\text{RA}\ (\%) = \frac{\text{Biocatalyst activity (U)}}{\text{Initial activity (U)}} \text{ x } 100 \qquad (3)$$

Immobilized activity is the difference between the initial activity and the activity determined in final supernatant. Initial activity is the activity of the enzyme offered to the support. Biocatalyst activity is the activity measured on the particles after immobilization.

2.7. Kinetic parameters

Kinetic parameters (K_M and V_{max}) of the soluble enzyme, Nano-CMag, Micro-CMag and Macro-CMag were calculated by using different substrate concentrations, varying between 1.39 mmol.L^{-1} and 55.5 mmol.L^{-1} the concentration of pectin at pH 4.8 employing 50 mmol.L^{-1} sodium citrate at 37 ºC. The K_M and V_{max} were calculated by non-linear regression of the initial reaction rates corresponding to different substrate concentrations using Solver extension (Microsoft Office Excel 2016).

2.8. Effect of pH and temperature on the enzyme activity

The total pectinase activity was measured for soluble and immobilized enzymes (Nano-CMag, Micro-CMag and Macro-CMag) at different values of pH and temperature. The

temperature varied from 30 ºC to 90 ºC, at pH 4.8 whereas the pH ranged from pH 3.0 to pH 6.0 at 60 ºC.

2.9. Thermal and storage stability

Thermal stabilities of the soluble and immobilized enzymes (Nano-CMag, Micro-CMag and Macro-CMag) were analyzed by incubating the samples in 50 mmol.L^{-1} sodium citrate buffer at pH 4.8 and 60 ºC. The storage stabilities were performed by incubating the samples in 50 mmol.L^{-1} sodium citrate buffer at pH 3.5 and 4 ºC. Periodically, the samples were taken to analyze total pectinase activity. The thermal inactivation was described by a first order reaction:

$$\frac{A}{A_0} = \exp(-kt) \qquad (4)$$

where A is the enzyme activity at time t, A_0 is the initial enzyme activity, t is the inactivation time, and k is the inactivation rate constant at the studied temperature. The half-life time ($t_{1/2}$) was calculated using the k values, according to the equation:

$$t_{1/2} = \frac{\ln(2)}{k} \qquad (5)$$

2.10. Operational stability

Operational stability of the biocatalysts was evaluated through reusing them in several consecutive batch cycles for clarification of apple, orange and grape juices. For this, 5 U of total pectinase activity of each biocatalyst were added to 1.0 mL of fresh juice and incubated at 40 ºC for 2 h. Between each clarification cycle, the immobilized enzymes were magnetically recovered from the juices and washed with 50 mmol.L^{-1} sodium citrate buffer at pH 4.8. Then, they were added to a new batch of fresh juice to start a new cycle. The decrease

in juice turbidity was determined for each cycle, considering the value of the first cycle as 100 %. Turbidity was measured spectrophotometrically by detection of the scattered light at 860 nm [52].

2.11. Materials characterization

Scanning Electron Microscopy (SEM) images were acquired using Zeiss microscope model Auriga microscope. The materials were dispersed on double side conduction tape on an aluminum support. The images were obtained with an accelerating voltage of 5 kV and different magnifications. X-ray diffractograms were obtained in a Siemens diffractometer model D500 using CuKα as X-ray source (l = 0.154056 nm) at a generator voltage of 40 kV and a generator current of 17.5 mA. Magnetic properties were investigated by using an EZ9 MicroSense vibrating sample magnetometer (VSM) at room temperature with a magnetic field (H) cycled between 22 kOe and +22 kOe. N2 adsorption-desorption isotherms were acquired at liquid nitrogen boiling point, using a Tristar II 3020 Kr Micromeritics equipment. The samples were previously degassed at 60 ºC, under vacuum, for 20 h. The specific surface areas were determined by the BET (Brunauer, Emmett and Teller) multipoint technique and the pore volume and pore size distribution were obtained by using the BJH (Barret, Joynerand Halenda) method [53]. Infrared spectra were acquired by using KBr supported disks (1 %). The equipment used was a Shimadzu FTIR Prestigie 21. The spectra were obtained at room temperature with 4 cm^{-1} of resolution and 120 cumulative scans.

3. Results and discussion

3.1. Morphological, structural, magnetic and textural properties

Fig.1 presents SEM images for Nano-CMag and Micro-CMag and a picture of the Macro-CMag. It is clearly seen that the materials presented size in the nano, micro and macro

levels, respectively. The average of particle size (w) and the standard deviation (s) were estimated by using Quantikov software as φ = 273 nm (σ =58 nm) for Nano-CMag, as φ = 82 mm (σ = 32 mm) for Micro-CMag and as φ =2.0 mm (σ = 0.2 mm) for Macro-CMag.

Additional SEM images obtained with higher magnification are presented in Supplementary Material as Figure S1. In these images it is possible to see the spherical shape of the magnetic particle (MP) (Fig. S1a) that is still observed after the functionalization with aminopropyl groups (MP-APTMS) (Fig. S1b). Moreover, it is also possible to see that the functionalization produced a coating on MP surface, making the MP particles more aggregated (Fig. S1b). The images of materials Nano-CMag, Micro-CMag and Macro-CMag are depicted in Fig. S1c, S1d and S1e, respectively, where it is possible to identify the organic moiety, assigned to chitosan, along with the spherical magnetic particles (MP). Roughness on surface of Nano-CMag and Macro-CMag materials can be seen, along with the presence of some macroporosity, *i.e*. pores with diameter higher than 50 nm [53]. The material Micro-CMag showed a more compacted surface, and no macroporosity can be identified. These analysis confirmed our hypothesis that using different methods for chitosan preparations we are able to obtain particles with different size (nano, micro and macro size), as well as different properties such as particle surface texture and porosity.

Infrared analysis of the materials is presented in Supplementary Material, as Figure S2. The spectra of Nano-CMag, Macro-CMag are very similar to the chitosan spectrum. The broad band with maximum around 3400 cm^{-1} presents contribution of N–H and O–H stretching from amine and hydroxyl groups of chitosan and also adsorbed water [54]. The band around 2900 cm^{-1} is due to C–H stretching of chitosan [54].The broad band around 1620 cm^{-1} has contribution of N–H_2 bending (1653 cm^{-1}) and N–H deformation of amide (1558 cm^{-1}) typical of chitosan [55]. The broad band with maximum at 1070 cm^{-1} was assigned to skeletal vibration of C–O stretching of chitosan [55]. Therefore, the infrared results confirm

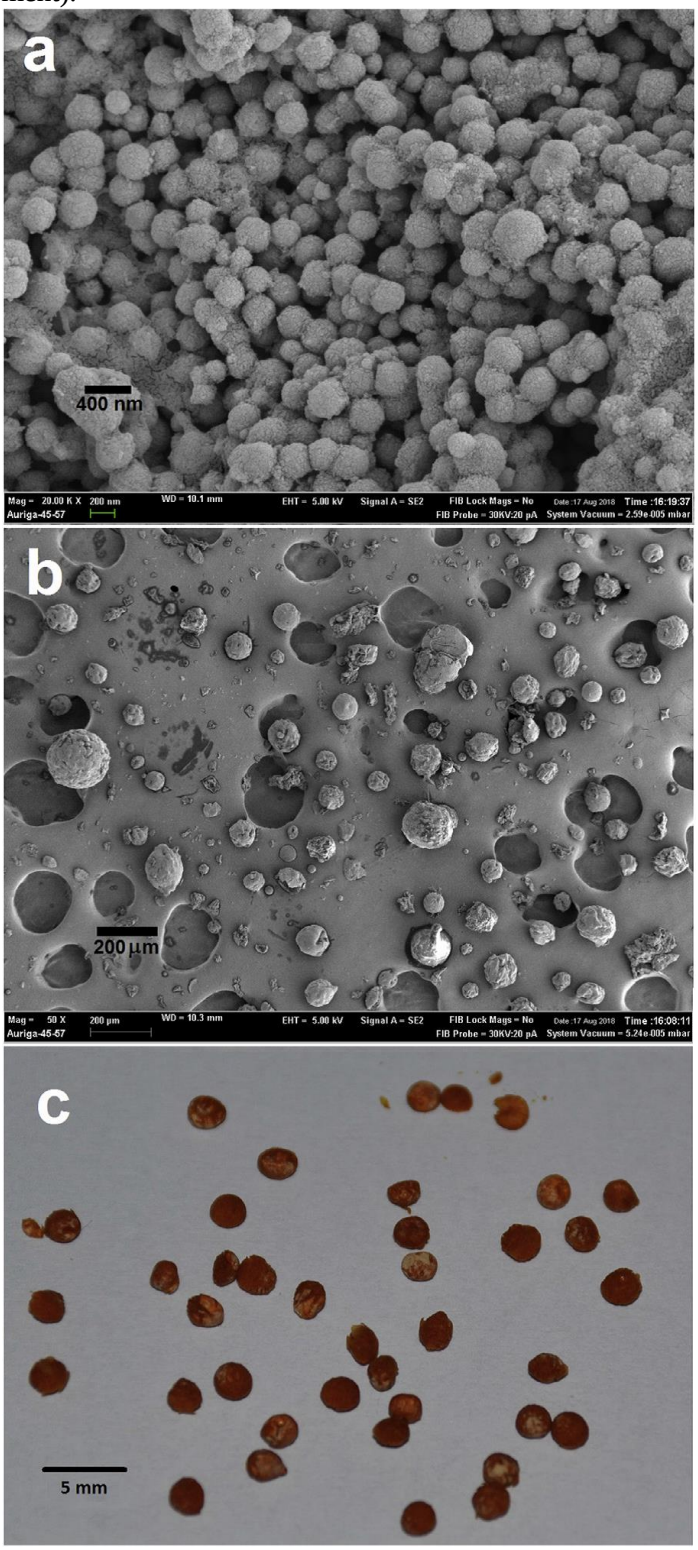
the presence of chitosan in the materials Nano-CMag and Macro-CMag. Unfortunately, the spectrum of the Micro-CMag sample was not obtained, because we were not able to disperse this sample in the KBr infrared support, as a matter of fact, this sample was not a powder, it presented characteristic such as a soft organic polymer.

The X-ray diffractograms of all materials are presented in Supplementary Material as Figure S3. The diffraction pattern (Fig. S3f) shows Bragg's reflections of typical face-centered cubic (fcc) inverse spinel structure of magnetite (JCPDS Nº 19-0629). These reflections are present in all diffractograms (Fig. S3a-e), indicating that the functionalization with APTMS, as well as, the subsequent experimental procedures, used to obtain Nano-CMag, Micro-CMag and Macro-CMag materials, did not affect the crystallinity of magnetite particles. The average crystallite size of magnetite for MP material was calculated by Scherrer's equation using the full width at half maximum for the (311) peak and the obtained value was 39 nm. This result indicates that MP particles are polycrystalline, because from the SEM images (Fig S1a) it was observed that they present diameter in around 200 nm.

The magnetization of the materials was measured as a function of the magnetic field and the non-normalized magnetization curves of all materials are shown in Supplementary Material (Fig. S4). It is clearly observed high magnetic saturation (MS) values for MP and MP-APTMS. The values for MP, MP-APTMS, Nano-CMag, Micro-CMag and Macro-CMag materials were 69, 60, 5.9, 5.8 and 11 $emu.g^{-1}$, respectively. The decreasing in MS for Nano-CMag, Micro-CMag and Macro-CMag materials can be interpreted as a consequence of the incorporation of non-magnetic chitosan moiety to the total mass of the materials. This result is in accordance with the SEM images of the Figure S1, where it was possible to identify both, spherical magnetite particles and organic moieties, which was assigned as chitosan. It is important to point out that, even presenting lower MS values, when compared to MP and MP-

APTMS, the Nano-CMag, Micro-CMag and Macro-CMag materials can be easily and rapidly separated by applying the external magnetic field.

Figure 1. Images of magnetic chitosan particles: a) Nano-CMag (SEM image, magnification of 20,000x); b) Micro-CMag (SEM image, magnification of 50x) and c) Macro-CMag (picture obtained using Nikon D3100 equipment).



The nitrogen adsorption-desorption isotherms are shown in Supplementary material as Figure S5, along with the pore size distribution curves (inset Fig. S5). The surface area and pore volume values are presented on Table 1. Figure S5a shows the curves for MP and MP-APTMS materials. From the isotherms, it is notice a decreasing in the amount of adsorbed nitrogen, in high relative pressures ($P/P_0 > 0.6$), after the MP functionalization with APTMS. This behavior suggested a decrease in both, surface area and pore volume (Table 1) that was interpreted as a consequence of partial closing of the mesopores, which are pores with diameter between 2 and 50 nm [53], produced by the APTMS coating on the MP material surface, in agreement with the SEM images (Fig. S1b). The isotherm curves for Nano-CMag, Micro-CMag and Macro-CMag materials are shown in Figure S5b, S5c and S5d, respectively. After the enzyme immobilization, there is a decreasing in the nitrogen adsorbed amount in all relative pressures that is markedly in $P/P_0 < 0.2$ for Micro-CMag and Macro-CMag materials (Fig. S5c and S5d, respectively) which corresponds to small mesopores and micropores [53]. In fact, the pore distribution curves confirm the closing of pores with diameter lower than 10 nm for these materials, probably produced by the enzyme coverage on the surface (inset Fig. S5c and S5d). Regarding the Nano-CMag material (Fig 5Sb), its textural characteristics seem not be markedly affected by the enzyme immobilization. All textural characteristics as isotherm profile, pore size distribution, surface area and pore volume remain almost constant.

Table 1. Textural analysis of the materials.

Material	**Before enzyme immobilization**		**After enzyme immobilization**	
	S_{BET} [a]	**PV_{BJH} [b]**	**S_{BET} [a]**	**PV_{BJH} [b]**
MP	15.7 ± 0.6	0.038 ± 0.001	-	-
MP-APTMS	12.6 ± 0.5	0.032 ± 0.001	-	-
Macro-CMag	11.5 ± 0.5	0.023 ± 0.001	5.4 ± 0.4	0.016 ± 0.001
Nano-CMag	1.7 ± 0.3	< 0.0005	0.46 ± 0.07	< 0.0005
Micro-CMag	18 ± 0.7	0.051 ± 0.001	18 ± 0.7	0.061 ± 0.001

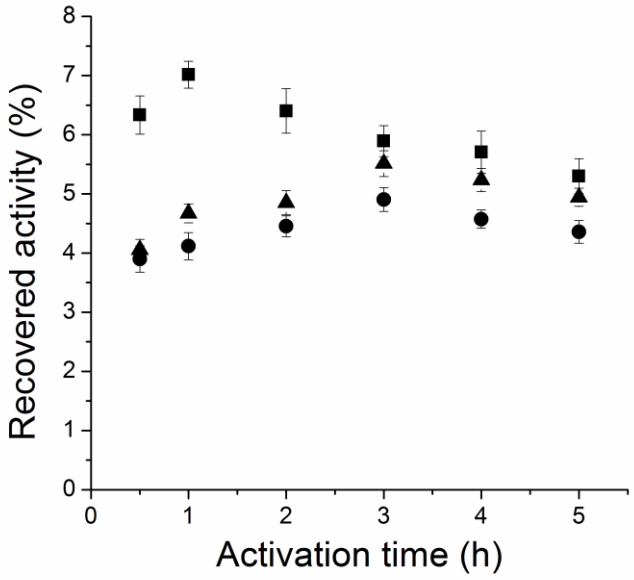
[a] = BET specific surface area ($m^2 g^{-1}$); [b] = BJH pore volume ($cm^3 g^{-1}$)

3.2. Effect of the activation time of the supports with glutaraldehyde on enzyme immobilization

The most common bi-functional cross-linking reagent used is glutaraldehyde because it is inexpensive, easily handled and capable to bind to different enzymes [41]. Thus, firstly, the supports were activated with glutaraldehyde and we evaluated the effect of the activation time on the support performance for pectinase immobilization. The glutaraldehyde concentration was set at 1 % (v/v), while the activation time ranged from 30 min to 5 h.

The recovered activity of the biocatalysts increased with the support activation time up to 1 h for Nano-CMag and 3 h for Micro-CMag and Macro-CMag, after these times the recovered pectinase activity decreased (Fig. 2).

Figure 2. Influence of the activation time on the recovered activity of the (■) Nano-CMag, (▲) Micro-CMag and (●) Macro-CMag biocatalysts, under the conditions 1 % of glutaraldehyde and 0.4 $mg.mL^{-1}$ of protein concentration. The immobilization was carried out during 15 h.



Similar results were found for the immobilization yield of the biocatalysts (Table S1). In very short activation times, the activation degree of the carrier is low, probably generating

fewer covalent attachment points, which resulted in lower immobilization yields, while longer activation times can favored the cross-linking between chitosan activated groups and reduce the possibilities of enzyme-support reaction. Srivatava and Anand [56] verified that higher glutaraldehyde concentrations generated multiple cross-linking points on the chitosan surface causing spatial hindrance among enzyme molecules that decreased the enzyme activity, thus decreasing immobilization yield. Nevertheless, according to our results, this will depend on the form that chitosan is in the support. Thus, the optimal activation time is different for each support.

3.3. Enzyme concentration

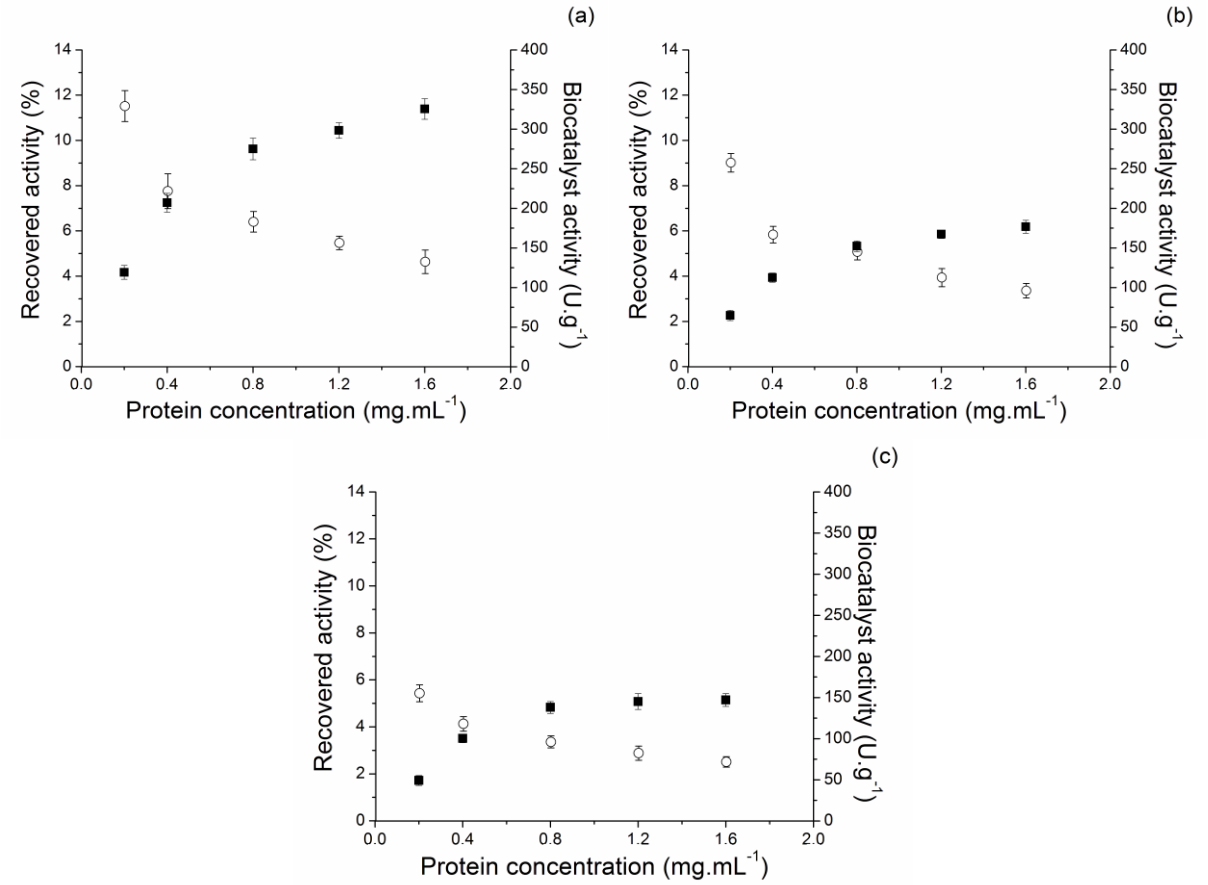
Different enzyme concentrations were tested for immobilization, ranging from 0.2 $mg.mL^{-1}$ to 1.6 $mg.mL^{-1}$ of protein (4 mg to 32 mg of protein per g of support) and the results are presented in Fig. 3.

The immobilization in all materials presented a similar behavior, where the highest recovered activity was obtained when the lowest enzyme concentration was used. The decrease in the recovered activity could be explained by diffusional limitations of the substrate into the pores, that will be mainly hydrolyzed by the enzymes located in the outer part of the pores and the external particle surface [42,57,58].

On the other hand, the activities of the biocatalysts increased when the enzyme concentration increased. Biocatalysts activity rapidly increased up to 0.8 mg $protein.mL^{-1}$, while enzyme concentrations higher than 0.8 $mg.mL^{-1}$ only promoted slight increments in the activity for Nano-CMag and Micro-CMag. These results may be related to the lower particle size, that will reduce the enzyme diffusional problems to the inner areas of the supports, allowing a more rapid immobilization of the enzyme. Using Macro-CMag, that has larger particle size, the enzyme diffusion problems increased and immobilization in the core of the

particle may become very slow. Moreover, also the substrate diffusion limitations will be higher in these larger particles, producing a decrement of the expressed activity [33]. Thus, to compare all biocatalysts, and in order to achieve high enzyme activity and satisfactory RA %, an enzyme concentration of 0.8 mg.mL^{-1} was chosen for the next experiments.

Figure 3. Influence of enzyme concentration on the (○) RA % and (■) biocatalyst activities of the (a) Nano-CMag, (b) Micro-CMag and (c) Macro-CMag. The immobilization was carried out during 15 h.



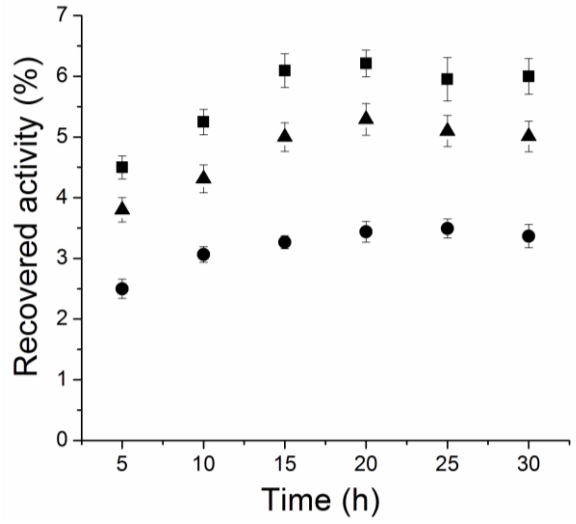
3.4. Immobilization time

Effects of immobilization time on the biocatalysts recovered activities were evaluated from 5 h to 30 h. (Fig. 4). Regarding the recovered activity, a large increase was observed up to 15 h for all biocatalysts, however, Micro-CMag and Macro-CMag biocatalysts showed a slight increase in activity up to 20 h. It could again be related to the diffusional problems of

the enzyme to be immobilized on these biocatalysts, whereas in Nano-CMag, the enzymes were immobilized more rapidly in the shorter pores, in the Macro-CMag, the enzymes needed a longer time to reach the inner part of the long pores.

Similar results were observed by Biró et al. [31]. The authors reported that the steric hindrance caused by the enzyme molecules on the macroparticles surface avoided immobilization inside the whole biocatalyst, whereas for the immobilization in smaller particles there was better distribution of the enzyme in whole particle. Thus, considering the highest recovered activities and immobilization yield, an immobilization time of 20 h for all biocatalysts was selected as optimal value for subsequent experiments.

Figure 4. Influence of immobilization time on the recovered activity of the (■) Nano-CMag, (▲) Micro-CMag and (●) Macro-CMag biocatalysts with of 0.8 mg.mL^{-1} of protein concentration.



Although the recovered activities were very low, they are similar to other immobilization strategies using the same enzymatic preparation [26,51,59,60]. This is mainly because the diffusion limitations presented by the large substrate on immobilized particles. Moreover, changing the immobilization support and the immobilization protocol it was possible to vary the enzyme orientation to the support, which leads to different activities and stabilities.

In the next steps, we characterized the immobilized preparations on chitosan-magnetic particles regarding activities, kinetic parameters, thermal and operational stabilities.

3.5. Kinetic parameters

The maximum reaction rate (V_{max}), Michaelis constant (K_M) and catalytic efficiency were determined by measuring initial reaction rates, varying the amount of substrate.

Regarding V_{max} and K_M values, all immobilized enzymes showed slower V_{max} and higher K_M values than the soluble enzyme (K_M, 0.96 $mg.mL^{-1}$ and Vmax, 143.40 $mg.min^{-1}$). The enzyme kinetic parameters exhibited different results as a function of the type a support, with the expected best properties for the smallest support particle. Thus, the enzyme immobilized on Nano-CMag had the lowest value of K_M (1.70 $mg.mL^{-1}$) and the highest value of V_{max} (78.95 $mg.min^{-1}$), followed by Micro-CMag (K_M, 2.23 $mg.mL^{-1}$ and V_{max}, 57.20 $mg.min^{-1}$), and Macro-CMag (K_M, 2.35 $mg.mL^{-1}$ and V_{max}, 46.03 $mg.min^{-1}$). Differences were significant but not decisive to discard some of the biocatalysts.

The increase in K_M when increasing the particle size can be attributed, again, to the diffusional limitations of the reacting species, although some structural variations of the immobilized enzymes on supports prepared by the different protocols may be not discarded, such as some enzyme distortions [61]. In fact, the decrease in V_{max} when the particle size increased should not be related to diffusional limitations but to a different structure of the enzyme that can drive to a lower catalytic capacity or some enzyme molecules whose active center is blocked by the support surface [62].

3.6. Enzyme activities of the soluble enzyme and biocatalysts

Total pectinase (PE), polygalacturonase (PG), pectin lyase (PL), pectin methyl esterase (PME) and cellulase (CE) activities were analyzed for each biocatalyst and compared to those of the soluble enzyme.

As can be seen in Table 2, the biocatalyst Nano-CMag presented the highest values for all the enzymatic activities analyzed. These results can be related to lower diffusion limitations on these small particles, facilitating the enzyme access to the macromolecular substrates. On the other hand, Macro-CMag biocatalysts presented the lowest activity recovery for all substrates, possibly because the higher diffusion limitations. Similar results were also found by Klein et al. [33] and Biró et al. [31], for immobilization of β-galactosidase, observing that the smaller the particle size, the higher the recovered activity.

Table 2. Enzymatic activities of the soluble enzyme, Nano-CMag, Micro-CMag and Macro-CMag biocatalysts.

Treatments	**Recovered activities** (%)				
	PE	**PG**	**PL**	**PME**	**CE**
Soluble enzyme	100	100	100	100	100
Nano-CMag	6.15	5.82	5.73	12.21	9.60
Micro-CMag	5.16	2.92	3.64	2.99	8.93
Macro-CMag	3.29	2.25	2.07	2.90	4.53

It has been reported that the immobilization of a mixture of enzymes with different sizes on porous supports may be a problem [13]. The protein with the largest size should determine the minimum support pore diameter, because if the support pore is not enough to allow the diffusion of the largest protein, this enzyme can be immobilized on the pore entrance, blocking the access to lower proteins [13]. Various crowding effects and pores

blocking effects, related to changes in diffusion rate, protein unfolding, self-association and protein binding enhancement, enzymatic activity alteration and reaction kinetics modification have been reported [63–65].

Moreover, the spatial arrangement and orientation of multiple enzymes in solid supports are very important, facilitating the cooperative catalytic mechanisms, where the reactive intermediates can be transported rapidly from one active site to the next to avoid diffusion losses, as in cascade reactions [66,67].

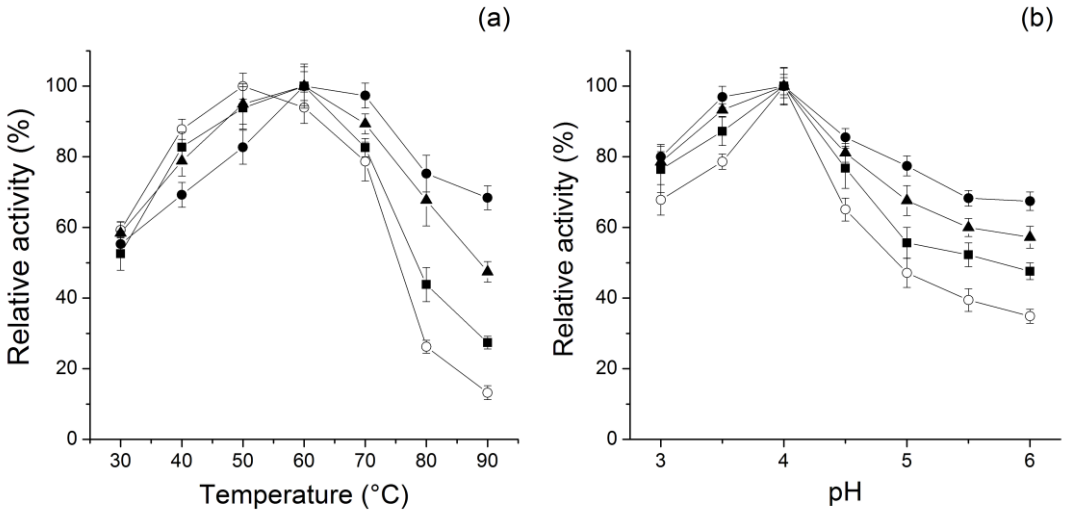
3.7. Effect of pH and temperature on enzyme activity

The reaction conditions are directly associated to the enzyme catalytic efficiency. Thus, the effect of different temperatures (30 ºC–90 ºC) and pH values (3–6) were analyzed for the soluble enzyme and immobilized biocatalysts activities. Regarding the temperature, the soluble enzyme presented maximum activity at 50 ºC, while the biocatalysts Nano-CMag, Micro-CMag and Macro-CMag showed maximum activity at 60 ºC (Fig. 5). This should be consequence of an improvement on enzyme stability after immobilization, where a more stable enzyme exhibits superior activity at higher temperatures [62]. Immobilized/stabilized biocatalysts presented higher activities at elevated temperatures compared to the soluble enzyme. Effect of temperature on enzyme activity is the result of several factors: enzyme activity should increase, but enzyme inactivation (negative conformational changes) may compensate this, moreover, viscosity of the medium may change and that way diffusion of substrate may be greatly altered.

The immobilized enzymes presented higher activities in a wider range of pH values than the free enzyme. The maximum activity was found at the same pH for all forms of enzymes (pH 4), however, the immobilized enzymes retained higher activities under extreme conditions. The biocatalysts prepared on the different particles presented higher activity at pH

3 and pH 6 than the soluble enzyme. Additionally, at pH 6, the activity of Macro-CMag was higher than Nano-CMag and Micro-CMag, as occurred at high temperatures. This suggests that the properties of the prepared particles by different methods affected not only the immobilization rate and yield, as presented above, but also the enzyme activity at different conditions.

Figure 5. Relative activity of (○) soluble enzyme, (■) Nano-CMag, (▲) Micro-CMag and (●) Macro-CMag under different conditions of (a) temperature and (b) pH.



3.8. Thermal and storage stability

A stable biocatalyst is essential for industrial application, so thermal and storage stabilities of the immobilized biocatalysts and the free enzyme were evaluated. At 60 ºC, the soluble enzyme presented the shortest half-life ($t_{1/2}$ = 0.257 min^{-1}) and the highest thermal inactivation rate (k = 2.69 min). For Nano-CMag, Micro-CMag and Macro-CMag, an improvement in the thermal stability was observed, since k decreased (0.117, 0.039 and 0.014 min^{-1}, respectively) and $t_{1/2}$ increased (5.92, 17.77 and 49.51 min, respectively), providing stabilization factors of 2.2, 6.6 and 18.4, respectively, when compared to soluble enzyme (Table 3). These stabilization results explain, at least partially, the wider range of temperatures and pH where the immobilized enzyme may be utilized.

Enzyme immobilization is one way to improve the enzyme stability [6,12,13]. If several linkages between the support and the enzyme are established, they will restrict conformational changes of the enzyme induced by heat and stabilizes the tertiary structure of the enzyme, which is essential to catalytic activity [41,68].

Moreover, a significant improvement in enzyme stability was once again observed for the Macro-CMag biocatalyst, presenting 8-times more thermal stability than the Nano-CMag biocatalyst. These results suggested that the three different support may have conferred different structures to the chitosan coating, developing different electrostatic interactions between the chitosan chains which may to favor the development of more open support, such as Nano-CMag, providing a lower possibility of getting an intense multipoint covalent attachment. Moreover, the rigidification achieved via multipoint covalent attachment also depends on the support rigidity (only a rigid support may produce enzyme rigidification), and this rigidification is likely different for each method of chitosan preparation. Results suggested that using Macro-CMag biocatalyst, the enzyme–support reaction may be more intense or the chitosan polymer may have a lower mobility, favoring the preparation of more rigid and compact support which helped to stabilize the enzymes.

A high thermal stability was also presented when the polygalacturonase from Rohapect 10 L was immobilized on MANAE-agarose using the glutaraldehyde chemistry [60]. Thermal stability was measured in four different pH and temperature conditions and in all of them immobilized enzyme was much more stable than free enzyme. Additionally, stabilization data suggested changing the immobilization pH from 5 to 8 the enzyme was probably attached by a different orientation on the support [60].

Related to the storage stability, the soluble enzyme and the three immobilized biocatalysts were fully stable when stored at 4 ºC, in sodium citrate buffer (50 mM), pH 3.5 (near to pH of fruit juices) during 1 month.

Table 3. Kinetic parameter of thermal deactivation for the soluble enzyme, Nano-CMag, Micro-CMag and Macro-CMag biocatalysts.

Treatments	**k (min^{-1})**	**$t_{1/2}$ (min)**	**Stabilization factors**
Soluble enzyme	0.257	2,69	1,0
Nano-CMag	0,117	5,92	2,2
Micro-CMag	0,039	17,77	6,6
Macro-CMag	0,014	49,51	18,4

3.9. Operational stability

Operational stability is another important parameter for identifying a suitable immobilized enzyme and it is one of the main reasons for immobilization. To evaluate the operational stability, several batch cycles of clarification of grape, orange and apple juices were performed.

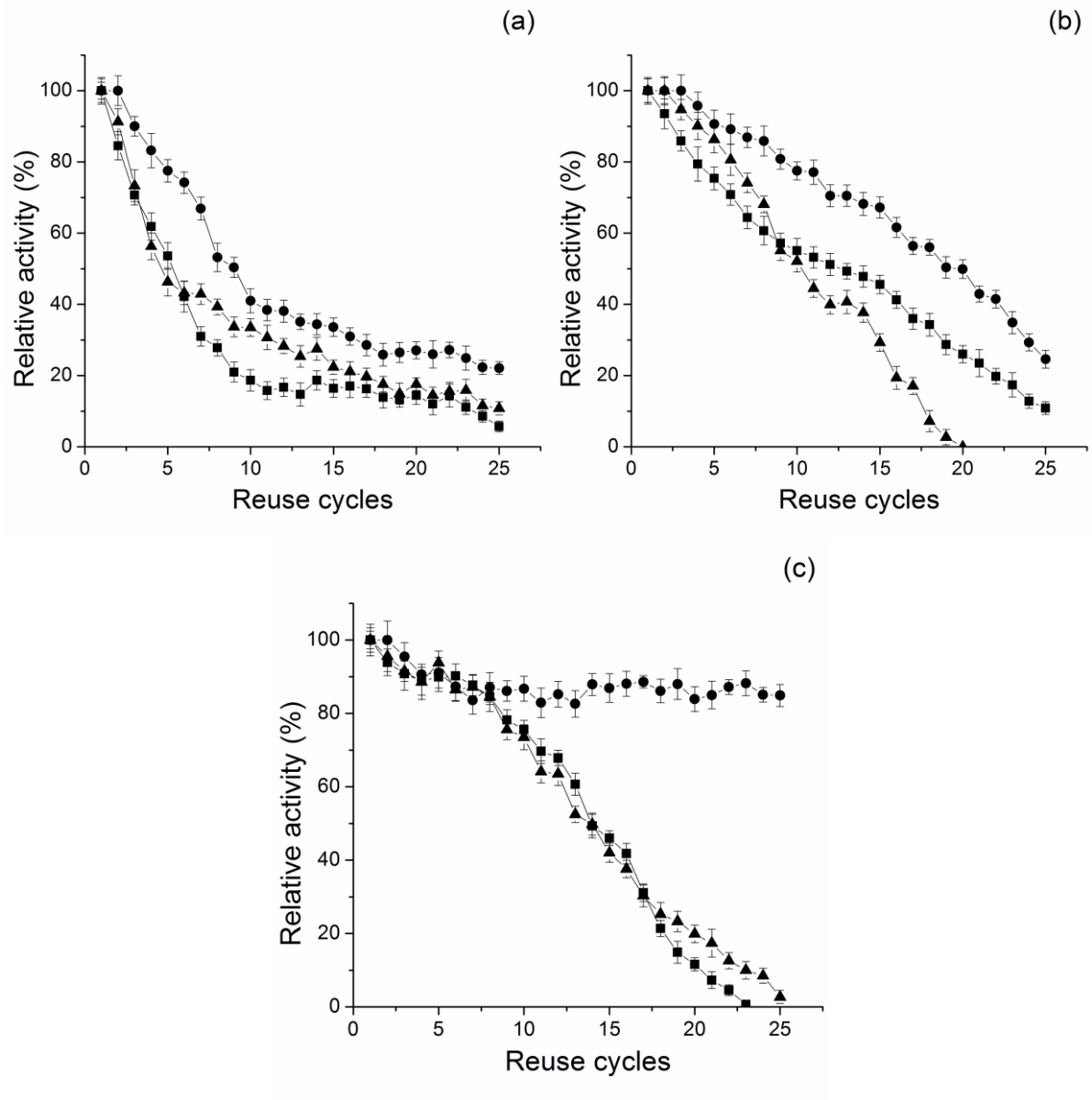
As can be observed in Fig. 6, all the biocatalysts were able to perform the clarification of the juices by several cycles of reuses. Due to the juices pH (around 3), the initial activity in the first cycle was lower than the maximal activity presented in Fig. 5b (at pH 4). Thus, the initial activity was around 80 % of the maximal one. Nevertheless, the decrease in each juice turbidity in the first cycle was considered as 100 %, and the subsequent cycles were compared to that, as this experiment was performed to check the biocatalyst stability during successive reaction cycles. However, the Macro-CMag biocatalyst presented the best results after 25 reuses, presenting in the last reuse a residual activity of 22 %, 85 % and 25 % for grape, orange and apple juices, respectively. On the other hand, the Nano-CMag and Micro-CMag biocatalysts presented a residual activity lower than 15 % for all the juices in the last reuse.

The more rapid loss of activity over the reaction cycles for the Nano-CMag and Micro-CMag biocatalysts may be related to the lower stability of the enzyme molecules immobilized in these supports when compared to the enzyme molecules immobilized into the pores of Macro-CMag.

Considering that the biocatalysts could be satisfactorily reused until the loss of 50 % of its initial activity, each biocatalyst will be used for different number of cycles until its replacement by a new immobilized enzyme. Thus, for grape and apple juices Nano-CMag should be replaced after 5 and 12 cycles, Micro-CMag after 4 and 10 cycles, and Macro-CMag after 9 and 19 cycles, respectively. In each case, to obtain the initial 5 U, it was used 18.93 mg, 29.43 mg and 37.30 mg of Nano-CMag, Micro-CMag and Macro-CMag, respectively, for 1 mL of juice. Then for each biocatalyst the relation mass/ cycle was 3.79 mg and 1.58 mg for Nano-CMag, 7.36 mg and 2.94 mg for Micro-CMag, and 4.14 mg and 1.96 mg for Macro-CMag, respectively for grape and apple juices. Then, it can be concluded that, despite the lower stability of Nano-CMag, its higher activity per gram allows the use of a smaller amount of the biocatalyst, reducing the overall cost.

For orange juice the result is even more expressive. The relation mass/cycle for Nano-Cmag and Micro-CMag was 1.26 mg and 1.96 mg, respectively. However, Macro-CMag biocatalyst can be used for at least 25 cycles in orange juice without significant losses of activity (over 80 % of the initial activity), that way, for this juice the advantages of this biocatalyst over the other two ones are very great. A proper calculation cannot be performed as the biocatalyst retained 85 % of the activity after 25 cycles and very likely inactivation did not follow first order kinetic.

Figure 6. Reusability of (■) Nano-CMag, (▲) Micro-CMag (●) and Macro-CMag biocatalysts assayed in grape (a), apple (b) and orange (c) juices.



The difference in the operational stability using different juices could be explained by the different composition of each juice. The ionic exchange adsorption of some compounds in chitosan, such as polyphenols, proteins, starch, among others, may have contributed to the increase of diffusion problems, reducing enzyme activity in grape and apple juices. These problems seem to be minimized using orange juice.

Pectin lyase from Rohapect 10 L presented high operational stability when immobilized on glutaraldehyde-activated agarose [59]. After 5 cycles of 72 h each, the

immobilized enzymes maintained more than 90 % of its initial activity, being a suitable strategy for the preparation of a biocatalyst with high potential for application in pectin hydrolysis, such as Macro-CMag.

4. Conclusions

This paper presents for first time the importance of the method of chitosan particles preparation on the properties of the final biocatalyst when used for enzyme immobilization. Moreover, the use of chitosan and magnetic particles as supports for enzyme immobilization showed to be an interesting strategy for fruit juice clarification. The coating with chitosan allowed the improvement of superficial area compared with the magnetic particles alone, while the magnetic particles facilitated the biocatalysts separation. We compared three different methods for preparation of chitosan particles, providing different particle sizes (nano, micro and macro particles), and also having different textural properties. The Macro-CMag biocatalyst presented the highest thermal stabilities among the biocatalysts prepared. In addition, Macro-CMag presented the highest retained activity after 25 cycles of reuse for all fruit juices, especially orange juice, where the final residual activity was 85 %. On the other hand, the Nano-CMag biocatalyst presented the best kinetic parameters and higher enzyme activity recoveries.

From a practical point of view, each biocatalyst presents its own advantages and disadvantages. For fruit juice clarification, it is necessary a smaller amount of Nano-CMag when compared to Macro-CMag, due to its higher activities. On the other hand, the separation of Macro-CMag is easier when compared to Nano-CMag, due to its larger size. Thus, our results bring new lights for immobilization of clarifying enzymes and to the development of an industrial process for juice clarification.

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TableS1. The recovered activity (RA), immobilization yield (IY) and immobilization efficiency (IE) during the preparation of the Nano-CMag, Micro-CMag and Macro-CMag biocatalysts.

	Nano-CMag			**Micro-CMag**			**Macro-CMag**		
	RA (%)	IY (%)	IE (%)	RA (%)	IY (%)	IE (%)	RA (%)	IY (%)	IE (%)
Activation time (h)									
0.5	6.33	72.68	8.71	4.06	64.04	6.33	3.91	60.65	6.45
1	7.01	73.56	9.53	4.67	65.60	7.12	4.12	62.50	6.59
2	6.40	71.35	8.97	4.85	66.54	7.29	4.45	62.02	7.18
3	5.89	69.70	8.45	5.51	67.57	8.16	4.90	64.46	7.61
4	5.71	68.12	8.38	5.23	64.66	8.09	4.57	64.39	7.10
5	5.30	69.00	7.68	4.94	63.99	7.72	4.36	62.70	6.95
Protein concentration (mg)									
0.2	11.52	77.39	14.88	9.01	73.50	12.26	5.43	82.81	6.56
0.4	7.76	71.57	10.84	5.84	67.88	8.60	4.13	60.94	6.77
0.8	6.40	67.71	9.45	5.07	56.15	9.03	3.36	43.66	7.70
1.2	5.47	49.65	11.01	3.94	40.81	9.64	2.88	30.37	9.48
1.6	4.64	36.51	12.70	3.36	30.14	11.14	2.51	23.90	10.50
Immobilization time (h)									
5	4.51	35.45	12.72	3.81	32.56	11.70	2.52	20.13	12.60
10	5.25	50.77	10.33	4.31	43.83	9.84	3.06	27.54	11.12
15	6.09	69.48	8.77	5.01	54.85	9.13	3.27	37.89	8.62
20	6.21	68.17	9.11	5.29	59.53	8.89	3.44	42.29	8.13
25	5.95	67.00	8.88	5.10	57.56	8.85	3.49	43.44	8.04
30	6.00	67.60	8.87	5.01	58.20	8.61	3.37	41.64	8.08

Figure S1. SEM images: (a) MP, (b) MP-APTMS, (c) Nano-CMag, (d) Micro-CMag and (e) Macro-CMag with different magnifications (from 10,000x to 50,000x).

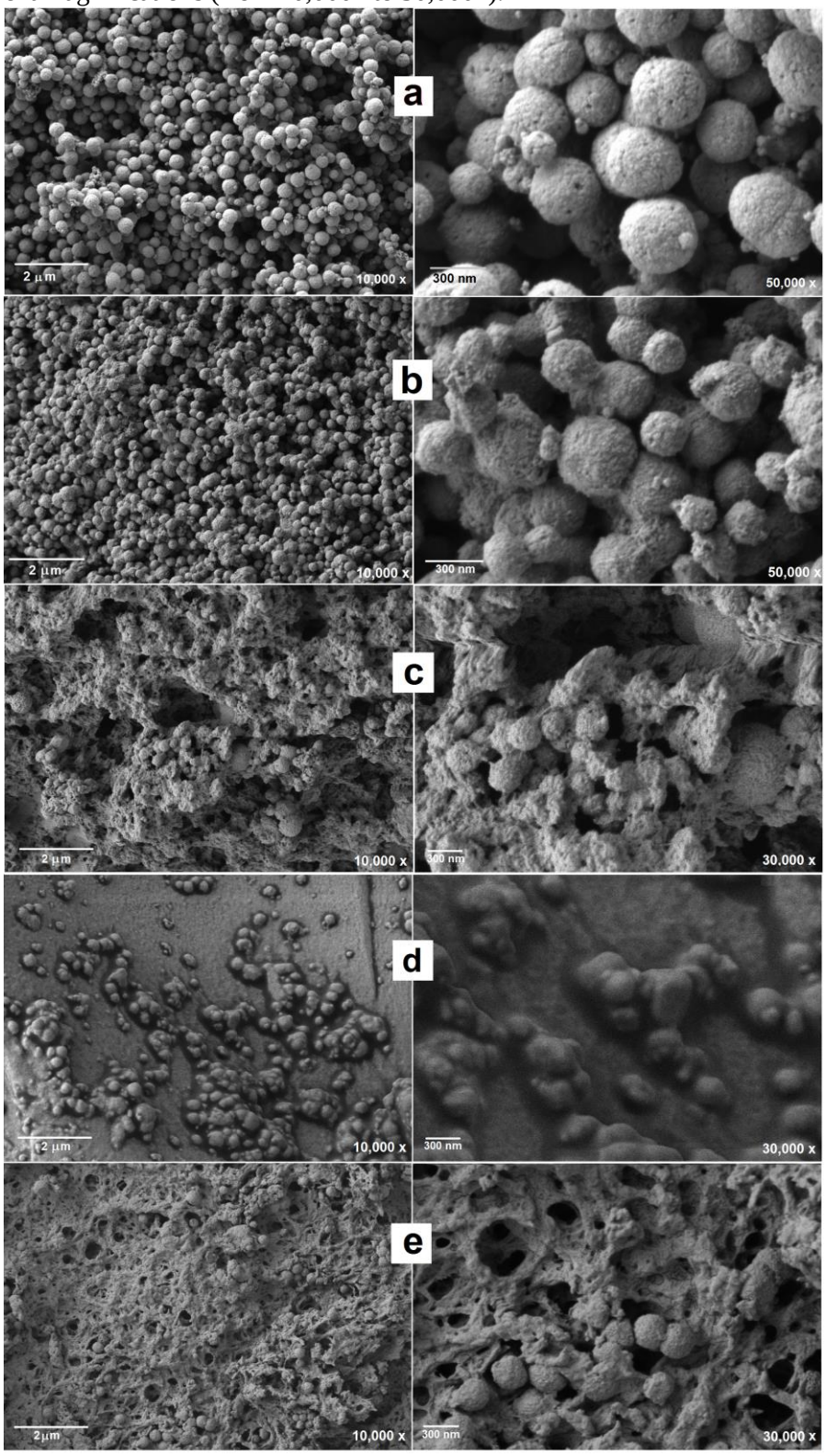


Figure S2: Transmission infrared spectra of materials. The bar value 10%.

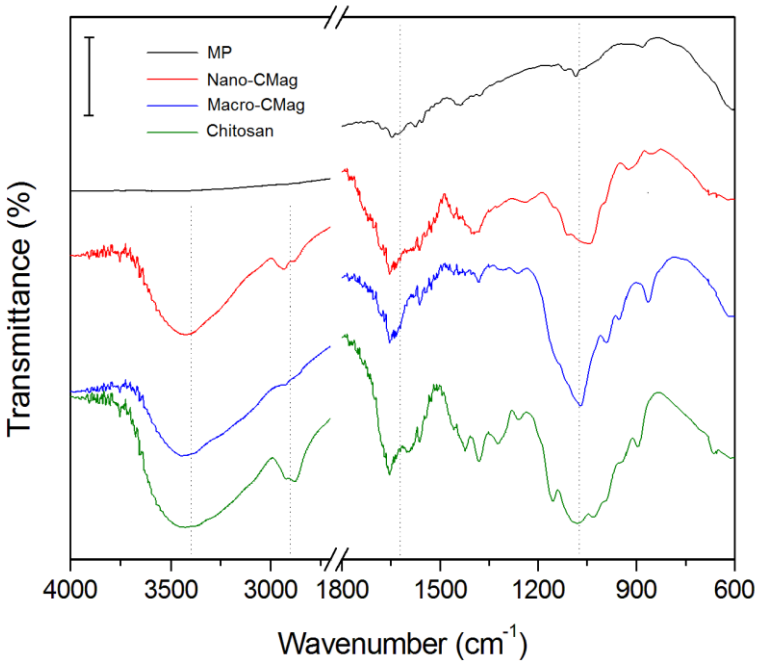


Figure S3. X-ray diffraction patterns of the materials: (a) MP; (b) MP-APTMS; (c) Macro-CMag; (d) Micro-CMag; (e) Nano-CMag and (f) Standard magnetite (JCPDS n° 19-0629).

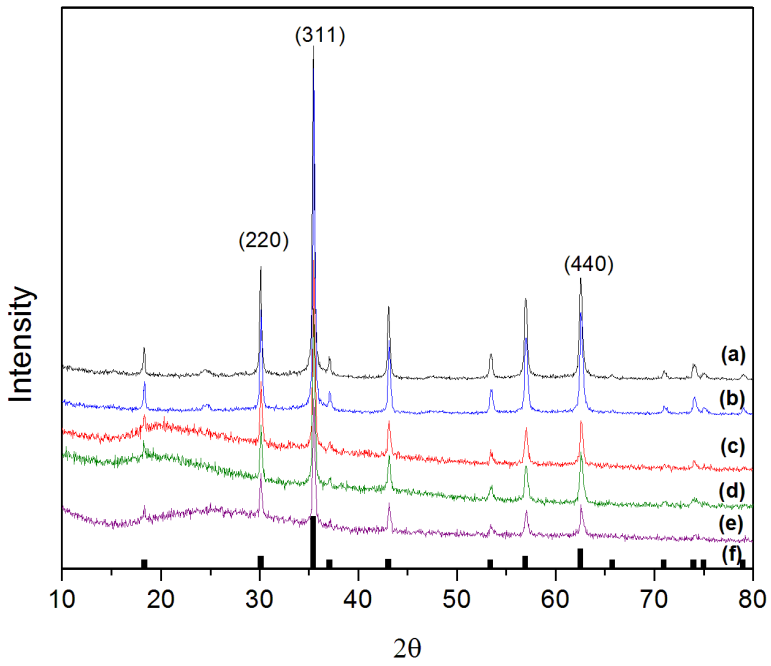


Figure S4. Non-normalized magnetization curves for MP, MP-APTMS, Macro-CMag, Micro-CMag and Nano-CMag materials.

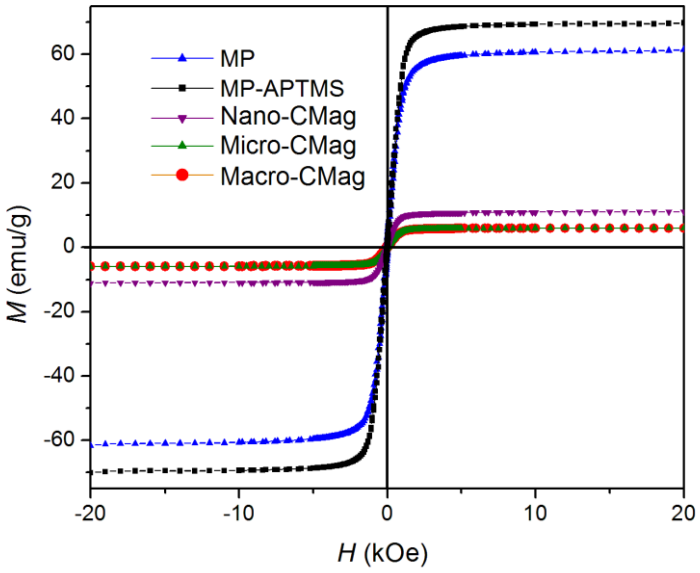
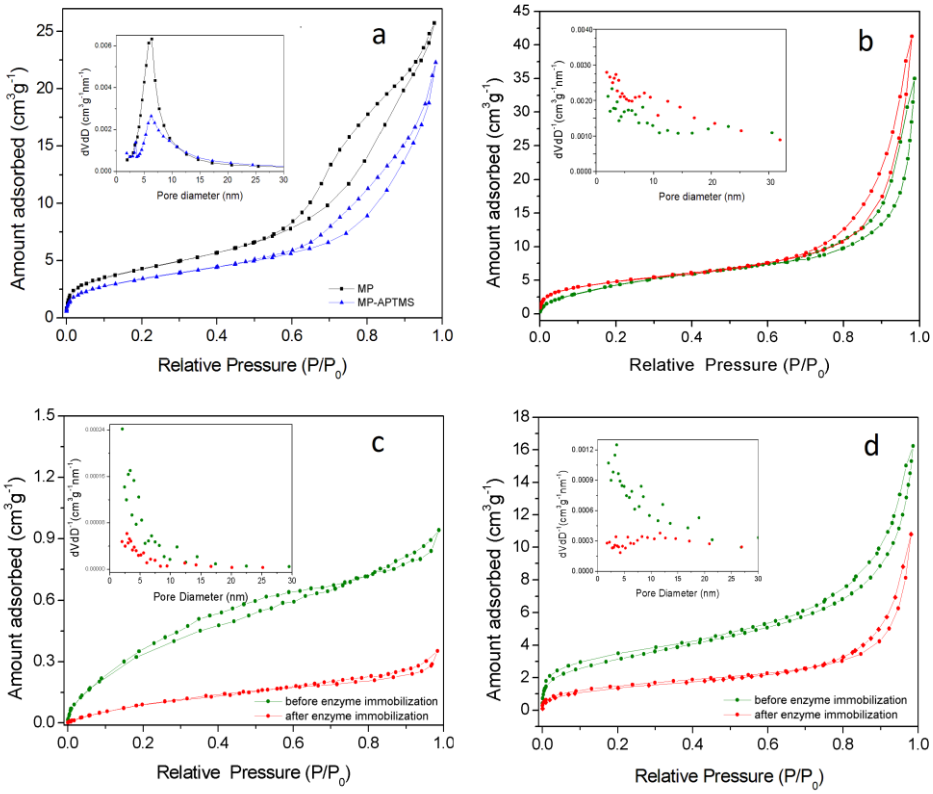


Figure S5. N2 adsorption-desorption isotherms for materials: (a) MP and MP-APTMS, (b) Nano-CMag, (c) Micro-CMag and (d) Macro-CMag. Inset Figures the BJH pore size distribution.



CAPÍTULO 5 – Stability/activity features of the main enzyme components of rohapect 10L

Muitos protocolos de imobilização exigem condições alcalinas para a ligação da enzima ao suporte. No entanto, algumas enzimas são extremamente sensíveis a estas condições. Assim, neste trabalho procurou-se entender como cada enzima do preparado enzimático Rohapect® 10L reage as diferentes condições de imobilização. As diferentes atividades enzimáticas, pectinase total (PE), poligalacturonase (PG), pectina liase (PL), pectina metil esterase (PME) e celulase total (CE), foram analisadas após a incubação das enzimas em diferentes condições de pHs e temperaturas, obtendo as estabilidades das mesmas. Em seguida, buscando alcançar a estabilidade de todas as frações enzimáticas em pH 10 a 25 ºC, o efeito de alguns agentes estabilizantes, como glicerol, dextrana e polietilenoglicol foram testados. Os resultados estão apresentados no manuscrito a seguir, publicado na revista *Biotechnology Progress*, v. 35, e2877, 2019.

CAPÍTULO 5 – Stability/activity features of the main enzyme components of rohapect 10L

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Abstract

Rohapect 10L is an enzyme cocktail commercialized for juice clarification. Here, we characterized the activity and stability of five enzymatic activities present in this cocktail: total pectinase (PE), polygalacturonase (PG), pectin lyase (PL), pectin methyl esterase (PME), and total cellulase (CE) activities. All these enzyme activities have the maximum activity and stability at pH 4, conditions near those found in most fruit juices. However, if the enzymes need to be handled under different conditions (e.g., to immobilize them), their stability becomes extremely low in some cases, just at pH values slightly higher than the optimal one. For example, at pH 10 only CE was reasonably stable at 25ºC, while many other enzyme activities were rapidly almost inactivated, even at 4ºC. For these cases, different additives were evaluated, and we found that polyethylene glycol was positive or very positive for all enzyme stabilities, allowing keeping reasonable activities after several hours at pH 10 and 25ºC. Another additive, that is, dextran, has a small positive effect for PE, PG, and CE, and a very positive effect for PL, albeit significantly destabilizing PME. Thus, the handling and use of this extract requires some care when is performed out of optimal conditions.

Keywords: cellulosic enzymes, enzyme cocktail activity, enzyme cocktail stability, pectinolytic enzymes, stabilization of enzyme activities, stabilizing additives

1. Introduction

Enzymes are extremely useful catalysts in many areas.[1-5] In some instances, a sequence of reactions is necessary to get the final product, the so-called cascade reactions, involving several enzymes.[6-9] That is, the case of polysaccharides modifying enzymes, where the substrate is complex, having different bonds and several enzymes are required to reach the final product (e.g., glucose).[10,11] In this kind of reactions, it is convenient to use an enzyme cocktail containing the different enzymes involved in the process.[10,12,13] The enzyme stability and activity in the final reaction conditions of each enzymatic component must be adequate. If one enzyme is less active than the others under the target conditions, a higher amount of that enzyme should be used to avoid kinetic limitations. If one enzyme is much less stable than the other ones, a strategy with a step wise addition of the least stable enzyme to maintain the activity (or the use of a much higher initial activity of this unstable enzyme) must be considered. This will not be possible if the only source of the different enzymes is the enzyme cocktail. Additives like polyols or ions that can stabilize one enzyme could be used only if they are not detrimental for some of the other cocktail components during the handling of the enzymes.[14-18]

When immobilized biocatalysts are designed using enzyme cocktails the preparation of the biocatalysts can be more difficult. In this case, the coimmobilization is the only way of preparing the biocatalyst (except if the extract is submitted to tedious purification steps). This can complicate taking full advantage of the enzyme immobilization improvement of enzyme features for all enzymes (e.g., stabilization, improved activity, selectivity or specificity, higher resistance to inhibitors, lower inactivation by drastic conditions or chemicals).[19-24] In fact, as a general rule it may be said that the individual enzyme immobilization will be better, even if some kinetic advantages of coimmobilization may be lost.[25-27] This way, optimal

immobilization protocols for each enzyme may be selected to have the most positive effects on the properties of each specific enzyme.[25]

Nevertheless, some enzyme supplier companies offer some enzyme cocktails that contain most of the enzymes involved in that process in a proportion designed for a specific use.[28-30] This is the case of enzymes utilized for juice clarification, where it is possible to find many different enzyme cocktails from different companies, such as Pectinex Ultra SP-L, Klerzyme 150, Pectinex Ultra Color, Rohapect 10L, Zimopec PX5, Pectinex Smash XXL, Novozym 33095, and Pectinex Ultra Clear.[29,31-34] In many instances, the lack of information on the individual enzyme properties may make the selection of the handling and reaction conditions complex if our target is a nonstandard process or requires different experimental conditions.

An example of these cascade reactions is in fruit juices production, where some commercially available enzyme cocktails are applied during processing.[35-46]

In this article, the commercial enzymatic cocktail Rohapect 10L has been characterized. It is commercialized for the extraction and clarification of fruit juices, and its main components are pectinolytic and cellulolytic enzymes that act in different ways catalyzing the hydrolysis of pectic and cellulosic substances of fruits.[29] The degradation of these long and complex polysaccharides from the fruit pulp requires that all enzymes act in combination to achieve an efficient hydrolysis as there are some synergetic effects.[10] Depending on their mechanism of action, pectinases can be classified as depolymerases and esterases. The first group includes hydrolases (polygalacturonases) that catalyze the hydrolysis of α-1,4 linkages, and lyases (pectin lyase), which are responsible for catalyzing the β-elimination of the main chain of the pectic polysaccharide. The group of esterases catalyzes the de-esterification of the methoxyl group of pectin forming pectic acid, and includes the pectin methyl esterase.[13,47-49] On the other hand, cellulases are highly specific

biocatalysts that work in synergy to release sugars, and they are often found together with pectinases in commercial enzymes preparations. These enzymes can be classified according to the attack position on the substrate as endoglucanases that catalyze the hydrolysis of internal linkages β-1,4 of the cellulose, exoglucanases, which act on the ends of the cellulose chain releasing glucose or cellobiose units, and β-glycosidases which hydrolyze cellobiose, releasing glucose units.[11,50-52]

Although these enzymes can be obtained from several sources such as plants or animals, enzymes from microbial sources generally satisfy the industrial requirements.[53] The most outstanding resource of these commercial biocatalysts is the filamentous fungi, particularly *Aspergillus niger*.[53]

Nevertheless, there is not a proper analysis of the stability/activity properties of the different enzyme components of Rohapect 10L to date. In this article, we have tried to advance in the study of the pH/activity and pH/stability of the different components. Moreover, we have analyzed the effect of the extract concentration on enzyme stability (this will be relevant if the enzymes are multimeric or depend on some compounds).[54,55] The effect of some additives on the stability of the enzymes has also been investigated so as not to be applied during operation but in the possible handling of the enzymes for any purpose, such as their immobilization (perhaps under drastic conditions such as alkaline pH values).[55] Polyols like glycerin or dextran, or polyethylene glycol in many instances have been used to stabilize enzymes with effect well described.[56-61] A final definition of the range of conditions where the enzyme cocktail may be used considering all individual components will be drawn.

2. Materials and Methods

2.1. Materials

Rohapect 10L was supplied from AB enzymes (Darmstadt, Germany). Pectin from apple, polygalacturonic acid, galacturonic acid, sodium dodecylsulfate (SDS), glycerin, and dextran were acquired from Sigma-Aldrich (St. Louis, MO). Polyethylene glycol (600 $g.mol^{-1}$) was from Merck (Hohenbrunn, Germany). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). All other reagents and solvents were of analytical grade.

2.2. Determination of enzyme activities

Total pectinase (PE) activity was determined using pectin as substrate. This activity evaluates pectin hydrolysis by the action of all pectinolytic enzymes, such as polygalacturonase, pectin lyase, and pectin methyl ester, which will also be evaluated individually. Then, 0.1 mL of diluted enzyme (0.05 $mg.mL^{-1}$ of protein) was added to 0.9 mL of substrate (1 $g.L^{-1}$) prepared in 50 mM sodium citrate at pH 4.8 and incubated at 37ºC, for 1 min. The amount of reducing groups formed was determined by the 3,5-dinitrosalicyclic acid (DNS) method according to Miller.[62] One PE unit was defined as the amount of enzyme that liberates 1 μmol of reducing groups per min under the reaction conditions.

Polygalacturonase (PG) activity was measured using polygalacturonic acid as substrate. Briefly, 0.1 mL of enzyme (0.05 $mg.mL^{-1}$ of protein) was added to 0.9 mL of substrate (1 $g.L^{-1}$) prepared in 50 mM sodium citrate buffer at pH 4.8, and it was incubated at 37 ºC during 2 min. The amount of reducing groups formed was determined by the 3,5-dinitrosalicyclic acid (DNS) method according to Miller.[62] One PG unit was defined as the amount of enzyme required to release 1 μmol of reducing groups per min under the reaction conditions.

Pectin lyase (PL) activity was determined spectrophotometrically measuring the increase in absorbance at 235 nm promoted by the formation of unsaturated products, as described by Albershein,[63] with slight modifications. Then, 0.2 mL of the enzyme solution (0.05 $mg.mL^{-1}$ of protein) were added to 2.3 mL of a solution of 2 $g.L^{-1}$ of pectin prepared in 50 mM sodium citrate at pH 4.8 and incubated at 25ºC for 1 min. One unit of PL was defined as the amount of enzyme which produces 1 μmol of unsaturated uronide (ϵ = 5500 $M^{-1}.cm^{-1}$ at 235 nm) per min under the reaction conditions.

Pectin methyl esterase (PME) activity was estimated by the titration of the carboxylic groups released by the hydrolysis of pectin, as described by Rouse and Atkins,[64] with some modifications. Then, 0.1 mL of the enzyme solution (0.18 $mg.mL^{-1}$ of protein) was added to 9.9 mL of 5 $g.L^{-1}$ of pectin solution prepared in 0.15 M NaCl at pH 4.5. The reaction was carried out at 30ºC for 10 min. After the reaction, the sample was titrated with a solution of 20 mM sodium hydroxide to the initial pH of 4.5. One unit of PME was defined as the amount of enzyme required to release one milliequivalent of carboxyl groups per minute under the reaction conditions.

Total cellulase (CE) activity was estimated using Whatman nº1 filter paper as substrate, following the method proposed by Ghose,[65] with some modifications. Briefly, 0.1 mL of enzyme solution (0.18 $mg.mL^{-1}$ of protein) were added to 0.9 mL of 50 mM sodium citrate at pH 4.8 containing 50 mg of filter paper. The reaction was conducted at 50ºC during 5 min. The released reducing sugars were determined by the DNS method according to Miller.[62] One unit of CE was defined as the amount of enzyme that liberates 1 μmol of reducing groups per min under the reaction conditions.

2.3. Activities of the different enzymes at different pH values

The effect of the pH on enzyme activities of the preparation Rohapect 10L was determined by incubation of the enzyme solution in the corresponding substrate solution according to section 2.2., prepared in 50 mM sodium citrate (pH 4), 50 mM sodium phosphate (pH 6 and 8), or 50 mM sodium carbonate buffers (pH 10), other specifications, such as reaction time and temperature, are described above.

2.4. Stability of enzymes under different conditions

To evaluate the stability of the enzymes contained in Rohapect 10L, the enzyme solution was subjected to incubation at different pH values using 50 mM of the respective buffer (sodium citrate at pH 4, sodium phosphate at pH 6 and 8, and sodium carbonate at pH 10) and temperatures (4 ºC, 25 ºC, and 45 ºC). Periodically, samples were taken and their respective activities analyzed according to section 2.2.

2.5. Effect of different additives on enzymes stabilities at pH 10

The effects of glycerin, dextran, polyethylene glycol, and glucose on enzymes stabilities were evaluated. For this, solutions with 20 % (concentration enough in previous reports to visualize the effect) of each stabilizer at pH 10 were prepared and incubated with 1 % enzyme solution (0.05 $mg.mL^{-1}$ of protein) at 25 ºC and 45 ºC to CE, the stability of all the enzymes was analyzed.

2.6. Effect of extract concentration on enzyme stability at pH 10

The stability of the different enzymes was determined at pH 10 using 0.05 $mg.mL^{-1}$ of protein or 0.5 $mg.mL^{-1}$ of protein. In some instances, the solution of protein (0.5 $mg.mL^{-1}$ of protein) was boiled for 10 min and after cooling it was mixed with fresh protein to have a

final concentration of 0.05 $mg.mL^{-1}$ of active protein to study if some component of the extract could have some effect on enzyme properties.

2.7. SDS-PAGE experiments

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli,[66] using a Miniprotean tetra-cell (Bio-Rad), 12 % running gel in a separation zone of 9 × 6 cm^2, and a concentration zone of 5 % polyacrylamide. The enzyme samples were resuspended in rupture buffer (2 % SDS and 10 % mercaptoethanol), boiled for 5 min and 14 μL of the supernatant were used in the experiments. Gels were stained with Coomassie brilliant blue. Low molecular weight markers from GE Healthcare were used (14,000–97,000 Da).

3. Results

3.1. Different activities contained in the protein extract

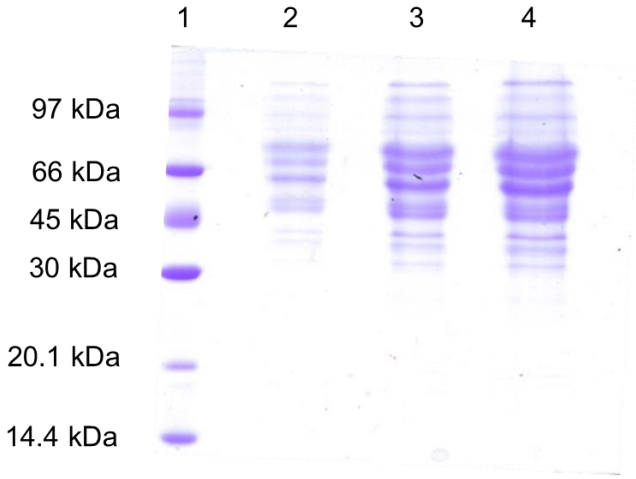
As shown in Table 1 and as also indicated by its supplier, this commercial preparation is characterized by having a main activity of pectinases, highlighting the activity of polygalacturonase, followed by pectin methyl esterase. This combination of enzymes is very important since the vast majority of fruit juices have in their composition pectin with a high degree of esterification, such as apple juices that have 91–92 % of esterified pectin.[13] Polygalacturonase is one of the main enzymes used for the pectin hydrolysis, however, this enzyme acts by hydrolyzing the α-1,4 bond between two nonesterified galacturonic acid residues, requiring the use of other enzymes with de-esterifying action for degradation of pectin, such as pectin methyl esterase that removes methyl groups from galacturonic acids, converting pectin into pectate (nonesterified polymer).[47-49,67]

Table 1. Activities of the different enzymes of the commercial preparation Rohapect 10 L.

	Enzyme Activity				
	PE	**PG**	**PL**	**PME**	**CE**
Total activity ($U.mL^{-1}$)	1259.79	2902.89	73.05	909.02	172.75
Specific activity ($U.g^{-1}$)	233.29	537.57	13.53	168.33	31.99

Figure 1 shows the SDS-PAGE of the extract. As expected, it shows many protein bands, even more bands than enzyme activities studied in this article, in which the major proteins components between 45 and 97 kDa in agreement with previous studies on this commercial protein extract.[68-78]

Figure 1. SDS-PAGE analysis of the enzyme preparation Rohapect 10L. Experiments were performed as described in the item 2.7. Lane 1: Low molecular weight protein standard from GE Healthcare. Lane 2: 0.5 $mg.mL^{-1}$ of protein of enzyme preparation Rohapect 10L. Lane 3: 1.0 $mg.mL^{-1}$ of protein of enzyme preparation Rohapect 10L. Lane 4: 1.5 $mg.mL^{-1}$ of protein of enzyme preparation Rohapect 10L



3.2. Effect of pH value on the activity of the different components of the extract

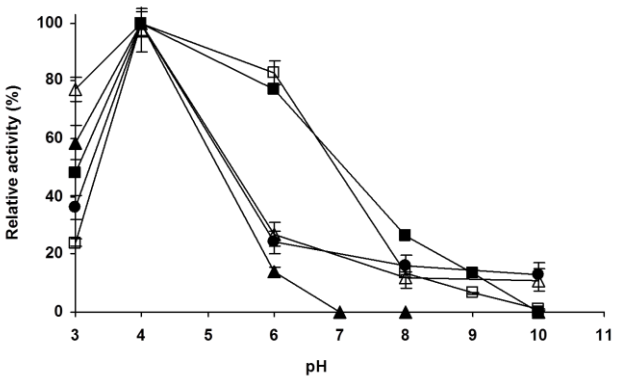
Figure 2 shows the curve activity/pH for the different components of the extract. Because the enzymatic extract has been designed to be used in acidic juices, all enzymes had maximum activities at pH 4, which is close to the pH of fruit juices. Although the maximum

activity is found at the same pH value for all enzymes, the decrease in activity is different with pH variation for each enzyme. PG showed the highest activity decrease when increasing the pH (its activity is negligible at pH 7). PE and PME activities are also very sensitive to the increase of the pH, but both activities are still significant at pH 10. PL and CE activities are initially more resistant to the increase of pH value (activity at pH 6 is near to 80 % of the activity at pH 4) but the activities of these enzyme are negligible at pH 10.

On the other hand, the decrease of pH at pH 3 also causes a high reduction in the activity of the enzymes. Total pectinase activity, PE, is curiously the better retained activity, more than any of the individual components, with a 77 % of the maximal activity. This could be due to a greater protection of the enzyme PG by pectin, than by polygalacturonic acid. Thus, when determining global PE activity with pectin, the activity is better maintained than when measuring specifically PG with polygalacturonic acid as substrate. Among the enzymes studied, PL presented a higher sensitivity to pH reduction, showing a decrease in activity at pH 3 by 76 %.

Thus, the activities of this crude seem to be prepared to be used at pH next to 4, while at moderately alkaline pH or very acidic pH some components show a drastic reduction in their activity.

Figure 2. PE (Δ), PG (▲), PL (□), PME (●), and CE (■) activities under different conditions of pH. It was performed as described in the items 2.2 and 2.3. All experiments were performed in triplicate and the values are given as mean value ± the experimental error.



3.3. Stability at different pH values of the different components of the protein extract

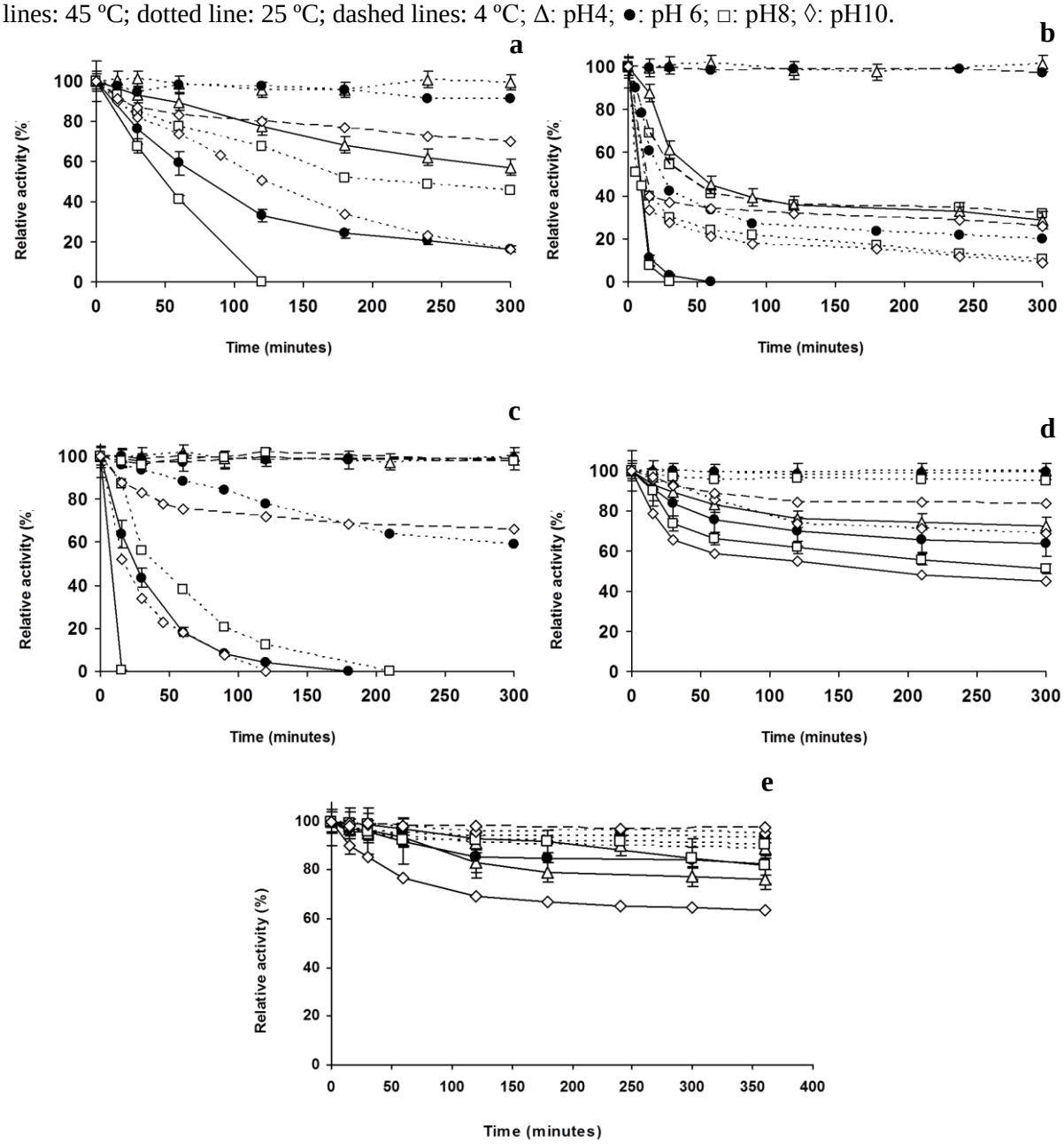
Figure 3 shows the stabilities of the different components of the extract at different pH and temperatures.

Starting with PE (that includes all pectinolytic enzymes), the activity stability at 45 ºC is maximal at pH 4 and decreased when the pH increased (e.g., after 2 hr the enzyme kept more than 75 % of the initial activity at pH 4, more than 30 % at pH 6 and is fully inactive at pH 8). At pH 10 it is not possible to take a reasonable value at this temperature. Using 25 ºC, PE activity was fully maintained at pH 4 and 6 after 5 hr, but presented a half-live of 3.5 hr at pH 8 and of 2 hr at pH 10. Unfortunately, this time is not enough to have a good immobilization process where the enzyme must be at pH 10 (e.g., glyoxyl), therefore it needs to be improved. To reach that goal, temperature was decreased at pH 10 to 4 ºC, where 70 % of the initial activity was maintained after 5 hr. Still this may require further improvement, but at least it was possible to handle the enzyme at pH 10 and 4 ºC for short times.

PG is clearly less stable than PE at all pH values. At 45 ºC, it is only possible at pH 4 to have a reliable half-live (around 50 min), at pH 6 and 8, the residual activity was almost null in the first determination. At 25 ºC, the enzyme retained all the activity after 5 hr only when incubated at pH 4, at pH 6 the half-life was under 25 min, at pH 8 around 5 min and at pH 10 less than 5 min. At 4 ºC, the enzyme remained full active after 5 hr at pH 6, but at pH 8 and 10, the activity decreased rapidly.

PL remained fully active at 45 ºC and pH 4, while at pH 6 the halflife was less than 30 min and at pH 8 it was readily inactivated. At 25 ºC, some inactivation was clear when incubated at pH 6 (60 % after 5 hr), while the stability remained very poor at pH 8 (less than 40 % after 1 hr) and it was even worse at pH 10 (less than 20 % after 1 hr). At 4 ºC there was only a significant inactivation when incubated at pH 10, with more than 60 % residual activity after 5 hr.

Figure 3. Inactivation courses of the PE (a), PG (b), PL (c), PME (d), and CE (e) under different pH and temperature conditions. It was performed as described in the items 2.2 and 2.4. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Solid lines: 45 ºC; dotted line: 25 ºC; dashed lines: 4 ºC; Δ: pH4; ●: pH 6; □: pH8; ◊: pH10.



In relation to PME, this showed to be the most stable enzyme of the pectinolytic complex, suffering the lowest inactivation by the variation of pH and temperature. However, at 45 ºC, the highest temperature used in this work, the inactivation was higher as the pH increased, after 5 hr, this enzyme still had 73 %, 63 %, 51 %, and 45 % of activity,

respectively, in pH 4, 6, 8, and 10. In fact, at 25 ºC, the enzyme kept above 95 % of the initial activity for pH 4, 6, and 8. At pH 10, the activity dropped to around 70 % after 5 hr of incubation. The stability at pH 10 was improved when the enzyme was incubated at 4 ºC, presenting still 84 % of activity after 5 hr.

CE was the most stable enzyme of the commercial preparation in all conditions studied, except at pH 4 where other enzymes were even more stable, keeping more than 80 % of the initial activity at pH 6 and 8, 76 % at pH 4, and 64 % at pH 10, after 5 hr at 45 ºC. At 25 ºC, the enzyme kept over 90 % under all the studied pH values, including pH 10. At pH 10 and 4 ºC the activity was almost 100 % after 5 hr.

Thus, in general, all enzymes have optimal stability at pH 4, only CE being reasonable stable at pH 10. In many cases the enzymes were too unstable even at 4 ºC when incubated at pH 10. Considering the relevance of this pH value in some immobilization protocols, we decided to analyze some ways to improve the enzyme stability under these conditions.

3.4. Influence of different additives in the stability of the different components of the protein extract at pH 10

The use of additives with stabilizing effects is reasonable to facilitate the handling of the enzyme, although it is not possible to use them in the reactions, as they will be incorporated to the juice. The stability of all components of this extract can be reasonable at pH 4 at room temperature; at pH 6 the crude can only be handled at 4 ºC. However, at pH 10 the problems were more serious, and this pH value may be necessary to immobilize the enzymes in glyoxyl agarose supports,[79,80] and the use of higher temperature (i.e., 25 ºC) is important to increase the enzyme support reactivity, to increase the number of enzyme supports bonds and that way, to have an intense multipoint covalent attachment.[81] Even if the support can immobilize an enzyme at neutral pH value, an alkaline incubation is convenient in

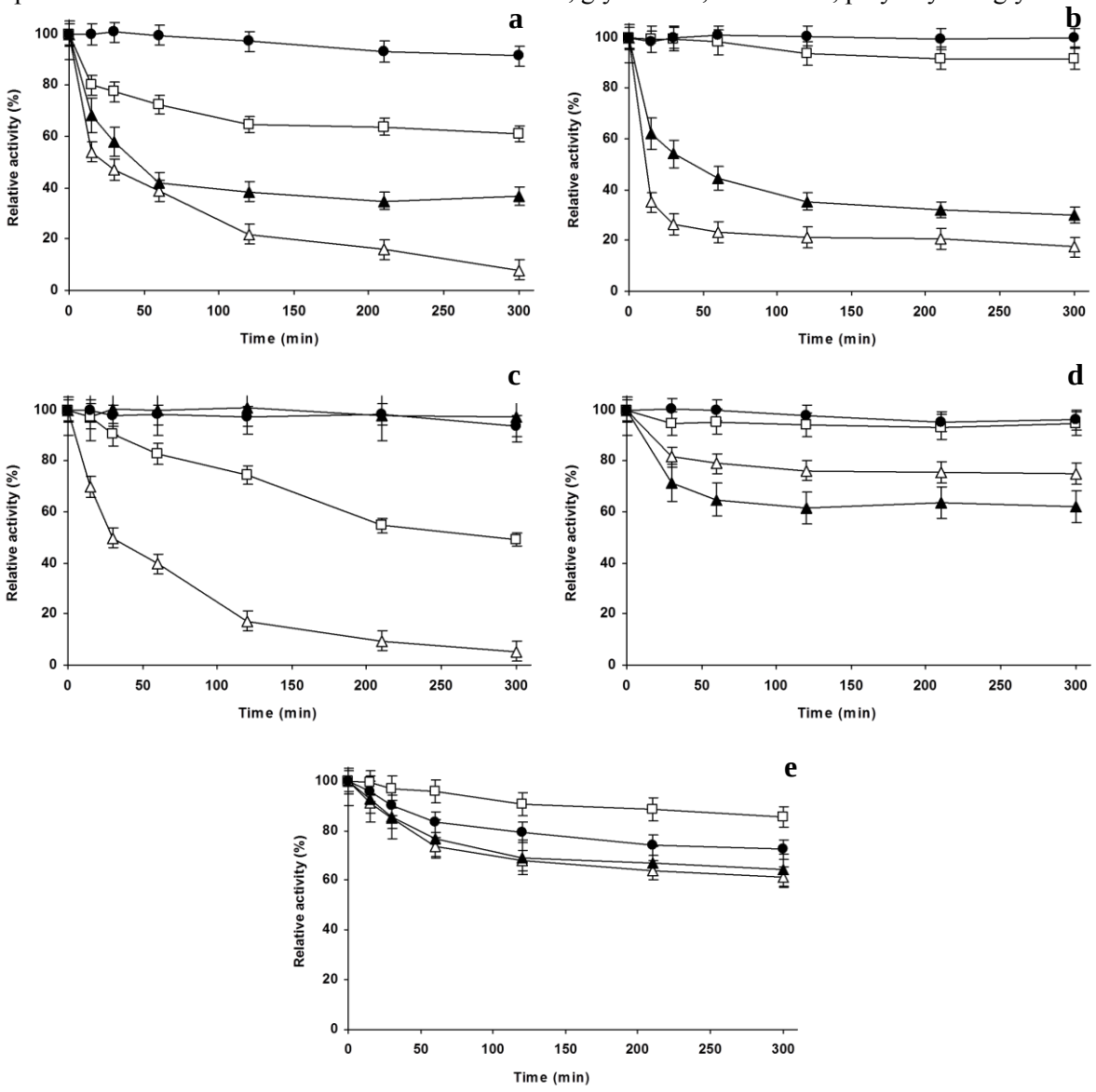
many instances to increase the enzyme-support reactivity, as for example, when using vinylsulfone activated supports.[82]

For these instances, it is possible to use some additives that can improve the enzyme stability at pH 10 at 25 ºC. However, if we desired to have a combi-biocatalyst having all the components of the extract, it is necessary that the additive does not present a negative effect on the stability of other enzyme components of the extract. Glycerin, dextran, and polyethylene glycol were analyzed (Figure 4).

Using PE, all the additives had a positive effect on enzyme stability. The effect of dextran was the lowest one, glycerin clearly stabilized the enzyme (e.g., after 5 hr of incubation, 61 % of the initial activity was maintained, while in absence of inhibitor the residual activity was under 10 %). However, the clearest effect was using polyethylene glycol, permitting to keep the enzyme activity almost unaltered after 5 hr.

In the case of PG activity, the enzyme was also stabilized for all additives. Again, the effect of dextran was very small, while polyethylene glycol gave the highest stabilization (99 % of activity) and glycerin in this case was also very positive (92 % of activity), after 5 hr of incubation. Using PL, again all additives produced a positive effect. While the enzyme without additives lost 95 % of the initial activity after 5 hr, the presence of dextran and polyethylene glycol permitted to maintain the enzyme activity almost unaltered. Glycerin permitted to maintain 50 % of the activity. For PME, the effect of dextran was even negative, while glycerin and polyethylene glycol increased enzyme stability, again almost preventing any a significant enzyme inactivation after 5 hr.

Figure 4. Inactivation courses of the PE (a), PG (b), PL (c), PME (d), and CE (e) with additives under conditions of pH 10 at 25 ºC and 45 ºC to CE. It was performed as described in the items 2.2 and 2.5. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Stabilizers: without stabilizers: Δ, glycerin: □, dextran: ▲, polyethylene glycol: ●



For the CE, the enzyme maintained the enzyme activity almost unaltered after 5 hr at 25 ºC, with or without stabilizers. However, this did not permit to state the reagent with the most positive effect for this specific enzyme. For this reason, the incubation temperature was increased to 45 ºC and the effect of the stabilizers was evaluated. Dextran had a small effect on enzyme stability, showing similar stability compared with the enzyme without additive (64 % of activity after 5 hr at 45 ºC), while glycerin and polyethylene glycol increased the

enzyme stability (72 % activity after 5 hr at 45 ºC), being glycerin the best stabilizer for this enzyme, (85 % initial activity under similar conditions).

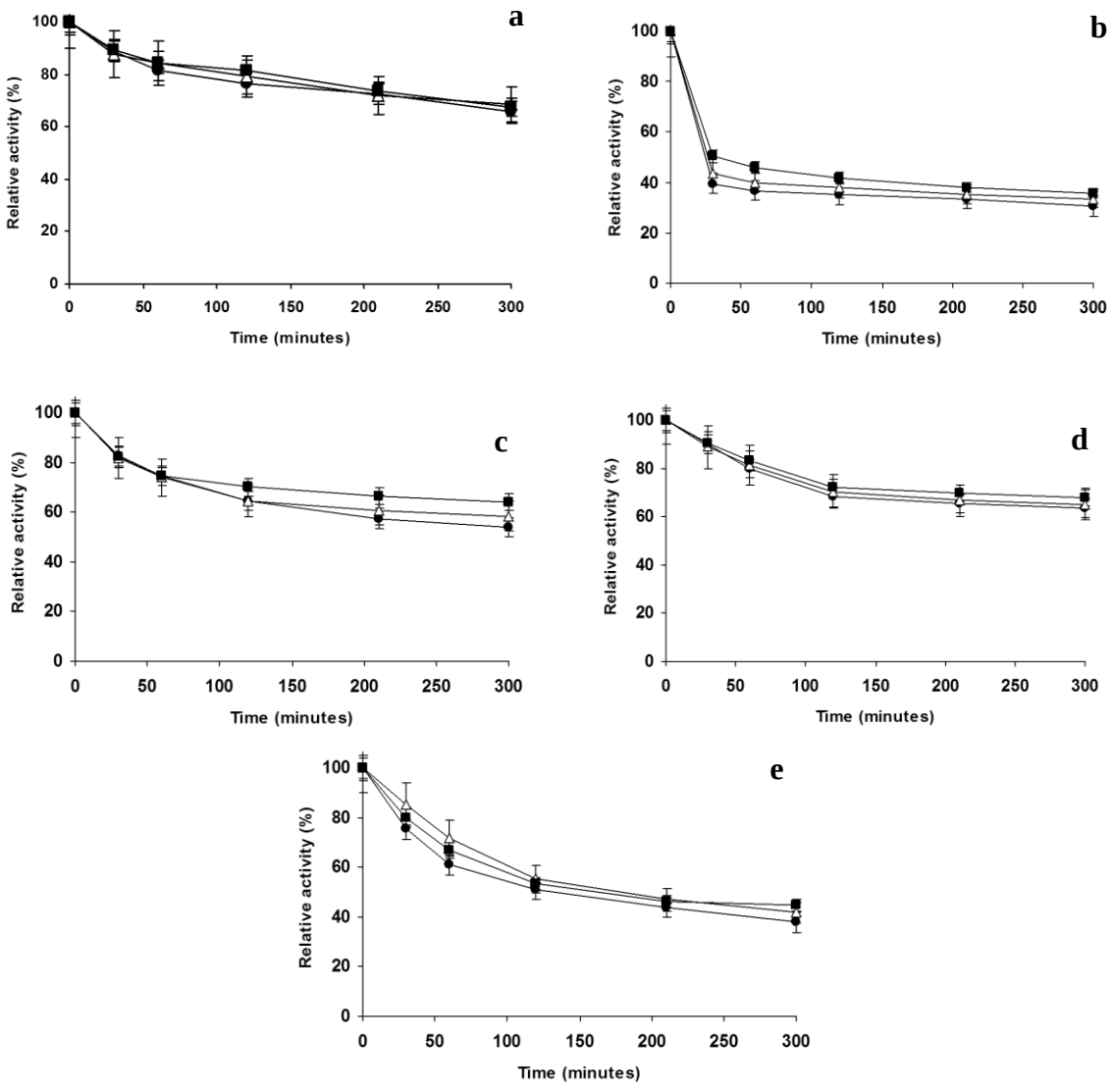
From these results, 20 % of polyethylene glycol seemed to be the ideal stabilizer, maintaining activities close to 100 % for all components of the extract, when these were incubated in pH 10 at 25 ºC. Nevertheless, glycerin presented good results for PG, PME, and CE (even under high temperature conditions) with activities close to 100 %, and gave reasonable stabilities for PE and PL with activities above 50 %. While the dextran was the ideal one for PL, for other components of the cocktail it produced a small improvement of enzyme stability (PE, PG, and CE), and even became negative for the stability of PME.

3.5. Influence of the enzyme concentration on the stability of the different components of the extract at pH

The effect of enzyme extract concentration on enzyme stability may come from different facts: if the enzyme is multimeric and the first step of the inactivation is enzyme dissociation (but in this case the enzymes are described to be monomeric),[83-87] if there are some enzyme–enzyme interactions with effect on enzyme stability (e.g., favoring inactivation by aggregation, or stabilizing the enzymes by forming some enzyme complexes), or if some component of the extract may have some effect on enzyme stability (some stabilizing reagent). To evaluate the effect of enzyme concentration on inactivation, two solutions with 1 % (0.05 $mg.mL^{-1}$ of protein) and 10 % (0.5 $mg.mL^{-1}$ of protein) of enzyme preparation were incubated at pH 10 at 4 ºC for PE, PG, and PL, at 25 ºC for PME and 50 ºC for CE. A third sample for each enzyme was prepared with 1 % enzyme preparation plus 9 % inactivated enzyme solution after boiling. We cannot find any significant difference on the inactivation courses of all these preparations (Figure 5). These results suggested that any enzyme dissociation process (subunit, metals, and other ions) is not the first step of the inactivation of

any of the components of the extract. Moreover, the extract seemed to not contain any stabilizer, as in that case a dilution should drive to a decrease in enzyme stability.

Figure 5. Inactivation courses of the PE (a), PG (b), PL (c), PME (d), and CE (e) in different concentrations under conditions of pH 10. It was performed as described in the items 2.2 and 2.5. All experiments were performed in triplicate and the values are given as mean value ± the experimental error. Sample: 1 % enzyme solution (0.05 mg.mL^{-1} of protein):●; 10 % enzyme solution (0.05 mg.mL^{-1} of protein):■; and 1 % of active enzyme (0.05 mg.mL^{-1} of protein) more 9 % of inactivated enzyme (0.5 mg.mL^{-1} of protein):Δ.



4. Conclusion

Rohapect 10L is an enzymatic extract bearing different activities including mainly pectinolytic and cellulolytic components. This extract is commercialized for juice clarification

and our results show that it is really quite focused on the conditions of most fruit juices: activity and stability data confirm that the set of enzymes contained in the extract exhibited maximal activities at pH near the usual conditions of fruit juices (acidic pH). This can make the handling of the extract at other pH values complex. For example, only CE is stable enough at alkaline pH, conditions necessary for enzyme multipoint immobilization. The use of polyethylene glycol showed to have positive effects for all enzymes involved, while dextran has low effects in general; however it was the most stabilizing agent in one case and was even negative for other case. Thus, the handling of this enzyme cocktail at conditions different from the target ones needs to be careful. This is important in the design of most immobilization strategies, as they use neutral or alkaline pH values.

Future work will include enzyme immobilization, as this way the enzymes can be reused many cycles, and also many other of their features may be improved, like stability, activity (mainly under harsh conditions), specificity, selectivity, or inhibition reduction.[19,20,88-90]

It should remarked that the immobilization of this extract means the coimmobilization of the five components, and this raises a kinetic advantage and also important problems: the operational stability of the biocatalysts will be marked by the stability of the least stable immobilized enzyme, all enzymes will be immobilized using the same chemistry, and the pore diameter of the support must permit to immobilize the largest of the enzymes.[25]

Moreover, immobilization may affect the activity of the different components in a different way, assuming that they have been optimized by the supplier for its main application, the final combi-biocatalyst hardly will have optimal activity/stability properties. The use of the individual enzymes may be better for building combi-biocatalysts, trying to use some of the recent strategies to coimmobilize enzymes that permit the reuse of the most stable enzymes when the least stable ones have been inactivated.[26,27,91,92]

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CAPÍTULO 6 – Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range

A partir dos resultados do trabalho anterior, utilizando o polietilenoglicol como agente estabilizante, a pectina liase (PL) foi imobilizada em diferentes suportes de agarose, variando o grupamento ativo para a ligação com a enzima, sendo as maiores atividades encontradas em MANAE ativada com glutaraldeído. Assim, neste trabalho foi avaliado diferentes condições de imobilização da PL em MANAE-glutaraldeído, buscando estudar a influência do pH e da força iônica sobre atividade e estabilidade da enzima. As imobilizações foram realizadas em pH 5, 6,5 e 8 e posteriormente incubada em pH 8 para aumentar as ligações entre enzima e suporte. Além disso, uma amostra foi imobilizada em pH 8 com alta força iônica (300 mM NaCl). Os biocatalisadores preparados foram caracterizados em relação as suas atividades recuperadas, estabilidades e potencial de reutilização. Os resultados estão apresentados no manuscrito a seguir, publicado na revista *Enzyme and Microbial Technology*, v. 132, 109397, 2020.

CAPÍTULO 6 – Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range

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Abstract

Pectin lyase (from Rohapect 10 L) was immobilized on glutaraldehyde supports at low ionic strength at pH 5, 6.5 or 8 and later incubated at pH 8 for 48 h. The activity recovery of the biocatalysts versus pectin was quite low, under 10 % for all of the immobilized biocatalyst at 20 °C. However, a high stabilization was found when the enzyme was immobilized at pH 5, (e.g., the immobilized enzyme kept 83 % of the activity when the free enzyme was fully inactivated (pH 4.8 and 55 °C in 5 h)). This biocatalyst increased the activity versus pectin in an almost exponential way when temperature increased until reach the maximum temperature used in the study (90 °C), conditions where the free enzyme was almost inactive. The immobilized biocatalyst was also active even at pH 9, where the free enzyme was fully inactive. This biocatalyst could be reused for pectin hydrolysis 5 times for 72 h reaction cycles at 40 °C maintaining more than 90 % of the initial activity.

Keywords: Pectin hydrolysis, diffusion limitations, steric hindrances, enzyme stabilization, enzyme hyperactivation under harsh conditions.

1. Introduction

The continuous increase in the consumption of natural fruit juices has attracted the attention of the industry to the investment in technologies that benefit their organoleptic quality [1,2]. In this sense, the application of commercial enzyme preparations (enzyme cocktails), is a crucial point to obtain clarified and stable fruit juices with a suitable extraction of compounds responsible for color and taste [3–6]. The target of this enzymatic treatment is the degradation of the long and complex structural polysaccharides of the fruit pulp, the main cause of juice turbidity [7,8].

Among these commercial enzymes cocktails, it is possible to highlight Rohapect 10 L (from AB Enzymes) [9]. Among their components, the pectin lyase from Aspergillus niger (a monomeric enzyme with a molecular weight of 36 kDa) is one of the main enzymes responsible for pectin hydrolysis, catalyzing β-elimination of the α-1,4 linkage in pecton, resulting in a galacturonate with an unsaturated bond between C4 and C5 at the non-reducing end of the formed galacturonic acid [10,11].

The industrial use of pectin lyase may be beneficed if the enzyme is in immobilized form, because the immobilized enzyme may be separated from the reaction medium and reused, if the biocatalyst is stable enough. However, nowadays the objectives of enzyme immobilization go beyond enzyme reuse, and try to increase enzyme stability, selectivity or specificity, purity or activity [12–20]. For example, a highly stabilized-immobilized enzyme may permit not only a greater number of enzyme reuses, but also to increase the range of conditions where the enzyme may be utilized. Stabilization after immobilization can be derived from different causes. For example, (i) any enzyme immobilized inside a porous support cannot aggregate [21], (ii) if all enzyme subunits of a multimeric enzyme are involved in the immobilization, the multimer cannot dissociate [22], (iii) if a specific microenvironment is generated around the enzyme, some deleterious compounds may be

secluded from the enzyme environments [23–26], (iv) if an intense multipoint covalent attachment is achieved, the enzyme structure will become more rigid [12,21,27,28].

In the case of pectin lyase, usually the immobilization on porous supports has a drastic negative effect on enzyme activity, because the large size of the pectin and the viscosity of the pectin solution can produce steric hindrances of the substrate te reach the active center of the enzyme if it is not properly oriented and substarte diffusional limitations in the entry of the substrate to the biocatalyst pores [29]. The immobilization on non-porous supports may eliminate the diffusion limitations [30–34], although the handling of nanomaterials (necessary to have suitable enzyme loadings immobilized only on external surfaces) in high volumes of viscous suspensions may become problematic. And in any case, if the enzyme orientation is not the appropriate (e.g., the enzyme active center is oriented towards the support surface), it is likely that the immobilized enzyme active center cannot even interact with pectin, at least in the first moments of the hydrolysis reactions, due to steric hindrances and the initial large size of the substrate [18,21]. Rohapect 10 L is a commercial enzyme extract supplied as aqueous solution and containing pectin lyase and other polysaccharides depolymerizing enzymes. Some features of the different enzymatic components of this enzyme extract have been recently reported [35].

To get a good stabilization after enzyme immobilization, the immobilization protocol is very important [20,21]. This includes the support [36], the active groups of the enzyme and support and the immobilization conditions and steps [37–39]. Pectin lyase from *Aspergillus niger* has been immobilized on different supports. For example, the enzyme has been immobilized on a nylon-polyethyleneimine copolymer support activated with glutaraldehyde [40]. Unfortunately, the immobilization yield was very low (8.6 %). Later, the enzyme was immobilized on epoxy activated supports, but also the immobilization yield was quite poor [41]. To improve the results, the same research group utilized a polyamide support that was

activated with glutaraldehyde or other activating reagents, but the immobilization results were not significantly improved [42]. Later on, this research group tried many different supports (cellulose, sulphides, γ-alumina, and bentonite XAD-amberlites) and immobilization strategies (physical adsorption or covalent bonds), improving the immobilization yields and with reasonable expressed activity, but the enzyme stabilization was scarce [43]. They compared epoxy-activated supports (Eupergit C) and glutaraldehyde-activated supports (Nylon 6), reaching the best results using the glutaraldehyde-activated support [44]. Later, the enzyme was immobilized on chitin (using glutaraldehyde to activate the support) and nylon supports, with activity retention under 20 % [45]. The enzyme has been also entrapped in alginate [46]. Activity recovery was competitive but stability was poor [46]. All immobilization protocols applied to this enzyme have some problems regarding immobilization yield (perhaps due to the glycosylation of the enzyme), expressed activity (usually under 20 %, perhaps due to the large size of the substrate) and immobilized enzyme stability (that was not significantly improved). Thus, we have considered that the immobilization of this enzyme still has some room for improvements.

As seen above, glutaraldehyde is the most used reagent for the immobilization of this pectin lyase [40,42,44,45]. Glutaraldehyde has revealed itself as a very interesting reagent for enzyme immobilization/stabilization, thanks to its great versatility [37,47,48], although the exact mechanism of the glutaraldehyde reaction versus proteins is not fully understand [48]. And this versatility was not utilized in any of the previous reports. The versatility comes from the fact that usually glutaraldehyde supports are prepared from supports bearing primary amino groups: therefore, they are heterofunctional supports [49]. That way, the enzyme is immobilized in a support having cationic groups (that is, it is an anion exchanger) and a chemically reactive moiety [37]. However, usually the direct covalent reaction is far slower than the ion exchange [49–53].

Moreover, it is possible to adsorb the enzymes on the anion exchanger and later modify the already immobilized enzyme with glutaraldehyde or to pre-activate the support with glutaraldehyde and to immobilize later the enzyme [49–54]. When modifying previously adsorbed enzyme, the objective is to introduce one glutaraldehyde molecule per amino group in the support and enzyme, as these aminoglutaraldehyde groups are very reactive among them [55]. In pre-activated supports, two glutaraldehyde molecules per amino group yield optimal reactivity versus amino groups in the protein [56]. This is easily controlled by the glutaraldehyde concentration, pH and time during the support activation step [56].

The glutaraldehyde modification of the adsorbed enzyme implies the full modification of the enzyme surface with glutaraldehyde (and this may have positive or negative effects on enzyme stability/activity) while the pre-activated supports only modify the enzyme groups involved in the immobilization [49–53]. Usually, when possible, the modification of previously ionically exchanged enzymes produces a higher stabilization, although this is not a fully general rule [29,49–54]. It must be also considered that the immobilization via ion exchange may offer very different results depending on the immobilization pH, as the enzyme orientation may be different [54,57]. In some instances, the cooperativity of ion exchange and covalent immobilization permits the full immobilization of the enzyme when each of them in an individual way are unable to give significant immobilization yields [52].

In this paper, pectin lyase, which uses the pectin as substrate (a large molecule), will be immobilized using the versatility of glutaraldehyde chemistry on aminated agarose. Agarose is a good support for enzyme immobilization, as it is inert in absence of any modification, that way the groups that interact with the enzyme are only those introduced by the researcher [58]. Using the advantages of glutaraldehyde chemistry, we expect to improve the activity recovery, which is usually low for this enzyme, as well as stability and activity under harsh conditions. The effect of different immobilization pH values on the immobilized

enzyme properties will be studied. The activity and stability in a wide range of pH and temperature conditions, as well as biocatalysts operational stabilities in pectin hydrolysis will be also studied. These wide studies cannot be found in the previous papers [40–46].

2. Materials and methods

2.1. Materials

The enzyme preparation used was Rohapect 10 L from AB Enzymes (Darmstadt, Germany). The extract contains 5.4 $mg.mL^{-1}$ of protein and 44.68 $U.min^{-1}$ of pectin lyase activity, determined according to the methodology described below. Sodium periodate, pectin from apple, sodium borohydride, 25 % (v/v) glutaraldehyde aqueous solution, ethylenediamine and glycidol were acquired from Sigma-Aldrich (St. Louis, MO, USA). 4 BCL Agarose beads were purchased from Agarose Bead Technologies (Madrid, Spain). All other reagents and solvents were of analytical grade.

2.2. Determination of pectin lyase activity

Pectin lyase (PL) activity was determined spectrophotometrically measuring the increase in absorbance at 235 nm promoted by the formation of unsaturated products, as described by Albershein [59], with slight modifications. Briefly, 0.2 mL of the diluted enzyme was added to 2.3 mL of pectin solution (2 $g.L^{-1}$) prepared in 50 mM sodium citrate at pH 4.8 and incubated at room temperature during 1 min, under agitation. One PL unit was defined as μmols of unsaturated uronide (ε=5500 $M^{-1}.cm^{-1}$ at 235 nm) produced per minute and mg of enzyme under the reaction conditions.

2.3. Preparation MANAE support

Monoaminoethyl-N-aminoethyl (MANAE) agarose beads were prepared starting from a glyoxyl support [38] and modifying it with ethylenediamine. For this reaction, 4 mL of 2.2 M ethylenediamine at pH 10.05 per gram of glyoxyl agarose beads were mixed and submitted to mild stirring for 24 h. After this, 10 $mg.mL^{-1}$ of solid sodium borohydride was added to reduce the supports, rinsed with high 100 mM sodium carbonate and sodium acetate buffers (pH 9 and 5 respectively) and finally with water [53,60].

2.4. MANAE activation with glutaraldehyde

The support was activated with glutaraldehyde by adding 10 g of support to 50 mL of a 10 % (v/v) glutaraldehyde solution in 200 mM sodium phosphate buffer at pH 7 and 25 °C [51,53] and left overnight under mild stirring conditions. This protocol enabled to introduce two glutaraldehyde molecules per primary amino group in the support [37,56].

2.5. PL immobilization

The immobilization of PL was performed on MANAE agarose beads, activated or not with glutaraldehyde. Subsequent to the preparation of the supports, 1 g of them was added to a solution containing 1 mL of the enzyme preparation (5.4 mg protein) and 9 mL of 5 mM sodium acetate (pH 5) or 5 mM sodium phosphate (at pH 6.5 and 8) containing 20 % polyethylene glycol as stabilizer [35]. The immobilization occurred at room temperature in a roller mixer to maintain a mild and continuous stirring. After immobilization, the biocatalysts were incubated at pH 8 for 48 h to permit an increase in the number of enzyme linkages with the support. The enzyme was also immobilized at pH 8 using 5 mM sodium phosphate/300 mM NaCl. The immobilization courses were monitored by following the enzymatic activity of the supernatants, suspensions and references with the enzyme under identical conditions to

that of the immobilization suspension, by using inert agarose. At the end of the immobilization process, the biocatalyst was filtered, rinsed with 50 mM sodium citrate buffer at pH 4.8 and stored at 4 °C. The recovered activity was calculated according to the following equation:

$$\text{RA}\ (\%) = \frac{\text{Pectin lyase activity in biocatalyst (U)}}{\text{Initial Pectin lyase activity (U)}} \text{ x } 100 \qquad (1)$$

2.6. Thermal inactivation of different PL preparations

The thermal inactivation of the soluble and immobilized enzymes was studied by incubating the biocatalysts in buffered solutions at different pHs, ranging from 4.0 to 8.0, using 50 mM sodium citrate (pH 4–6) or sodium phosphate buffers (pH 6–8), at different temperatures, ranging from 35 °C to 60 °C, according to the pH used to select inactivation velocities that offered reliable inactivation courses. Periodically, samples were withdrawn and the PL activity was measured.

2.7. PL activity at different pH values and temperatures

The effects of the pH and the temperature on the enzyme activity of soluble and immobilized biocatalysts were determined using pectin as substrate (see above). The temperature was varied from 30 °C to 90 °C, using 50 mM sodium citrate at pH 4.9. The effect of the pH on enzyme activity was studied at room temperature using 50 mM sodium citrate pH 4–6, 50 mM sodium phosphate (pH 6–8) and 50 mM sodium carbonate buffers at pH 9.

2.8. Operations stability of the immobilized biocatalysts

To evaluate the efficiency in pectin hydrolysis of the different enzyme formulations, free enzyme or the enzyme immobilized at pH 5 were incubated in a 0.5 % (w/v) pectin solution prepared in 50 mM sodium citrate buffer at pH 4.8 and 70 °C, where the rate of unsaturated uronide formation by pectin hydrolysis was monitored as previously described.

Then, to evaluate the reuse of immobilized biocatalysts, for each 1 mL of 0.5 % (w/v) pectin solution in 50 mM sodium citrate buffer (pH 4.8) was added 0.5 g of biocatalyst. The reaction was conducted at 40 °C for 72 h when total pectin hydrolysis was obtained. After the reaction, the biocatalyst was filtered, washed with 50 mM sodium citrate at pH 4.8 and used in a new hydrolysis cycle.

3. Results

3.1. Immobilization of PL on MANAE supports

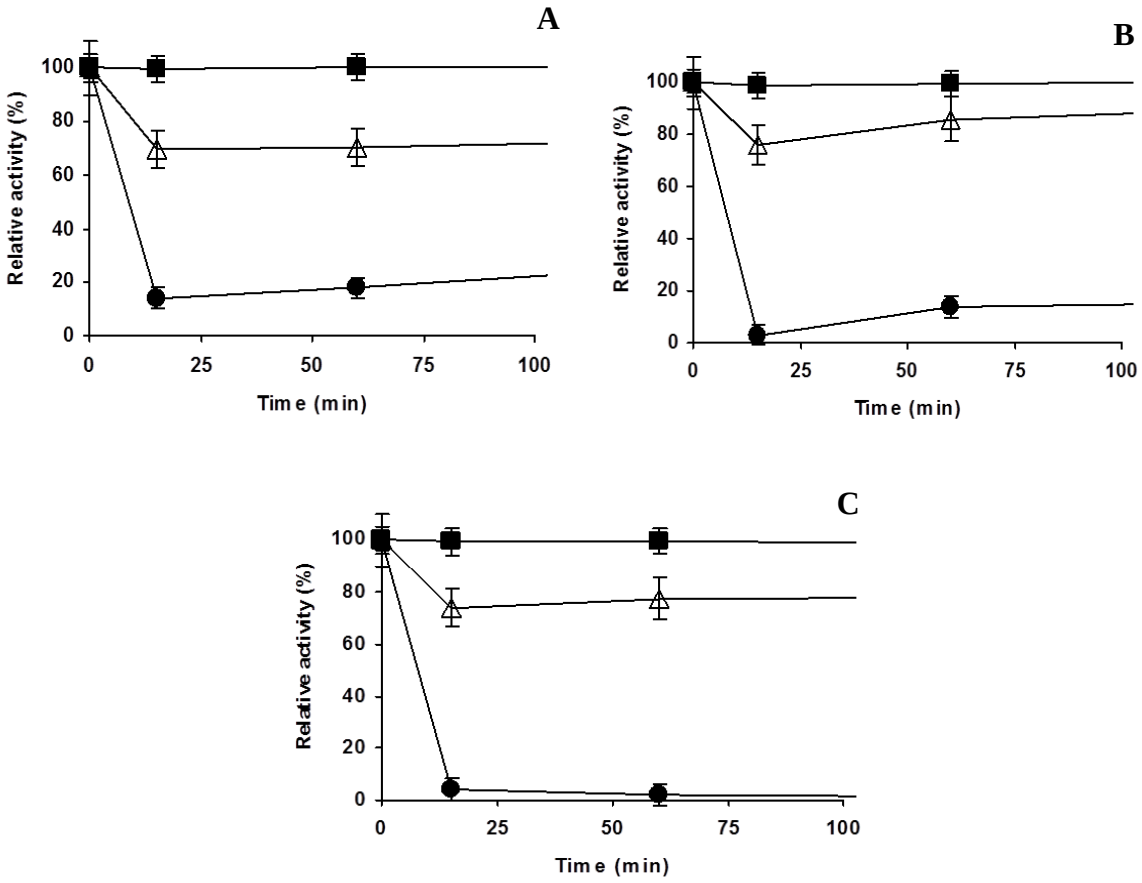
Immobilization of PL on MANAE agarose beads was very inefficient at pH 5 and 6.5. However, at pH 8 the immobilization was complete after some minutes and the enzyme remained in the support (Fig. 1). This should be related to the distribution of the ionic groups of the enzyme (that have an isoelectric point under 6) [61], as the support cationic character is lower at pH 9 (the pK of the MANAE is 6.9 (for the primary amino groups) and 10.2 (for the secondary amino group)) [60].

Activity recovery was apparently very high, but we checked that this high activity was due to the enzyme desorption during the activity measurement at pH 4.8. In fact, after washing with water the activity of the biocatalysts become almost 0.

In order to prevent enzyme desorption, the immobilized enzymes were incubated with 1 % of glutaraldehyde to have covalent bonds, but the activity almost fully disappeared. It was checked that the incubation of the free enzyme with glutaraldehyde had a similar very

negative effect on enzyme activity. Therefore, the strategy of adsorption plus glutaraldehyde treatment was unsuitable for this enzyme.

Figure. 1. Effect of pH value on the immobilization course of PL on MANAE agarose beads pH 5 (A), pH 6.5 (B) and pH 8 (C). Experiments were performed in triplicate and the values are given as mean value ± the experimental error as described in Section 2.5. PL activity was measured on reference (■), suspension (Δ) and supernatant (●).



3.2. Immobilization of PL on glutaraldehyde-MANAE agarose beads

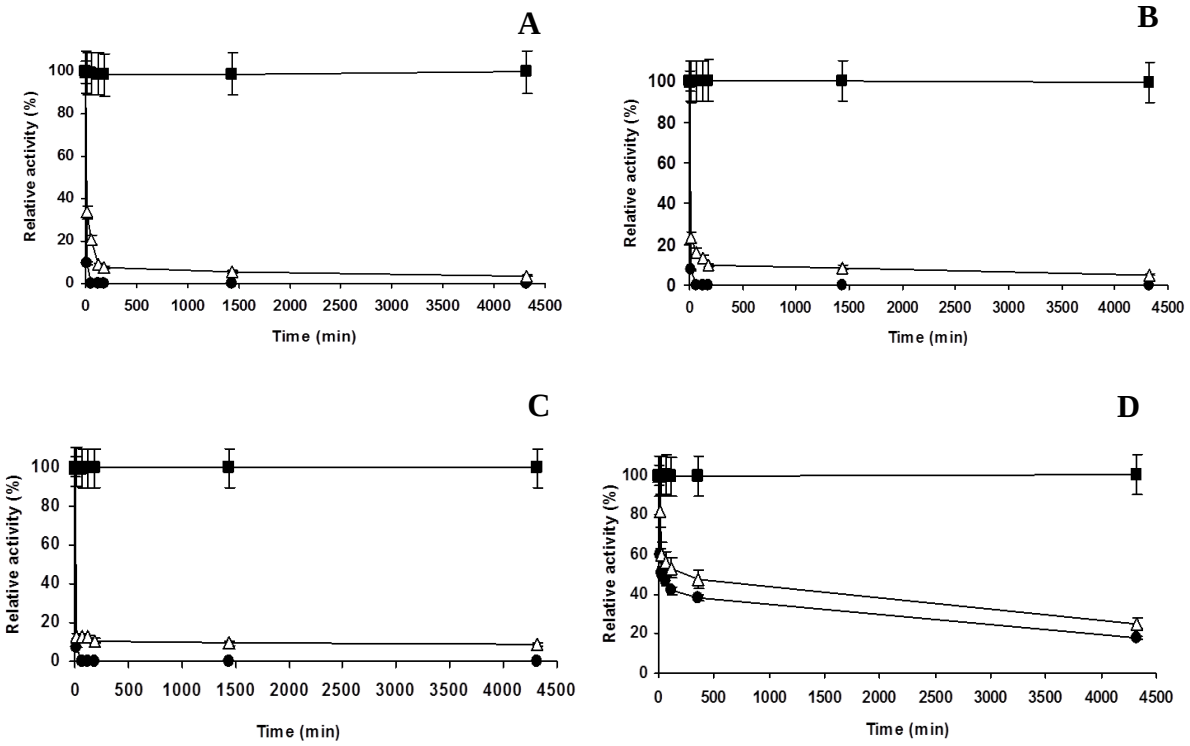
Fig. 2 shows that the immobilization of PL on glutaraldehyde preactivated supports was quite rapid at pH 5–8. However, the immobilization at pH 8 with high ion strength (300 mM NaCl) to prevent ion exchange almost stopped after 30 min of immobilization, becoming very slow after this time with a large amount of free enzyme remaining in the solution (Fig. 2 D). The faster immobilization at low ionic strength should be related to the cooperative effect

of ion exchange and covalent immobilization [52], the covalent immobilization of the enzymes adsorbed produced a shift in the enzyme adsorption/desorption equilibrium and permitted the final complete enzyme immobilization.

However, the expressed activity was quite poor just after immobilization, around 13 % if the enzyme was immobilized at pH 8 or around 8 % if the enzyme was immobilized at pH 5. Further incubation at pH 8 to permit an increase in the number of enzyme support linkages produced biocatalysts with expressed activities ranging from 9 % for the enzyme immobilized at pH 8 to 4 % for the enzyme immobilized at pH 5.

The first drop in activity may be caused by the generation of substrate steric hindrances and substrate diffusion limitations, due to the substrate large size and viscosity of the substrate solution [1,29]. The further decrease in enzyme activity may be related to the promotion of some additional reaction between enzyme and support, that is, a multipoint covalent attachment may be generated, and this may have as a positive effect an increase in enzyme stability [20]. The difference in enzyme activity when immobilized at different pH values should be related to the different orientation of the enzyme on the support, that modulate the substrate access to the active center and the different reactivity of the enzyme with the support [54,57]. Curiously, the highest activity is recovered when immobilization is performed under the hardest conditions for the enzyme, at pH 8, and where the enzyme support reaction should be maximized by increasing the reactivity of the enzyme primary amino groups. These results are different to other results published in literature with optimum immobilization pH varying between 4 and 4.5 [29,62–64].

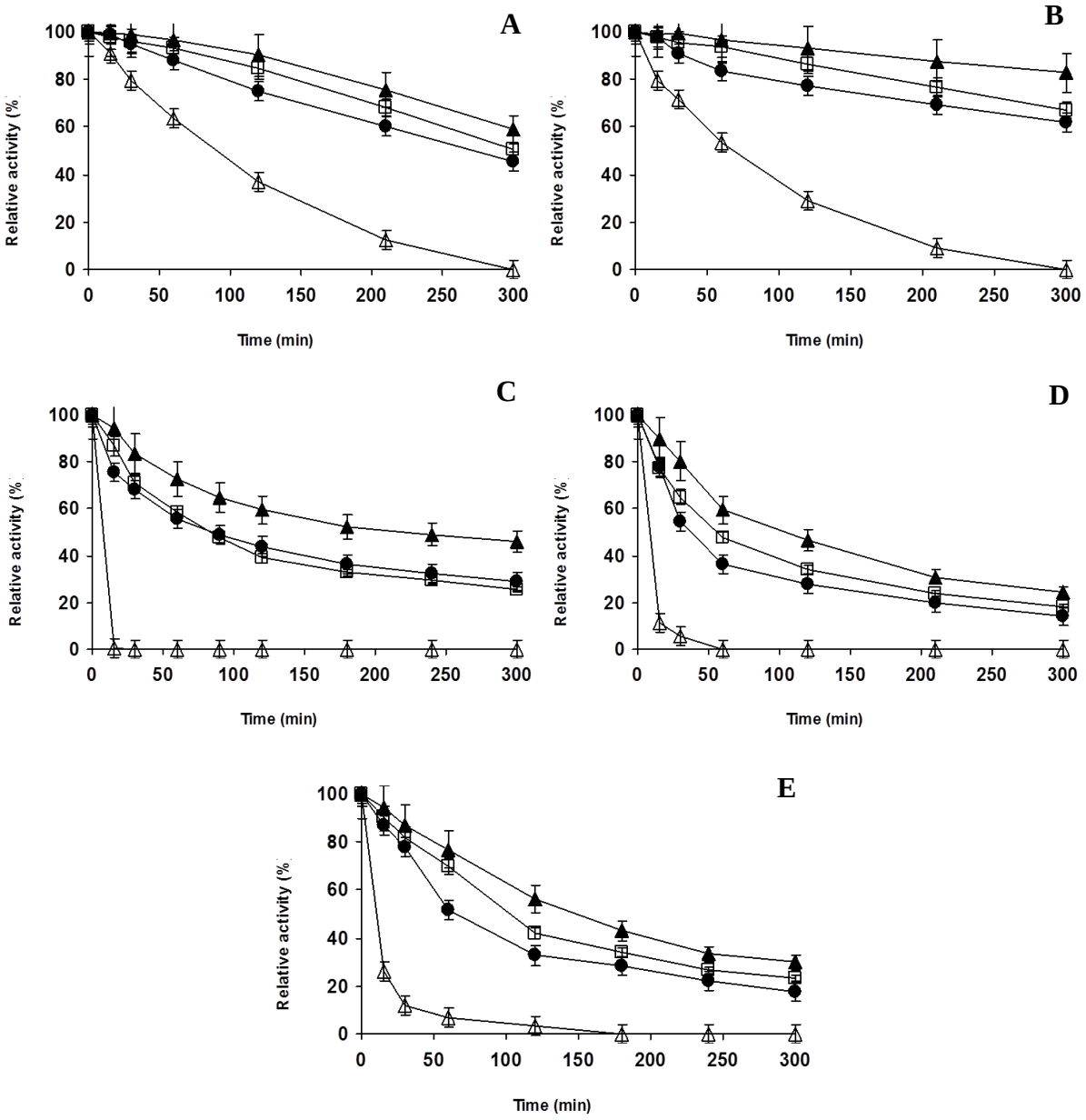
Figure 2. Effect of experimental conditions (pH value and ion strength) on the immobilization course of PL on glutaraldehyde-MANAE agarose beads, pH 5 (A), pH 6.5 (B), pH 8 (C) and pH 8 with 300 mM NaCl (D). The immobilizations were carried out as described in methods. Experiments were performed in triplicate and the values are given as mean value ± the experimental error as described in section 2.5. PL activity was measured on reference (■), suspension (Δ) and supernatant (●).



3.3. Thermal stability of the immobilized enzymes

Fig. 3 shows the thermal inactivation of the different preparations compared to the free enzyme at different pH values. Note that temperature should be decreased when the pH increased to have reliable inactivation courses. As expected, the enzyme showed higher stabilities at more acidic pHs, in which higher temperatures were used to observe the activity decrease, while lower stabilities were observed at neutral and basic pHs, in which a more pronounced activity decrease was observed even at lower temperatures.

Figure 3. Inactivation courses under different pH values and temperatures of the soluble enzyme (Δ) and enzyme immobilized at pH 5 (▲), pH 6.5 (□) or pH 8 (●).The immobilization was performed as described in methods. Experiments were carried out in triplicate and the values are given as mean value ± the experimental error as described in Section 2.6. Conditions: pH 4 at 60 °C (A); pH 5 at 55 °C (B); pH 6 at 50 °C (C); pH 7 at 40 °C (D); pH 8 at 35 °C (E).



However, at all studied pH values the immobilized enzymes were far more stable than the free enzyme and the enzyme immobilized at pH 5 was the most stable whereas the enzyme immobilized at pH 8 was the least stable immobilized biocatalyst. For example, at pH 4.8 and 55 °C, the free enzyme was completely inactivated when the most stable preparation,

immobilized at pH 5, retained more than 80 % of the activity and the enzyme immobilized at pH 6.5 and 8 showed a very similar residual activity (around of 60 %) (Fig. 3A). Similar results were found at other pH values, where the inactivation at pH 8 and 35 °C showed a pronounced decrease of the free enzyme activity in the first minutes of incubation, while the biocatalyst immobilized at pH 5 still presented 30 % of activity, followed by the biocatalyst at pH 6.5 (23 %) and pH 8 (18 %), after 5 h of inactivation (Fig. 3D).

For all the conditions tested, the biocatalyst prepared at pH 5 presented the highest stability, while the biocatalyst prepared at pH 8 showed the lowest, although much higher than that of the free enzyme. This may be related to a different orientation of the enzyme on the support depending on the immobilization pH, as the 48 h of incubation at pH 8 should permit similar levels of enzyme-support reaction [57].

Thus, even if the enzyme immobilization caused a drastic decrease in enzyme activity, its stability was quite improved. However, it is not easy to justify for applied purposes this great reduction of activity after immobilization just by the high stability. Nevertheless, the situation may be different if this stabilization really increases the range of conditions where the enzyme may be used exhibiting a reasonable activity.

3.4. Effect of pH and Temperature on the activity of different PL preparations

Fig. 4A shows the activity/temperature curve for free and immobilized PL at pH 4.8. The free enzyme reached a maximum of activity at 60 °C, decreasing at 70 °C by almost 50 % and was almost inactive at 90 °C. The behavior is very different for the immobilized preparations; activity increased continuously until to the maximum temperature tested (90 °C). The increase in the activity was very high at very high temperature, perhaps because there were several positive factors in this reaction acting together when the temperature increased, if the enzyme was stable at these temperatures. At high temperatures, the viscosity

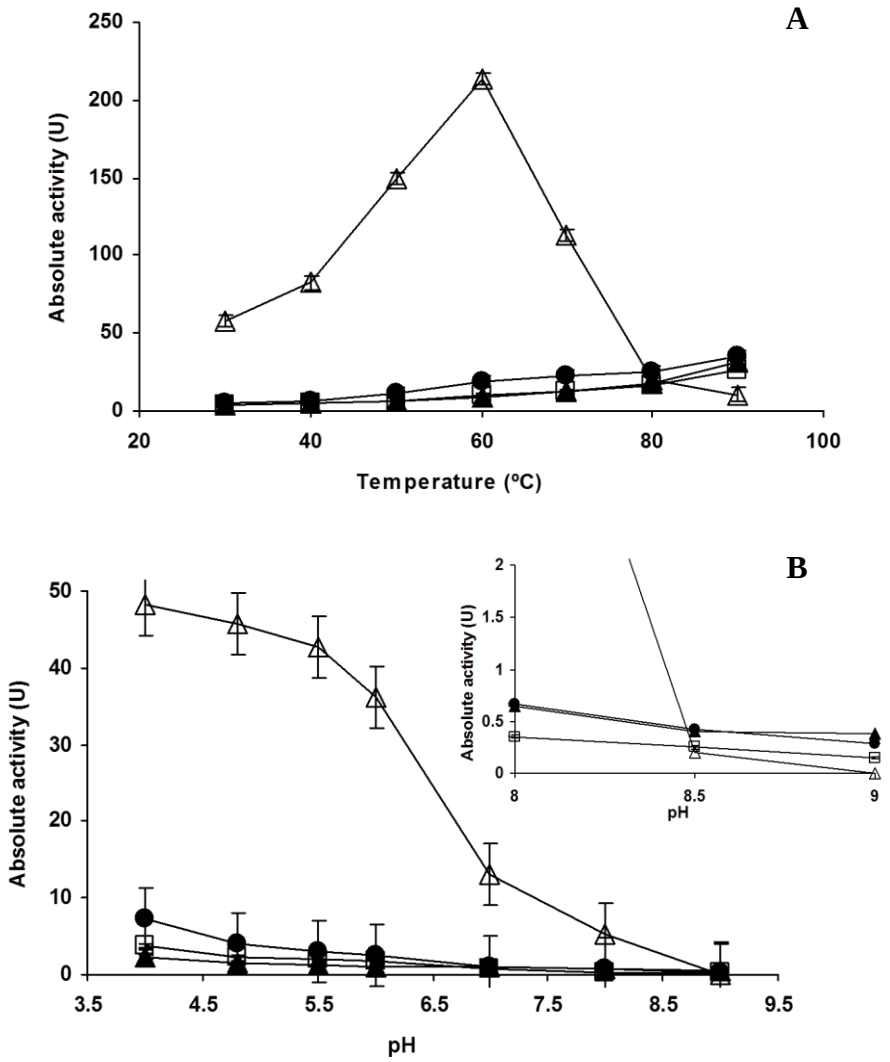
of the pectin solution decreases and the mobility of substrate inside the support pores will be increased, decreasing diffusional and steric limitations, thus the catalytic capacity of the immobilized enzyme greatly increased. Consequently, at 80 °C the activity of the immobilized enzyme was similar to that of the free enzyme, and at 90 °C, the activity of the immobilized biocatalyst was almost twice that of the free enzyme. That way, at 90 °C the PL activity after immobilization not only did not decrease, but significantly increased. The activity at 90 °C was higher when the enzyme was immobilized at pH 8, followed by the enzyme immobilized at pH 5 and the lowest activity was exhibited by the enzyme immobilized at pH 6.5. However, the differences between the enzymes immobilized at pH 5 and 8 were much reduced at this temperature than at 20 °C.

Fig. 4b shows the effect of pH on the enzyme activity. Optimal activity is found at the lowest assayed pH value (pH 4) for all enzyme preparations, characteristic that makes the biocatalysts excellent for application in fruit juices. However, the most remarkable fact is found when focusing at alkaline pH values, showing that these immobilized biocatalysts may also be an option for processes that use higher pHs, such as degumming and retting of fiber crops, paper making and pretreatment of pectic wastewater [11]. The free enzyme was fully inactive at pH 9, while the immobilized enzymes still exhibited a significant activity. Moreover, at pH 9, the enzyme immobilized at pH 5 become more active than the enzyme immobilized at pH 8.

Thus, although the effects of the immobilization were very negative for enzyme activity under mild conditions, this was not the case if the conditions were more drastic, and the immobilized enzyme exhibited some activity under conditions where the free enzyme was fully inactive. This is a good example on how the effect of immobilization on enzyme activity may be fully different depending on the measurement conditions, measuring at pH 4.8 and 25 °C the effect of immobilization is very negative, while measuring at 90 °C or at pH 9, the

effect become positive [18]. This is not only an example on how the enzyme immobilization may enlarge the range of conditions where the enzyme may be used, but also may have some applied repercussions as explained above.

Figure 4. Effect of Temperature (A) and pH (B) on the activity of the soluble enzyme (Δ) and enzyme immobilized at pH 5 (▲), pH 6.5 (□) or pH 8 (●). The hydrolysis of 0.2 % pectin in citrate was performed as described in methods. Experiments were performed in triplicates and the values are given as mean value ± the experimental error as described in Section 2.7.

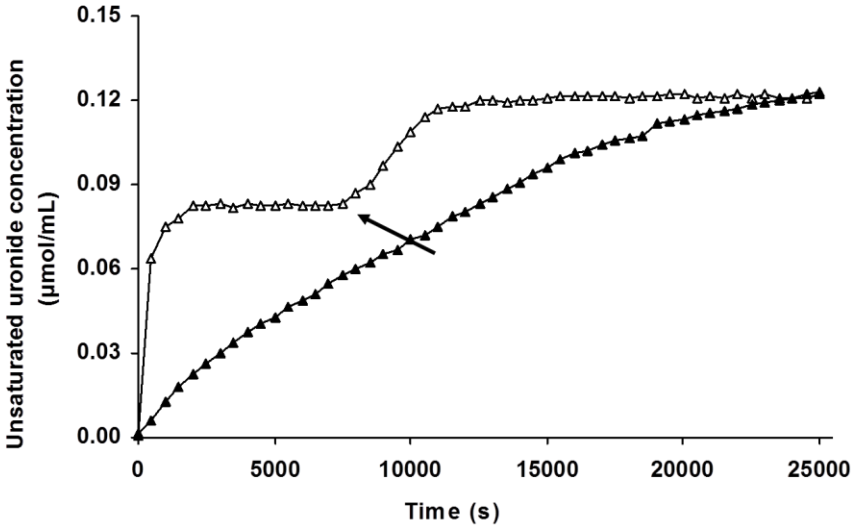


3.5. Operational stability of the optimal immobilized PL biocatalyst

Fig. 5 shows the reaction course of pectin modification at pH 4.8 and 70 °C catalyzed by free and PL that has been immobilized at pH 5. It is clear that the initial rate of the reaction was much higher using the free enzyme. However, after some time the reaction course started

to show a slowdown, until a reaction full stop. It was added a new amount of fresh enzyme at this moment, and the reaction re-started over, suggesting that the reason for the reaction stops was the enzyme inactivation and not an enzyme inhibition. Using the immobilized enzyme, the initial rate was much slower, but the reaction course was very linear. This shows that while the free enzyme became inactivated during the reaction, the immobilized enzyme was able to stand under these drastic conditions for a long time. This way, the use of free enzyme makes necessary a step wise addition of enzyme along the reaction, while the immobilized enzyme allowed reaching the end of the reaction.

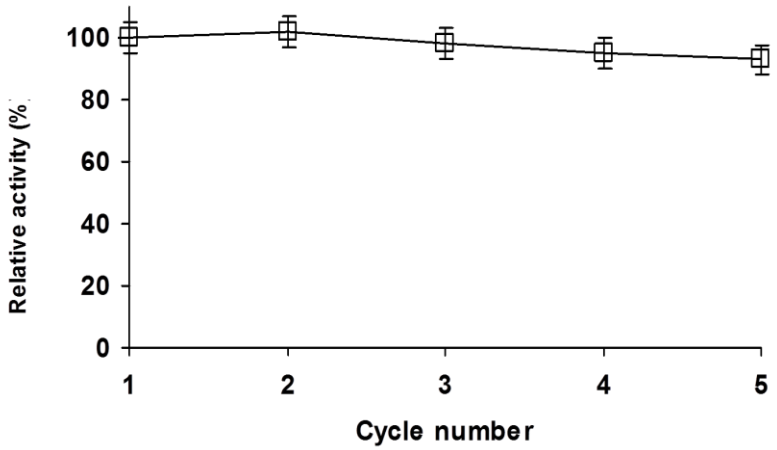
Figure 5. Reaction course of pectin modification by soluble PL (Δ) and PL immobilized at pH 5 (▲). 2.5 mL of 0.5 % pectin in 50mM sodium citrate buffer (pH 4.8) at 70 °C was incubated with 10 mg of the biocatalyst as described in methods. The arrow indicates the addition of fresh enzyme when using free enzyme.



Reusability of immobilized enzymes is an important parameter for identifying a suitable immobilized enzyme and it is one of the main reasons for enzyme immobilization. As it can be seen in Fig. 6, the immobilized enzyme at pH 5 can be reused for 5 cycles of total pectin hydrolysis at pH 4.8 at 40 °C (72 h each cycle), maintaining more than 90 % of the initial activity, thus showing that immobilization of pectin lyase on glutaraldehyde-activated agarose may be a suitable strategy for the preparation of a biocatalyst with high potential for

application in pectin hydrolysis. This reusability can be attributed to the strong stabilization achieved after immobilization and the good properties of the utilized enzyme [65].

Figure 6. Operational stability of enzyme immobilized at pH 5. The reaction was carried out by following the hydrolysis of 0.5% pectin in 50mM sodium citrate buffer at pH 4.8 and 40 °C for 72 h, as described in Methods section.



4. Conclusions

As may be expected from the large size of the substrate and medium viscosity, immobilization of the enzyme in a porous support produced a decrease on the enzyme activity. The results obtained in this research are similar to those described in literature regarding expressed activity but significantly improve the previously reported immobilization yields. Moreover, the new biocatalysts significantly overpass the previously reported stability results. Our immobilized biocatalysts have a perfect operational stability and activity under drastic conditions that the other studies did not report [40–46]. The first immobilization pH plays an important role in both, recovered activity and stability, and this variable in not considered in any of the previous papers.

The immobilization also produced an increase in enzyme stability. That makes that the range of conditions where the enzyme may be used is greatly enlarged: at 90 °C, the

immobilized enzyme continue increasing its activity compared to lower temperatures while the free enzyme is almost fully inactive.

The effect of immobilization is even clearer if focusing on the effect of the pH value on enzyme activity, the immobilized PL is active at pH 9, conditions where the free enzyme is fully inactive. The immobilization of PL on glutaraldehyde supports is a clear example on how a partial study of the biocatalysts properties may drive to wrong conclusions. If we only analyze the activity at 25 °C and at acidic pH value, the immobilized PL looks with a very poor activity recovery, while at higher temperature the immobilized enzyme becomes more active than the free enzyme. At pH 9, it looks that the extract immobilization induces a "new activity" on the extract, as under these conditions the free enzyme is fully inactive. Thus, an overall study of the biocatalyst properties is the only way to understand the real effects of immobilization on them, the use of just a specific activity determination condition can drive to very wrong conclusions, sometimes negative, sometimes positive, but equally false.

All authors certify that they have seen and approved the final version of the manuscript being submitted. We warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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CAPÍTULO 7 – Optimized immobilization of polygalacturonase from *Aspergillus niger* following different protocols: Improved stability and activity under drastic conditions

Na mesma linha de experimentos do trabalho anterior, neste artigo, a imobilização da poligalacturonase (PG) foi avaliada em suportes glioxil-agarose, vinilsulfona-agarose, MANAE e MANAE-glutaraldeído. Novamente, visto os melhores resultados alcançados em MANAE-glutaraldeído, a imobilização neste suporte foi estudada mais detalhadamente, variando as condições de pH, entre pH 5, 6,5 e 8, as quais foram posteriormente incubadas em pH 8 para aumentar as ligações entre enzima e suporte. Além disso, uma amostra foi mantida em pH 5 até o final da imobilização e outra amostra foi imobilizada em pH 8 com 300 mM de NaCl. Os biocatalisadores obtidos foram caracterizados em relação as suas atividades recuperadas, estabilidades e potencial de reutilização. Os resultados estão apresentados no manuscrito a seguir, publicado na revista *International Journal of Biological Macromolecules*, v. 138, p. 234-243, 2019.

CAPÍTULO 7 – Optimized immobilization of polygalacturonase from *Aspergillus niger* following different protocols: Improved stability and activity under drastic conditions

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Abstract

Polygalacturonase (PG) from *Aspergillus niger* was immobilized using glyoxyl, vinylsulfone or glutaraldehyde activated supports. The use of supports pre-activatedwith glutaraldehyde presented the best results. The immobilization of PG on glutaraldehyde-supports was studied under different conditions: at pH 5 for 24 h; at pH 5, 6.5 or 8 for 3 h and then incubated at pH 8 for 24 h; at pH 8 in the presence of 300 mM NaCl for 24 h, to prevent ion exchange. The immobilization under all conditions showed a significant increase in the enzyme thermal stability under inactivation conditions at pH 4–10. As a result, at temperatures over 70 °C or pH values over 7, the immobilized PG maintained significant levels of activity while the free PG was fully inactivated. The immobilization conditions presented a clear effect on enzyme activity, thermostability and operational stability, suggesting that the different conditions permitted to get immobilized PG having different orientations. Varying the immobilization protocol it is possible to achieve high activity or stability, and the optimal biocatalyst depends on the conditions where it will be utilized. The immobilized PG biocatalysts could be reused 10 times without a significant decrease in enzyme activity and offered very linear reaction courses.

Keywords: Glutaraldehyde, enzyme immobilization, enzyme stabilization, enlarging utilization range, enzyme orientation.

1. Introduction

Polygalacturonases (PGs) act on pectic acid hydrolyzing the α-1,4 bond between two non-esterified galacturonic acid residues [1,2]. They can be endo-PGs or exo-PGs [1,2]. Fungal PGs have high activity and usually have optimum activity at slightly acidic pH values and optimum temperature between 30 and 50 °C [3,4]. These enzymes prefer substrates with low degree of pectic acid methylation [3–5]. PGs are usually monomeric with a molar mass close to 35 kDa [6,7]. They have been used in the beverages industry for juice clarification. The addition of PGs in fruit and vegetable juices reduces their viscosity and gives a crystalline appearance to the final product, in addition to increasing the juice yield and reducing the filtration time by up to 50 % [3,4,8,9].

Immobilization of the enzyme may help in its industrial implementation. A proper immobilization may improve enzyme stability and activity, and tailor enzyme selectivity or specificity [10–15]. Moreover, it can reduce inhibition problems, resistance to chemicals or improve enzyme purity [16–18]. The immobilization of enzymes that act versus large substrates, like cellulose, starch or pectin, presents one additional requirement to be considered; if the enzyme active center is oriented towards the support surface, the enzyme may be inactive by steric hindrances even if the active center is fully functional [11,19]. Therefore, enzyme orientation during immobilization is critical for these enzymes.

To take full advantage of immobilization, an appropriate design is required [20–24]. Immobilization should permit a high enzyme-support reaction to yield an intense multipoint covalent attachment, and thus to increase enzyme rigidity [17,19,25,26]. In order to reach this intense multipoint attachment, all aspects of the immobilization must be considered.

First, the support [27]. It must offer a suitable geometrical congruence to allow the enzyme-support multi-interaction. Moreover, the final surface of the support should as inert as possible [27]. Agarose beads have been described as suitable supports from all points of view

[28]: the support is formed of thick fibers of very hydrophilic agarose polymers, permitting to incorporate 17–20 reactive groups per 1000 $Å^2$ (using just the anomeric hydroxy groups in the activation), and the polymer is very similar to water; in fact it is used in gel filtration chromatography because agarose does not interact withmost proteins [28].

Second, the immobilization protocol must be addressed to facilitate the enzyme-support reaction [10,11,21,29]. This means utilizing pH values (usually alkaline) where the reactivity of the enzyme with the support is favored [30]. Moreover, temperatures (room temperature if possible) should facilitate enzyme mobility. Additionally, long reaction times are also required: after the first immobilization, the enzyme-support reaction will continue [30]. Lastly, some way to inactivate all the remaining reactive groups in the support is also convenient, to avoid an uncontrolled reaction between enzyme and support during operation and to permit a fine control of the intensity of the enzyme-support interaction [31].

Finally, selecting a reactive group that really permits this intense multipoint attachment is also required. That means groups that have low steric hindrances for the enzyme-support reaction, that react with good nucleophiles from the proteins, stable under immobilization conditions (long times at alkaline pH values), and compatible with some end-point strategy [31]. The spacer arm is also very important; if it is too large, the induced rigidity on the enzyme structure will be low, if it is too short, the multi-interaction will be very difficult [31].

Among the described active groups that fulfil these requirements, we can mention three. Glyoxyl agarose presents very interesting properties such as the ability to react with primary amino groups without steric hindrances, it is stable at pH 10, the spacer arm is reasonably short, and the enzyme-support imino bonds [32,33] and remaining aldehyde can be reduced to stable secondary amino bonds and inert hydroxy groups using different reagents [32–34]. They have a peculiarity; the low energy of the imino bond makes the formation of

several enzyme-support bonds necessary to fix the enzyme to the support. Thatway, immobilization is directed towards the region of the enzyme surface that is richest in amino groups [33].

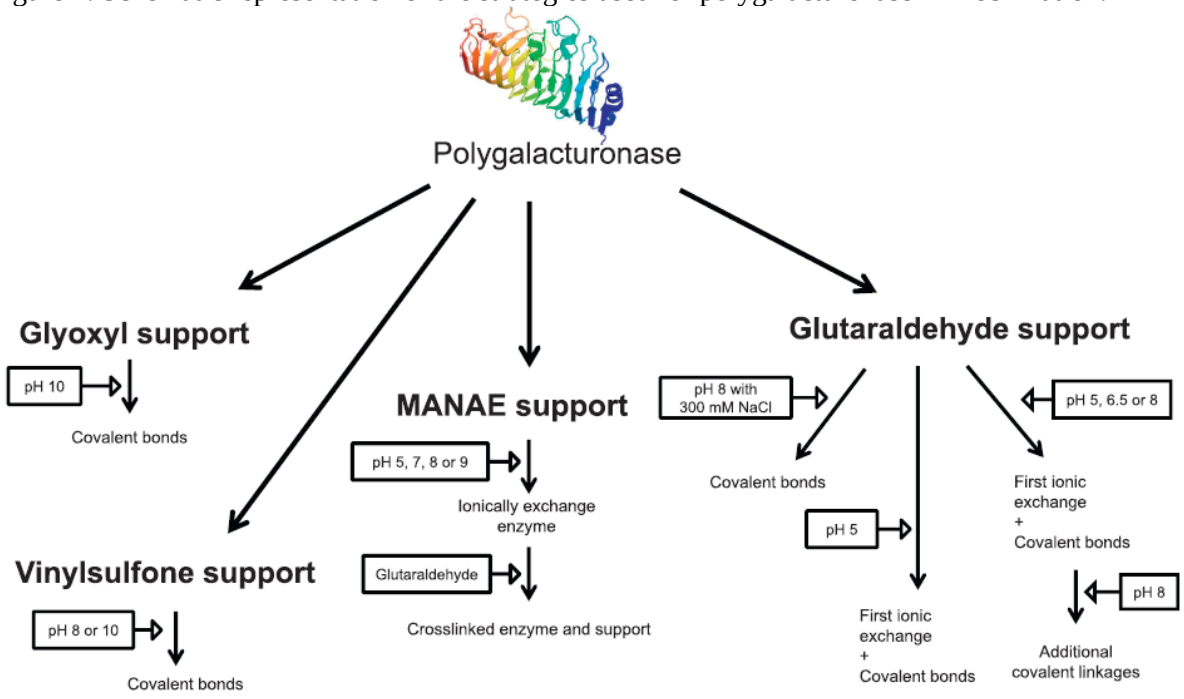
Glutaraldehyde is another good method to immobilize enzymes via multipoint covalent attachment [35]. Glutaraldehyde may be used to preactivate aminated supports (becoming a heterofunctional support) [31]; or to treat enzymes adsorbed on aminated supports [31,36–40]. Each method requires different glutaraldehyde structures, as previously described [35]. Here it is not possible to get a directed orientation towards the richest region of the enzyme in Lys groups, but we can alter the immobilization orientation by altering the pH or the ion strength during immobilization, trying to find the area where the multipoint covalent immobilization is easier [31,36–42]. The chemical reactivity of the support disappeared after a relatively short time, making any end point for the immobilization unnecessary, even though the support will be never physically inert [31,35].

The last option to get an intense multipoint covalent attachment is the support activated by reaction with divinylsulfone, used over a long period of time to immobilize enzymes [43–50]. However it has been only recently when its great potential for getting an intense multipoint covalent attachment has been reported [51,52]. This support can react with primary or secondary amino groups, with hydroxy groups (mainly phenol),with imidazole groups orwith thiol groups [51,52]. The groups may be blocked by using diverse agents [51–54], enabling to tailor the enzyme environment [55–57]. They can give a more intense multipoint covalent attachment than glyoxyl groups, but the longer spacer arm reduces the impact on enzyme stability [51].

Thus, in this new research, we have immobilized PG contained in a commercial extract supplied for juice clarification by the company AB Enzymes, Rohapect 10L. This enzymatic mixture is produced by *Aspergillus niger*, and contains cellulolytic and pectinolytic

enzymes and was previously characterized regarding the activities and stabilities [58]. We have assayed the different immobilization protocols described above using agarose 4BCL as support, represented in Fig. 1, and the best catalysts have been characterized in their activity and stability features.

Figure 1. Schematic representation of the strategies used for polygalacturonase immobilization.



2. Materials and methods

2.1. Materials

The enzyme preparation used was Rohapect 10L from AB Enzymes (Darmstadt, Germany). Sodium periodate, pectin from apple, sodium borohydride, 25 % (v/v) glutaraldehyde aqueous solution, divinylsulfone (DVS), ethylenediamine and glycidol were acquired from Sigma-Aldrich (St. Louis, MO, USA). 4-BCL agarose beads were purchased from Agarose Bead Technologies (Madrid, Spain). All other reagents and solvents were of analytical grade.

2.2. Determination of polygalacturonase activity

Polygalacturonase (PG) activity presented in Rohapect 10 L was measured using polygalacturonic acid as substrate. To this purpose, 0.1 mL of enzyme solution (free or immobilized) was added to 0.9 mL of substrate (1 $g.L^{-1}$) prepared in 50 mM sodium citrate buffer at pH 4.8, and it was incubated at 37 °C for 1 min. After the reaction time, the samples were incubated in an ice bath for 5min to stop the enzymatic reaction and 1 mL of 3,5-dinitrosalicylic acid (DNS) was added to each sample. The amount of reducing groups formed was measured by the DNS method according to Miller [59]. One PG unit was defined as the amount of enzyme required to release 1 μmol of reducing groups per min under the reaction conditions.

2.3. Preparation of glyoxyl-agarose support

The preparation of the glyoxyl agarose support was done according to the procedure described by Mateo et al. [32]. 50 g of 4 % agarose beads were suspended in 85 mL of distilled water. Then, 23.8 mL of NaOH (1.7 N) containing 1.6 g of sodium borohydride was added to the agarose solution. This suspension was kept in an ice bath under mechanical stirring, while 17.2 mL of glycidol was added dropwise. The suspension was gently stirred overnight (18 h) at room temperature. After this time, the activated gel was washed with 20 volumes of distilled water and suspended in 720 mL of distilled water containing 60 μmol of $NaIO_4$ per wet gram of glyceryl-agarose. The oxidation reaction was performed for 3 h under stirring at room temperature. Then, the glyoxyl support was washed with distilled water and stored at 4 °C under wet conditions.

2.4. Preparation of vinylsulfone-agarose support

The preparation of the vinylsulfone-agarose support was performed as described by dos Santos et al. [52]. Briefly, a DVS solution was prepared mixing 7.5 mL of DVS and 200 mL of sodium carbonate buffer (333 mM) at pH 12.5, until the solution became homogeneous. After that, 10 g of agarose (4BCL) were added to the solution under gentle agitation for 35min. At the end, the support was washed with 20 volumes of distilled water and stored at 4 °C.

2.5. Preparation of MANAE support

Monoaminoethyl-N-aminoethyl (MANAE) support was prepared starting from a glyoxyl support, as described above, and modified with ethylenediamine. For this reaction, the proportions were 4 mL of 2 M ethylenediamine at pH 10.05 per gram of glyoxyl agarose beads and submitted to mild stirring 24 h. After this, 10 $mg.mL^{-1}$ of solid sodium borohydride was added to reduce the supports, rinsed with 100 mM carbonate and 100 mM acetate buffers and finally with water [39,60].

2.6. MANAE activation with glutaraldehyde

The support was activated with glutaraldehyde by adding 10 g of MANAE-support to 50 mL of a 12 % (v/v) glutaraldehyde solution in 200 mM sodium phosphate buffer at pH 7 [37,39], and left overnight under mild stirring conditions. This enabled to introduce two glutaraldehyde molecules per primary amino group in the support [35,61].

2.7. PG immobilization

Immobilization of PG preparationwas performed in glyoxyl agarose, vinylsulfone-agarose, MANAE and MANAE-glutaraldehyde using 1 g of support. The support was added

to a solution containing 1 mL of the preparation (5.4 mg of protein) and 9 mL of 5 mM buffer (occasionally containing 20 % of polyethylene glycol, as it has been described to stabilize PG [58]. The buffers used for immobilization were 5 mM sodium citrate (pH 5), 5 mM sodium phosphate (pH 6–8) or 5 mM sodium carbonate (pH 9–10). Immobilizations were carried out at room temperature in a roller mixer.

Immobilization on glyoxyl-agarose was performed at pH 10, with or without 20 % polyethylene glycol. After 5 h of immobilization, 1 mg of solid sodium borohydride per mL of immobilization suspension was added and after 30 min the biocatalysts the sample was vacuum filtered using a sintered glass filter and washed with an excess of distilled water. For vinylsulfone-agarose, the immobilization was performed at pH 8 and pH 10, after 5 h of immobilization the biocatalysts were incubated in 1 M ethylenediamine at pH 8 overnight to block the remaining active groups in the support.

Immobilization on MANAE-agarose was carried out at pH 5, pH 7, pH 8 and pH 9 using PEG as stabilizer during all steps [58]. After the ion exchange of the enzyme in the support, the pH was adjusted to 7 and 1 % of glutaraldehyde was added to the suspension to promote the modification of the amino groups in the enzyme and the support for 1 h. The biocatalysts were washed with distilled water to eliminate the remaining glutaraldehyde, and incubated for a minimum of 24 h to permit the enzyme-support reaction. The immobilization in MANAE-glutaraldehyde pre-activated supports was performed at pH 5, pH 6.5 and pH 8. After 3 h of immobilization, all samples were incubated at pH 8 to allow an increase in the number of enzyme linkages on the support. One sample was maintained at pH 5 until the end of immobilization (24 h). An immobilization at pH 8 adding 300 mM NaCl was also performed.

The immobilization courses were monitored by the enzymatic activity of the supernatant, suspension and a reference with the enzyme under the same conditions as the

immobilization suspension, but using inert agarose. At the end of immobilization, the biocatalyst was filtered, rinsed with 50 mM sodium citrate buffer pH 4.8 and stored at 4 °C. The recovered activity (RA) was calculated according to the following equation:

$$\text{Recovered Activity (\%)} = \frac{\text{Pectin lyase activity in biocatalyst (U)}}{\text{Initial Pectin lyase activity (U)}} \text{ x } 100 \qquad (1)$$

2.8. PG activity at different pH values and temperatures

The influence of different pH and temperature conditions on PG activity was studied for the free enzyme and the selected immobilized biocatalysts in a pH range from 3 to 10, using 50 mM sodium citrate, 50 mM sodium phosphate or 50 mM sodium carbonate buffers, depending on the pH used, at 37 °C.

The effect of the temperature on enzyme activity was analyzed from 30 °C to 90 °C, using 50 mM sodium citrate buffer at pH 4.8.

2.9. Thermal inactivation of different PG preparations

Thermal inactivation was performed by incubating the free enzyme and the selected biocatalysts at pH 4 and 50 °C, pH 6 and 40 °C, pH 8 and 30 °C or pH 10 at 25 °C. During the inactivation, samples were withdrawn and the PG activity was determined immediately. The combinations of temperature and pH were selected to have reliable inactivation courses.

2.10. Courses of polygalacturonic acid hydrolysis catalyzed by PG

The hydrolysis of a solution of polygalacturonic acid was monitored for the free enzyme and selected immobilized biocatalysts. Briefly, the enzymes were incubated in 50 mM sodium citrate buffer at pH 4.8 and at 50 °C containing 1 % (w/v) polygalacturonic acid.

The amount of reducing groups formed was periodically determined by the 3,5-dinitrosalicyclic acid (DNS) method according to Miller [59].

2.11. Operational stability of the immobilized biocatalysts

Operational stability of the immobilized biocatalysts was analyzed by the hydrolysis of polygalacturonic acid. 8 U of polygalacturonase were added to 10 mL of substrate (1 % w/v of galacturonic acid) prepared in sodium citrate buffer (50 mM, pH 4.8), and incubated at 37 °C for 1 h under stirring. The biocatalysts were recovered from the reaction mixture by filtration using sintered glass filter under vacuum and subsequently washed with sodium citrate buffer (50 mM, pH 4.8) before the addition of fresh substrate to be used in a new cycle. The concentration of the product formed after each cycle was determined by the DNS method according to Miller [59], considering the concentration of the first cycle as 100 %.

2.12. Results treatment

All experiments were performed by triplicate and the values are given as mean value ± the experimental error.

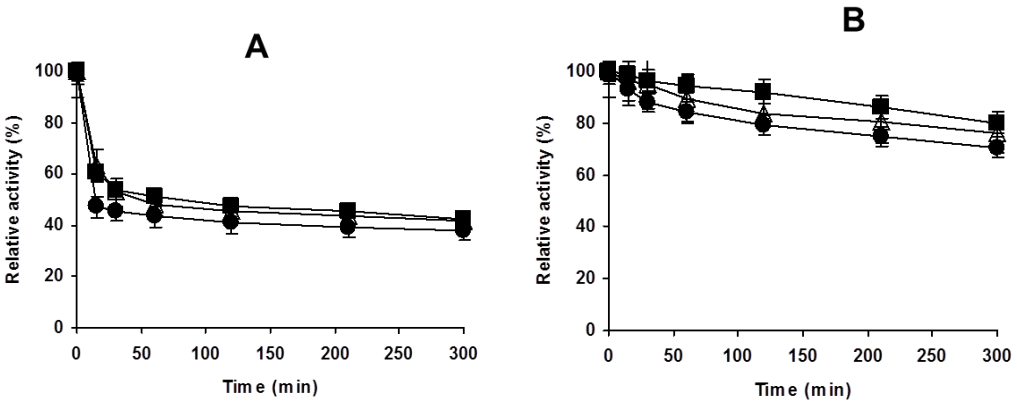
3. Results

3.1. Immobilization of PG on glyoxyl agarose beads

Fig. 2 shows the immobilization course of PG on glyoxyl agarose at pH 10. Immobilization was low and the activity decreased, for both reference and enzyme suspension, probably due to the alkaline pH. Therefore, 20 % PEG was added. This chemical has been reported as a stabilizer for this enzyme [58]. The results improved, regarding the activity, but still, immobilization yield was very short. Around 75 % of the enzyme remained

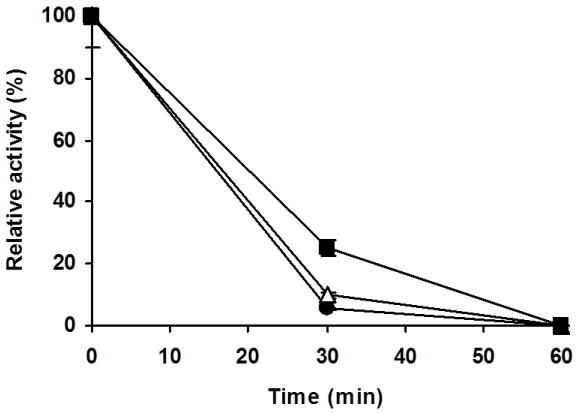
in the supernatant after 5 h, and the recovered activity of the immobilized enzyme was below 20 %.

Figure 2. Immobilization course of PG on glyoxyl-agarose support at pH 10 without (A) or with 20 % polyethylene glycol (B). Experiments were performed as described in Materials and methods. PG activity was measured on reference (■), suspension (△) and supernatant (●).



The PG-glyoxyl biocatalysts stabilities were compared to those of the free enzyme (Fig. 3), finding a very low stabilization. Therefore, it seems that immobilization on glyoxyl agarose could not be very suitable for this enzyme. Perhaps its glycosylation could reduce the possibilities of an enzyme-support multipoint covalent attachment, which is compulsory for immobilization on this support. Thus, immobilization is slow following this protocol [33].

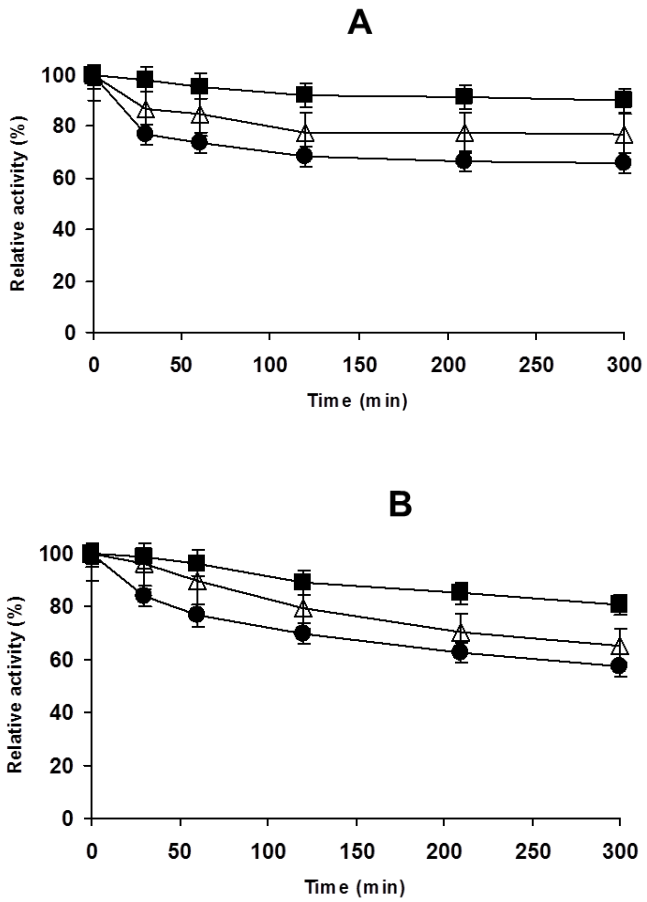
Figure 3. Inactivation courses of the soluble enzyme (●) and biocatalysts immobilized on glyoxyl-agarose support prepared in the presence (■) or absence (△) of polyethylene glycol. Experiments were carried out as described in Materials and methods.



3.2. Immobilization of PG on vinylsulfone agarose beads

Fig. 4 shows the immobilization courses of PG on DVS activated agarose at different pH values, using PEG to avoid enzyme inactivation. Again, immobilization yield was not satisfactory, and it was not very dependent of the immobilization pH (pH 9 or 10), only around 25–40 % of the enzyme was immobilized after 5 h. At pH 9, 50 % of the activity of the immobilized enzyme could be found, while at pH 10 only around 35 % of the immobilized enzyme activity could be detected. Unfortunately, the blocking step almost fully inactivated the enzyme and even the residual activity presented a stability very similar to that of the free enzyme (results not shown). Therefore, this support did not seem very adequate to immobilize-stabilize PG.

Figure 4. Immobilization courses of PG on DVS activated agarose beads at pH 8 (A) or pH 10 (B). Other specifications are described in Materials and methods. PG activity was measured on reference (■), suspension (△) and supernatant (●).

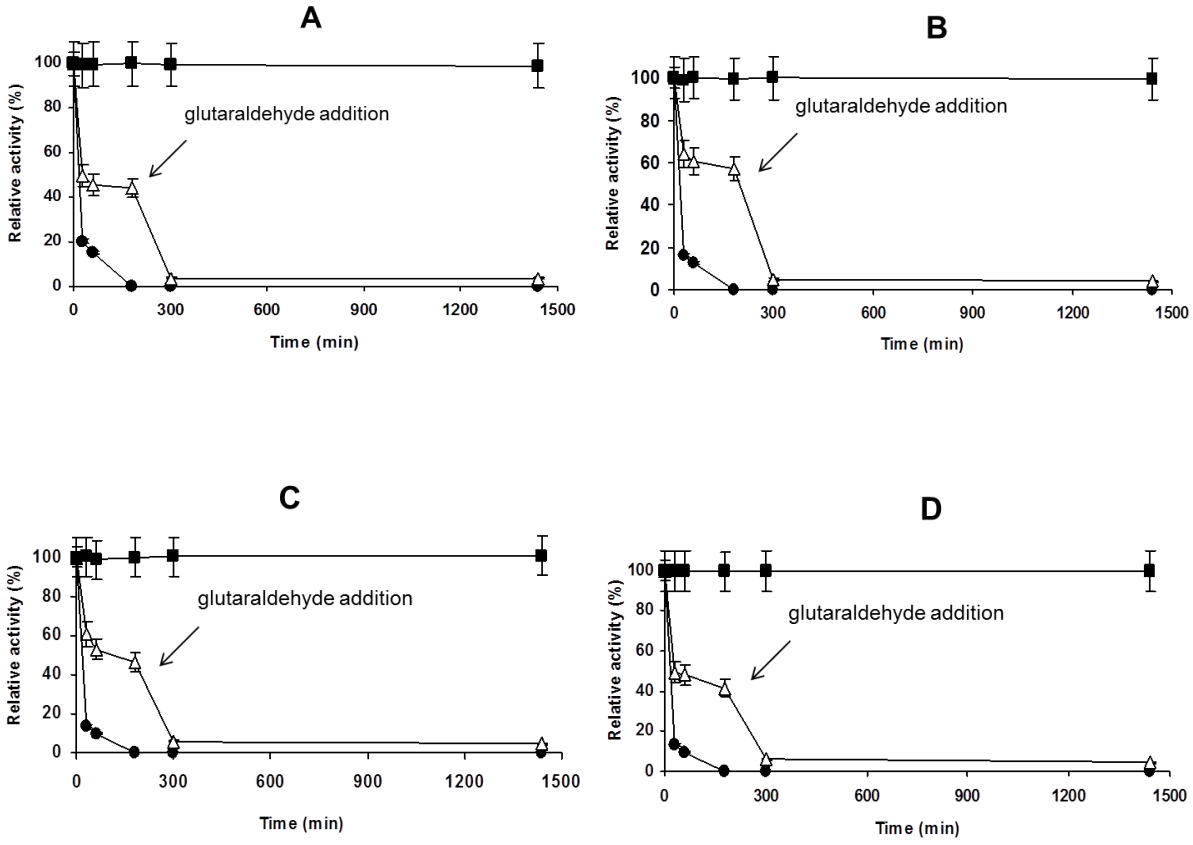


3.3. Immobilization of PG using glutaraldehyde chemistry

3.3.1. Glutaraldehyde crosslinking of enzyme ionically exchanged in MANAE support

Fig. 5 shows that PG was ionically exchanged in MANAE at pH 5, 7, 8 and 9. In all cases, immobilization was quite rapid and around 40–50 % of the enzyme activity can be detected, showing that the ion exchange was a good protocol for the PG immobilization. However, the addition of glutaraldehyde produced the almost full inactivation of the enzyme, leaving less than 5 % of residual activity. Thus, this immobilization protocol was also discarded.

Figure 5. Immobilization course of PG onMANAE agarose support at pH 5 (A), pH 7 (B), pH 8 (C) or pH 9 (D). After 3 h of immobilization, 1 % of glutaraldehyde was added to the solutions. Experiments were performed as described in Materials and methods. PG activity was measured on reference (■), suspension (△) and supernatant (●).

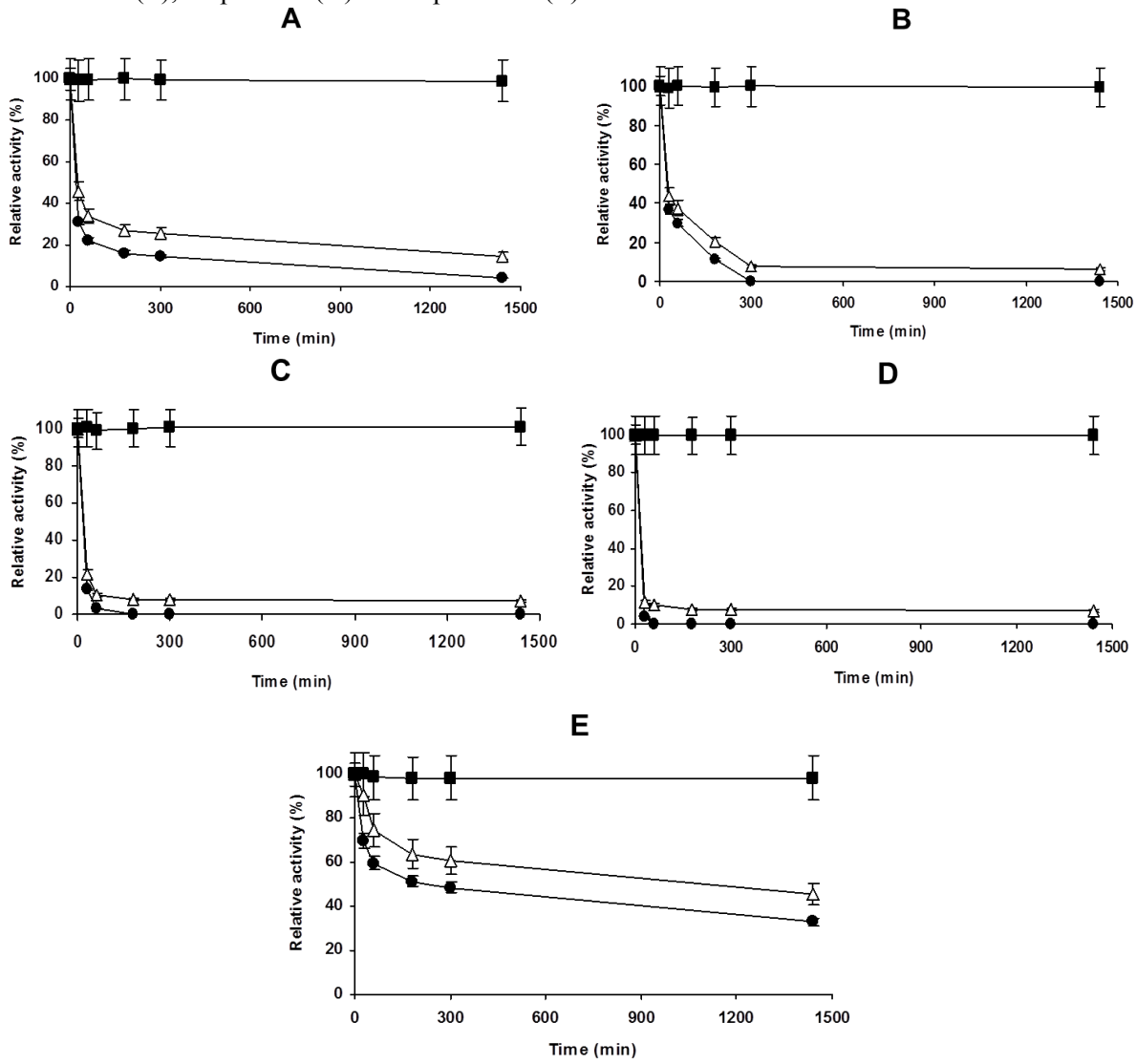


3.3.2. PG immobilization on glutaraldehyde pre-activated MANAE agarose beads

Fig. 6 shows the immobilization courses of PG on glutaraldehyde activated MANAE agarose at pH 5, 6.5 and 8 using low ionic strength to permit a first anion exchange of the enzyme on this cationic support. The highest immobilization rate was found at pH 8 and the lowest at pH 5. A sample was left throughout immobilization at pH 5, and it was found that even after 24 h, some enzyme had not been immobilized. It should be remembered that at pH 5 the enzyme was fully immobilized by ion exchange at pH 5 on MANAE agarose (Fig. 5). This slower immobilization can be due to the masking of the amino groups by the glutaraldehyde groups, which somehow make the enzyme-support interaction required to immobilize a protein by ion exchange more difficult. The addition of NaCl 300 mM at pH 8 in the immobilization suspension produced a significant slowing down of the immobilization rate, suggesting that the ion exchange was the first step in the immobilization in the other cases.

The final activities of the different biocatalysts ranged from 10 % for the immobilization at pH 5 for the whole immobilization process to 7 % when the biocatalysts were incubated at pH 8 for 24 h. The addition of 300 mM NaCl reduced the immobilization yield (to just over 65 %), but allowed to recover the highest expressed activity, around 15 %. Thus, these biocatalysts prepared using glutaraldehyde preactivated supports, even showing a low activity, were the most promising ones among the studied. Next, these biocatalysts were characterized.

Figure 6. Immobilization course of PG on MANAE-glutaraldehyde agarose support at pH 5 for 24 h (A), at pH 5 (B), pH 6.5 (C), pH 8 (D) for 3 h and then incubated 34 h at pH 8, or at pH 8 adding 300 mM NaCl (E). Other specifications are described in Materials and methods. PG activity was measured on reference (■), suspension (△) and supernatant (●).



3.3.2.1. Effect of the experimental conditions on the activity of different PG preparations. Fig. 7A shows the activity of the different PG biocatalysts at different temperatures at pH 4.8. The free enzyme reached the maximal activity at 50 °C. At temperatures under this value, the activity of free enzyme was significantly higher than that of all immobilized enzymes. Thus, at 50 °C the free enzyme was 2.5-folds more active than the enzyme immobilized at pH 5 for 24 h or the enzyme immobilized at pH 8 (in both, absence or presence of 300 mM NaCl) and almost 4-folds more active than the enzyme immobilized at pH 5 or 6.5 and incubated at pH 8

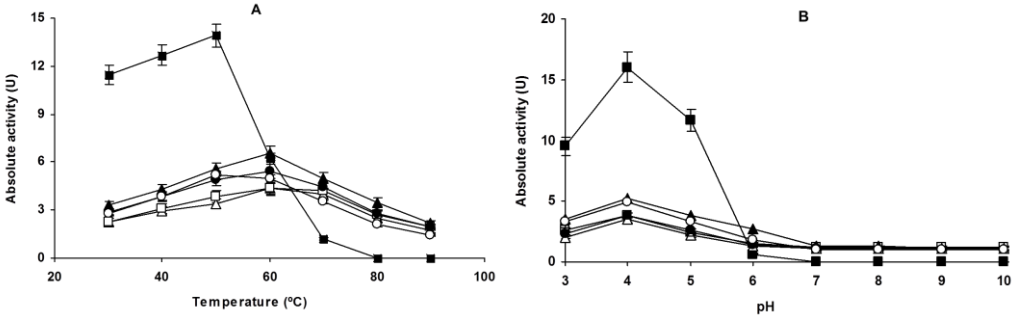
for 24 h. At 60 °C, all immobilized catalyst showed the highest activity, while the free enzyme activity started to decrease. That way, at this temperature the activity of all preparations became quite similar (ranging from 6.5 to 4.5 units/mg). This higher optimal temperature for the immobilized preparations may be related to a higher rigidity of the enzyme structure after immobilization, and also to the reduction of the viscosity of the substrate solution and a higher mobility of the pectic acid that can facilitate the interaction with the active center of not fully properly oriented enzyme molecules. At 70 °C all immobilized biocatalysts reduced their activity in a very small percentage compared to 60 °C. All of them are clearly more active than the free enzyme, that strongly reduced its activity. The enzyme immobilized at pH 5 remained the most active one. Interestingly, at 80 and 90 °C, where the free enzyme is fully inactive, all immobilized biocatalysts maintained a significant percentage activity, with around 2 U/mg at 90 °C, except PG immobilized at pH 8 in the presence of 300 mM NaCl that kept just under 1.5 U/mg. This is around 30 % of the maximum activity (at 60 °C). Thus, although at low temperature the recovered activity may be considered low, at 60 °C and over, the new biocatalysts offers a very significant expressed activity and even an apparent “hyperactivation” may be found over 60 °C. Obviously, this is an artifact caused by the high stabilization of the enzyme after immobilization and not due to a real improved enzyme conformation [11].

Fig. 7B shows the effect of pH on the activity of the different PG preparations at 37 °C. The optimum pH was in all cases pH 4, with around 70 % activity retention in the 3–5 pH range. The observed activity in this pH range was much higher for the free enzyme than for the immobilized enzymes. At pH 3, the most active immobilized preparations were the biocatalyst prepared at pH 5 for 24 h and the enzyme immobilized at pH 8 and in the presence of 300 mM NaCl (more than 35 % of the activity of the free enzyme), while the least active immobilized biocatalyst was that immobilized at pH 5 and them incubated at pH 8 for 24 h

(just over 20 % of the activity of the free enzyme). The situation was similar when the activity was measured at pH 4 and 5. However, when the activity was determined at pH 6, the free enzyme was less active than all immobilized preparations. The most active preparation was that immobilized at pH 5 (4 folds more active than the free enzyme). The other derivatives have a similar activity (from 2.1 to 2.7- times more active than the free enzyme). At pH 7 and higher, the free enzyme becomes almost fully inactive, while the immobilized enzymes maintained a significant percentage of activity even at pH 10, with very slight decreases in activity when determined from pH 7 to pH 10. At pH 10, the most active biocatalysts was the enzyme immobilized at pH 6.5, while the enzyme immobilized at pH 8 was the least active one, but differences were not very significant (1 to 1.3). Again, while at optimal conditions it is clear that the immobilization has a negative effect on enzyme activity, if the activity is determined only at pH 6 or higher, an apparent hyperactivation of the enzyme emay be "discovered" [11]. This activity under conditions where the free enzyme is fully inactive may be due to an improved stability [58].

Thus, although initially the enzyme immobilization on glutaraldehyde preactivated supports seemed not to be positive for enzyme activity, a deeper study showed the significant enlargement of the range of conditions where the immobilized enzyme can be utilized, even conditions where the free enzyme was fully inactive.

Figure. 7. Effect of temperature (A) and pH (B) on the activity of the soluble PG (■) and PG immobilized at pH 5 for 24 h (▲); immobilized at pH 5 (△), pH 6.5 (□), pH 8 (●) for 3 h and then incubated at pH 8 for 24 h; and immobilized at pH 8 in the presence of 300 mM NaCl (○) on MANAE-glutaraldehyde agarose beads. Other specifications are described in Materials and methods.



3.3.2.2. Thermal stability of the different PG preparations. Fig. 8 shows the inactivation courses of the different enzyme preparations at 4 different pH values (from 4 to 10). Under all studied conditions, the great stabilization achieved upon immobilization becomes clear. All immobilized enzymes were far more stable than the free enzyme. For example, when inactivating at pH 4, the free enzyme kept under 10 % of the initial activity while the enzyme immobilized at pH 5 and incubated later at pH 8 (the most stable immobilized biocatalysts under these conditions) kept more than 85 % of the initial activity. The following most stable biocatalysts were those immobilized at pH 5 all time or at 6.5 (and incubated later at pH 8). In inactivations at pH 6, the free enzyme was fully inactive when the most stable immobilized biocatalysts. Again, the enzyme immobilized at pH 5 and incubated later at pH 8, kept almost 90 % of the activity, shortly followed by the enzyme immobilized at pH 6.5. The inactivation at pH 8 showed a slightly lower stabilization, the most stable enzyme maintained 70 % of the activity when the free enzyme maintained under 10 %. At this pH, the enzymes immobilized at pH 6.5 and at pH 5 (and incubated later at pH 8 for 24 h) presented an almost identical stability. Finally, when inactivating at pH 10, the free enzyme maintained under 5 % of the activity when the enzyme immobilized at pH 8 maintained 45 %, followed by the enzyme immobilized at pH 6.5.

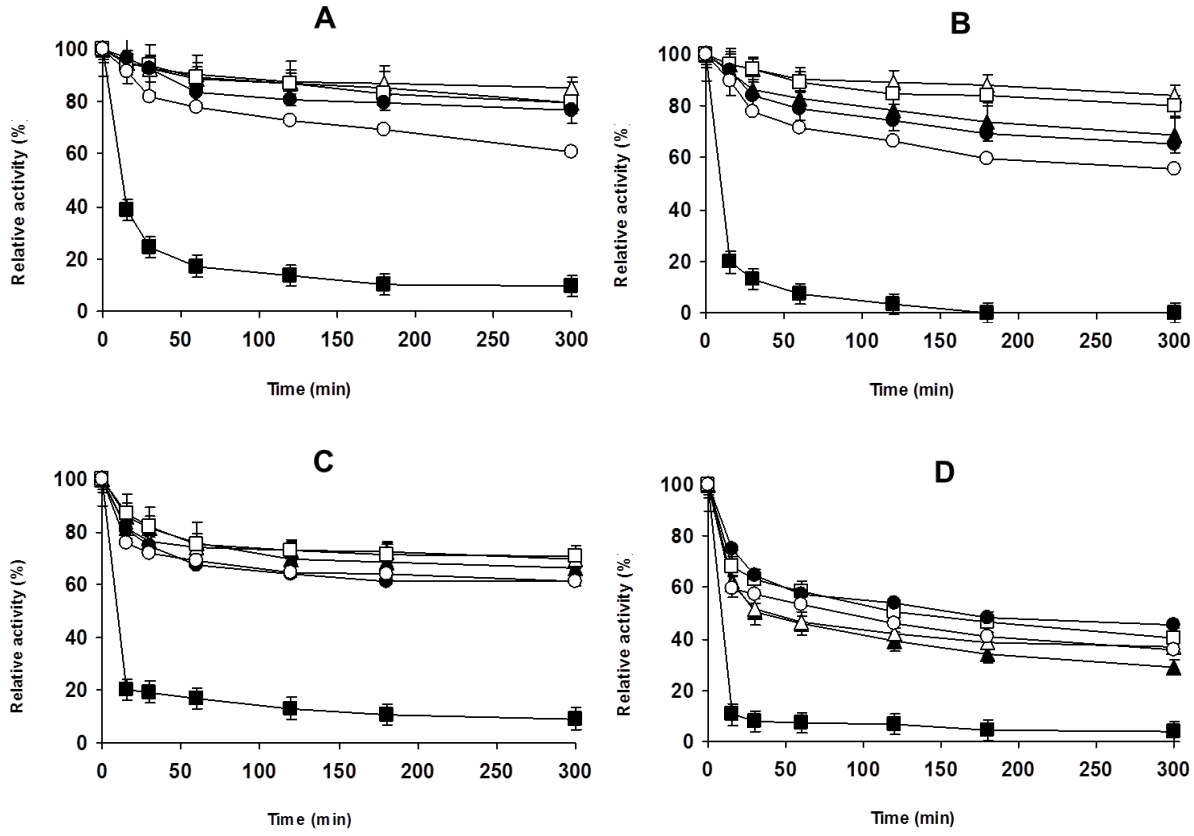
The least stable immobilized enzyme also changes with the inactivation pH. At pH 4 and 6, it is quite clearly the enzyme immobilized at pH 8 at high ionic strength, at pH 8 both enzyme preparations immobilized at pH 8, while atpH 10 the least stable immobilized PG biocatalyst is that immobilized at pH 5.

Thus, although differences are not very large, it can be stated that the immobilization conditions alter the final stability/activity of the immobilized PG. While the incubation at pH 8 should make similar enzyme-support multipoint reactions, the differences based in the immobilization pH suggested a different orientation of the enzyme on the support. Curiously,

in many instances the PG stability is higher when the enzyme is immobilized for 24 h, at pH 5 than when the enzyme is immobilized for 24 h, at pH 8, where the enzyme support reaction should be easier (therefore, a higher multipoint covalent attachment can be expected). However, as expected, the incubation at pH 8 of the PG immobilized at pH 5 always permitted an increase in enzyme stability, as a more intense multipoint covalent attachment may be achieved by incubation under this more alkaline pH condition.

These stability data, again, suggest that the enzyme immobilized at pH 5 and at pH 8 may have different orientation on the support.

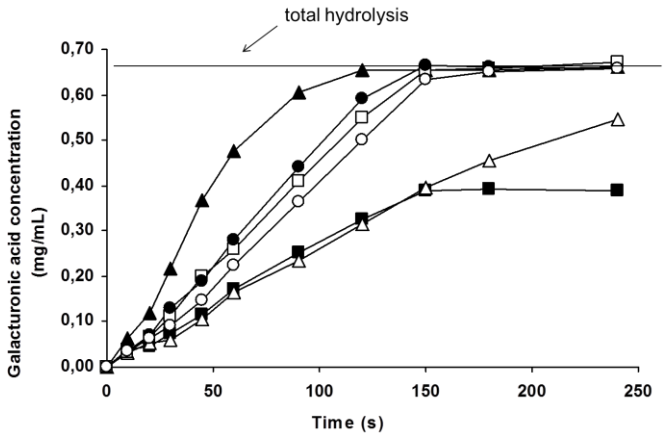
Figure 8. Inactivation courses of the soluble PG (■) and PG immobilized at pH 5 for 24 h (▲); immobilized at pH 5 (△), pH 6.5 (□), pH 8 (●) for 3 h and then incubated at pH 8 for 24 h; and immobilized at pH 8 in the presence of 300 mM NaCl (○) on MANAE-glutaraldehyde agarose beads under different conditions. Inactivation conditions: pH4 at 50 °C (A); pH 6 at 40 °C (B); pH 8 at 30 °C (C); pH 10 at 25 °C (D). Other specifications are described in Materials and methods.



3.3.2.3. Performance of the different PG biocatalysts in hydrolysis of polygalacturonic acid.

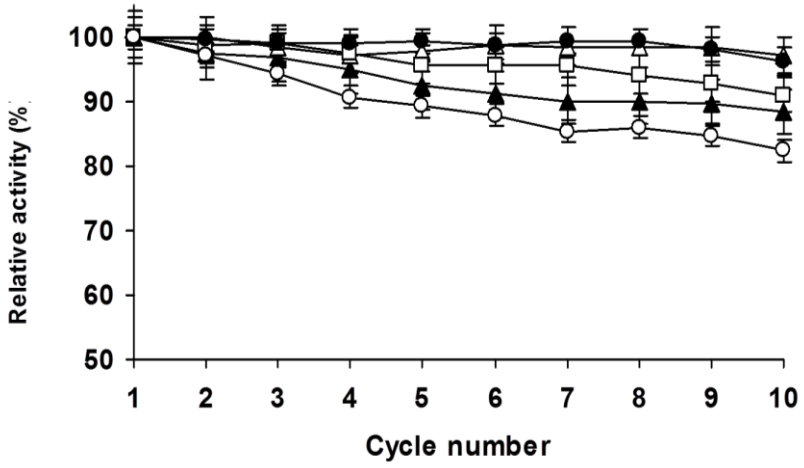
Fig. 9 shows the hydrolysis of polygalacturonic acid with the different PG preparations at 50 °C (optimal conditions for the free enzyme) and pH 4.8. The figure shows that the free enzyme is able to hydrolyze just over 55 % of the substrate in 150 min, and then the reaction stopped. Addition of fresh enzyme permitted to increase the yields, suggesting that the problemwas caused by enzyme inactivation and not by enzyme inhibition. On the contrary, all immobilized biocatalysts reached 100 % of hydrolysis. The biocatalyst prepared at pH 5 and then incubated at pH 8 presented a similar initial performance to that of the free enzyme; however, it was not inactivated and reached almost 100 % hydrolysis after 250 min. The enzyme immobilized only at pH 5 presented the highest activity, reaching 100 % in 2 h, while the other 3 biocatalysts exhibited a quite similar performance. The linear courses observed using the immobilized biocatalysts should be related to the high stability of the immobilized biocatalysts in the utilized conditions. The differences in the PG activity are more visible in this long reaction courses than when just 1 min of reaction was used (in standard activity determination), but qualitatively, they fit the previous results.

Figure 9. Reaction course of polygalacturonic acid modification by soluble PG (■) and PG immobilized at pH 5 for 24 h (▲); immobilized at pH 5 (△), pH 6.5 (□), pH 8 (●) for 3 h and then incubated at pH 8 for 24 h; and immobilized at pH 8 in the presence of 300 mM NaCl (○) on MANAE-glutaraldehyde agarose beads. A solution of 10 mL of 1 % polygalacturonic acid in 50 mM sodium citrate buffer (pH 4.8) at 50 °C was used as substrate, adding 40 mg of the each immobilized biocatalyst and the equivalent free enzyme (0.05 mg·mL^{-1} of protein): Experiments were performed as described in Materials and methods.



3.3.2.4. Operational stability of the different PG immobilized biocatalysts. The main target of immobilization is to reuse the biocatalysts. Thus, all immobilized PG biocatalysts were reused for 10 cycles in hydrolysis of polygalacturonic acid at 37 °C and pH 4.8. Fig. 10 shows that the enzyme immobilized at pH 5 and further incubated at pH 8 or that immobilized at pH 8 maintained almost full activity for the 10 cycles. The biocatalysts prepared at pH 6.5 maintained over 90 % of the initial activity in tenth cycle. The preparation with lower operational stability was that prepared at pH 8 in 300 mM NaCl (82 %). Again, the immobilization conditions determine the final properties of the biocatalysts even though all of them underwent 24 h incubation at pH 8.

Figure 10. Operational stability of PG immobilized at pH 5 for 24 h (▲); immobilized at pH 5 (△), pH 6.5 (□), pH 8 (●) for 3 h and then incubated with glutaraldehyde at pH 8 for 24 h; and immobilized at pH 8 in the presence of 300 mM NaCl (○) on MANAE-glutaraldehyde agarose beads. The reactionwas carried out by total hydrolysis of 1 % polygalacturonic acid in 50 mM sodium citrate buffer at 37 °C and pH 4.8. 8 U of the biocatalysts was added to 10 mL of the substrate, reaction time was fixed in 1 h. Experiments were carried as described in Materials and methods.



4. Conclusion

PG has been immobilized following 4 different protocols, and the best results were obtained using glutaraldehyde preactivated supports. Using these supports, 5 different immobilized biocatalysts were prepared under different conditions to exploit the versatility of

the method. The effect of the incubation pH on enzyme stability was clear when comparing the biocatalyst prepared at pH 5 with or without incubation at pH 8; the alkaline incubation improved enzyme thermal stability at all studied inactivation pH values, as well as the operational stability, but it had a cost in terms of activity. The immobilization pH presented also a very important role in enzyme features, even though the 24 h incubation at pH 8 of all of the immobilized biocatalysts should compensate the differences on support reactivity at lower pH values in the first moments of the immobilization, suggesting a different enzyme orientation. This becomes quite clear comparing the enzymes immobilized at pH 5 and pH 8. The results also showed how the comparison between the free enzyme and the immobilized enzyme must be quite complete to understand the effect of immobilization on enzyme activity. A fixed condition can drive to wrong interpretations. At low temperature, the immobilized enzyme is less active than the free enzyme, very likely due to enzyme distortion caused by the immobilization, but also because of the diffusion problems for the viscous solution and steric problems caused by the large substrate size. However, at temperature over 60 °C the immobilized enzyme remains active while the free enzyme becomes inactivated. Enzyme stabilization should reduce the enzyme distortion caused by this higher temperature, and this temperature reduced the substrate solution viscosity and increased the mobility of the large substrate (reducing steric problems), that way the immobilized enzyme activity increased with temperature.

The effect of the pH on enzyme activity is even stronger and can drive to misunderstandings, at pH 3–5 the free enzyme is clearly more stable than any immobilized enzyme preparation, but at pH 7–10 the free enzyme is inactive while the immobilized enzyme biocatalysts retained a significant percentage of activity. That is, measuring under unfavorable conditions, the conclusion may be a hyperactivation of the enzyme, that is not

real (undermild conditions the immobilized enzyme is less active), but the result of a much more stable enzyme biocatalysts.

The biocatalysts prepared in this paper may have interest for the industrial use of PG, and considering all factors, perhaps the enzyme immobilized at pH 8 could be considered the recommended biocatalysts, but if a higher activity is demanded and stability is not so relevant, the enzyme immobilized at pH 5 without any further inactivation may be a better solution.

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CAPÍTULO 8 – Enzymatic clarification of orange juice in a continuous bed reactors: fluidized-bed versus packed-bed reactor

A partir das diferentes metodologias de imobilização testadas, bem como das características dos biocatalisadores obtidos, as pectinases e celulases foram imobilizadas em macro-esferas de quitosana, devido a elevada estabilidade em conjunto a uma fácil separação do meio reacional deste biocatalisador, e aplicadas em reatores de leito empacotado e leito fluidizado para clarificação contínua do suco de laranja. Além disso, devido ao maior tamanho deste suporte, a inclusão das partículas magnéticas na preparação do mesmo não foi necessária, assim uma maior concentração de enzima pôde ser imobilizada. O biocatalisador obtido foi caracterizados em relação as suas atividades recuperadas, pH e temperatura ótima de atividade e estabilidade térmica. Por fim, foi avaliada a capacidade de clarificação do biocatalisador em dois reatores enzimáticos de fluxo contínuo, com leito empacotado e com leito fluidizado. Os resultados estão apresentados na forma de um manuscrito aceito para publicação na revista *Catalysis Today*.

CAPÍTULO 8 – Enzymatic clarification of orange juice in a continuous bed reactors: fluidized-bed versus packed-bed reactor

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Abstract

In this study, a commercial enzyme cocktail designed for fruit juice clarification was immobilized on chitosan beads activated with glutaraldehyde and used for setting-up continuous packed-bed and fluidized-bed reactors. Firstly, the enzyme cocktail concentration in the immobilization process was tested to get maximum biocatalyst activity in the reaction. Then, the effect of pH and temperature on the free and immobilized enzyme cocktail stability and activity were studied. Despite the low enzyme cocktail activity recovery after immobilization (1 %), the stability of immobilized enzyme was greater compared to free enzyme, even under extreme reaction conditions. For example, when activity was measured at 90 °C and pH 4.8, the immobilized enzyme cocktail retained 80 % of its initial activity while the free enzyme retained only 35 %. Finally, the clarification capacity of the biocatalysts using orange juice was tested, providing clearer juices when performed in the fluidized-bed reactor, showing 60 % of its initial clarification capacity after 72 h of continuous use. Otherwise, the clarification rate in the packed-bed reactor decreased linearly during 54 h, probably due to the formation of dead zones and preferential paths along the bed. Pectinase immobilized on chitosan particles and used in fluidized-bed reactor seems to be a good alternative for large scale application on juice clarification.

Keywords: juice clarification, enzyme cocktail immobilization, fluidized reactor, packed bed reactor.

1. Introduction

The use of enzymes has been attracting attention from many industries and researchers, mainly because of their high selectivity, specificity and activity under environmental benign conditions (Pollard and Woodley, 2007; Siar et al., 2018). However, the poor stability of enzymes associated with harsh industrial conditions is limiting the wide use of these biocatalysts.

Among other strategies, enzyme immobilization is a promising tool for obtaining biocatalysts with improved properties. A suitable immobilization protocol may improve the enzyme stability, activity, purity, selectivity and specificity, apart from reducing inhibition problems, increasing the resistance to inactivating chemicals or the reaction products (Mateo et al., 2007; Garcia-Galan et al., 2011; Liese and Hilterhaus, 2013; Rodrigues et al., 2013; Sheldon and Van Pelt, 2013; Bilal et al., 2019). Besides, it must not be forgotten that the original objective of enzyme immobilization is to make the separation of the biocatalyst from the reaction medium easy, allowing the reuse of the biocatalyst for multiple cycles, or its use in continuous processes (Cao, 2005; Mateo et al., 2007; Garcia-Galan et al., 2011; Liese and Hilterhaus, 2013; Sheldon and Van Pelt, 2013; Asgher et al., 2014).

The development of a system using immobilized enzymes for the continuous clarification of fruit juices may be of great industrial interest (Lozano et al., 1990). One of the major challenges in fruit juice production is the elimination of turbidity without losing its sensory characteristics (Deng et al., 2019). Currently, the traditional practices used to remove suspended particles from juices consist in the addition of clarifying agents, such as gelatin, bentonite, albumin, among others, as well as physical methods such as filtration and centrifugation. Although these present an effective result, these practices may also remove some essential compounds of the color, aroma and flavor of juices (Erkan-Koç et al., 2015; Benucci et al., 2019). Thus, pectinases and other depolymerizing enzymes have been

extensively applied in fruit juice processing, as well as promoting the hydrolysis of compounds responsible for turbidity, they improve the efficiency of the production process, the visual aspect and stabilization of the juice, maintaining the quality characteristics in the final product (DiCosimo et al., 2013; Cerreti et al., 2016; Cerreti et al., 2017; Benucci et al., 2019).

The enzymes used for fruit juice clarification are usually commercialized as enzymatic cocktails, where diverse pectinolytic and cellulolytic enzymes are the main components (Dal Magro et al., 2019b). These enzymes act in different ways catalyzing the hydrolysis of pectic and cellulosic substances that cause the turbidity of fruit juices (Kashyap et al., 2001; Jayani et al., 2005; Tapre and Jain, 2014; Dal Magro et al., 2016c). The degradation of these long and complex polysaccharides from fruit pulp requires that all enzymes act together, as some synergistic effects exist among them (Wilkins et al., 2007). However, immobilized pectinolytic and cellulolytic enzymes generally exhibit very low recovered activity, due to the large size and colloidal characteristics of the substrate that produce a high viscosity in the medium (Kmínková and Kučera, 1983; Lozano et al., 1990; Dal Magro et al., 2016c; Dal Magro et al., 2018; Dal Magro et al., 2019c; Dal Magro et al., 2020). Thus, the preparation of a biocatalytic reactor that reduces the hydrodynamic limitations and allows obtaining high rates of substrate conversion along with high operational stability of the biocatalyst opens the way for new research efforts.

Reactions performed by immobilized enzymes can be made in reactors of different configurations. The most used enzymatic reactors are packed-bed and fluidized-bed reactors, which can be operated continuously. In packed-bed reactors, the immobilized enzymes are located in a fixed-bed, while the solution is pumped through the biocatalyst bed (Poppe et al., 2015). These reactors are suitable for their use in industrial scale, because they have a higher surface area per unit of reaction volume, lower operational cost and the damage of the

biocatalyst is minimized due to the lower shear stress compared to fluidized-bed reactors. However, they also have some drawbacks. For example the possibility of bed compaction and formation of preferential paths, thus limiting mass and heat transfer (Watanabe et al., 2001; Laudani et al., 2007; Illanes, 2008). In fluidized-bed reactors, the immobilized biocatalyst is maintained in suspension by recirculating air or the substrate solution in the system with the inlet in the bottom of the reactor, providing the free circulation of the particles throughout the bed, avoiding the decantation of the immobilized enzymes in the reactor bottom (Kosseva et al., 2009). In addition, the use of this type of reactor avoids the formation of preferential paths and mass transfer is facilitated (Ray, 2012). However, fluidized-bed reactors can withstand smaller amounts of solid enzyme biocatalyst per unit volume of the reactor than packed-bed reactors, reducing the overall efficiency of the reactor per volume unit, making the scale-up process more difficult (Kosseva et al., 2009).

In this work, chitosan was used for enzyme immobilization. Chitosan, the principal derivative of chitin, has desirable characteristics for enzymes immobilization (Krajewska, 2004; Biró et al., 2008). Chitosan has reactive amino and hydroxyl groups in its linear polyglucosamine chains. These groups can be used to immobilize enzymes or to modify the structure of the support, for example using glutaraldehyde (Krajewska, 2004; Adriano et al., 2008).

Based on these aspects, the objective of this research work was to study the continuous clarification of orange juice using two different biocatalytic reactor systems: a packed-bed and a fluidized-bed reactor. For the biocatalyst preparation, the enzymatic cocktail Novozym 33095®, composed of pectinolytic and cellulolytic enzymes, was immobilized on chitosan beads activated with glutaraldehyde. This enzymatic mixture is produced by Novozymes, with declared activity of pectin lyase and polygalacturonase, but some other activities, such as pectin methyl esterase and cellulase, were also detected (Dal Magro et al., 2016a). The

immobilized enzyme was characterized by their enzymes activities, thermal stability, optimum pH and temperature, and applied in the construction of the two bioreactors for the continuous clarification of orange juice.

2. Materials and methods

2.1. Materials

The freshly pressed orange juice without any treatment was donated by Vitivinícola Jolimont (RS, Brazil). The commercial enzyme preparation Novozym 33095® was kindly donated by Novozymes (Spain). Pectin from apple, polygalacturonic acid, galacturonic acid, glucose and chitosan (from shrimp shells, ≥75% deacetylated) were acquired from Sigma Aldrich (St. Louis, MO). Glutaraldehyde 50 % was purchased from Dinâmica (SP, Brazil). All other reagents and solvents were of analytical grade.

2.2. Determination of enzyme activities

The total pectinase (PE) and polygalacturonase (PG) activities were determined by pectin and polygalacturonic acid hydrolysis, respectively, according to Dal Magro et al. (2016a), in which the amount of reducing groups released was quantified by the 3,5-dinitrosalicyclic acid (DNS) method, as described by Miller (1959). The pectinlyase (PL) activity was determined by formation of unsaturated uronide (ϵ=5500 $M^{-1}.cm^{-1}$), measuring the increase of the absorbance at 235 nm, as proposed by Albersheim (1966). The pectin methyl esterase (PME) activity was quantified by titration of carboxylic groups released by the de-esterification of pectin, as proposed by Rouse and Atkins (1952). The total cellulase (CE) activity was measured using Whatman no. 1 filter paper as substrate, as described by Ghose (1987), in which the amount of reducing groups released was quantified by DNS method.

2.3 Protein determination

The protein was determined by the Bradford method (Bradford, 1976). 20 μL of diluted sample was mixed to 1 mL of Bradford reagent. After color stabilization, the samples were measured by spectrophotometer at 595 nm. Absorbance values were converted to protein using a standard curve of BSA solutions (protein standard, bovine serum albumin) from 0.1 $mg.mL^{-1}$ to 1.0 $mg.mL^{-1}$. Results were expressed as $mg.mL^{-1}$ albumin.

2.4. Preparation and activation of chitosan beads

Chitosan beads were prepared by the precipitation method, as described by Klein et al. (2012). A solution of chitosan (2 % w/v) was dissolved in acetic acid (0.35 M) and sonicated for 30 min to remove the air bubbles. Then, it was added dropwise into the coagulation solution (1 M sodium hydroxide) under gentle agitation. The obtained chitosan beads were separated and rinsed with distilled water until neutrality was achieved.

For activation of the chitosan beads, they were incubated in a solution of glutaraldehyde 5 % prepared in 0.1M phosphate buffer (pH 7.0) for 3 h under gentle rotation. Finally, the beads were washed abundantly with distilled water to remove the excess glutaraldehyde.

2.5. Enzyme immobilization

Enzyme immobilization on the chitosan beads was performed by incubating the particles with different enzyme solutions, varying the concentration from 12.5 to 300 mg of protein per gram of support, prepared in sodium citrate buffer (50 mM, pH 4.8). The enzyme solution was placed in contact with the beads for 15 h at room temperature and under gentle stirring in a roller mixer. After this time, the immobilized enzymes were separated from the

solution and washed with sodium citrate buffer to remove the unbound enzymes. Then, the immobilized enzymes were suspended in sodium citrate buffer (50 mM, pH 4.8) and stored at 4 °C until its use. The recovered activity was determined according to the following equation:

$$\text{Recovered Activity (\%)} = \frac{\text{Biocatalyst activity (U)}}{\text{Initial activity (U)}} \text{ x } 100 \qquad (1)$$

2.6. Optimal pH and temperature

The influence of different pH and temperature conditions on free and immobilized enzymes activity was studied. For the evaluation of pH, the pectin solution was prepared in 50 mM sodium citrate buffers, varying the pH from 3 to 6. The activities were measured at 37 ºC. The effect of the temperature on the enzyme activity was analyzed from 30 ºC to 90 °C, using 50 mM sodium citrate buffer at pH 4.8. The temperature of 37 ºC and pH 4.8 were fixed based on literature data that presents these values as optimal condition for this enzymes class activity, as well as in previous experiments (Dal Magro et al., 2019b).

2.7. Thermal inactivation

The thermal inactivation was performed by incubating the free and immobilized enzyme preparations in sodium citrate buffer (50 mM, pH 4.8) at 60 °C. During the inactivation, samples were withdrawn and the PE activity was determined immediately.

2.8. Packed-bed and fluidized-bed reactors

The packed-bed reactor was composed of a glass tube of 1 cm internal diameter and 18 cm in length, covered with an external jacket for water recirculation at a controlled temperature of 40 ºC. The internal volume of the reactor was of 14 mL, which was filled with

650 beads (0.22 g dry mass with activity of 1340 $U.g^{-1}$), being the bed porosity 0.5. A schematic representation is presented in Figure 1a.

For the fluidized-bed reactor, a larger glass tube was used in order to provide the beads (650 beads, 0.22 g dry mass with activity of 1340 $U.g^{-1}$) fluidization. Then, the fluidized-bed reactor was composed of a glass tube of 1 cm internal diameter and 39 cm length, covered with an external water jacket for water recirculation at a controlled temperature of 40 ºC. The internal volume of the reactor was of 31 mL. A second peristaltic pump, with high flow rate, was used for juice recirculation and suspension of the beads, as observed in Figure 1b.

2.9. Flow rate

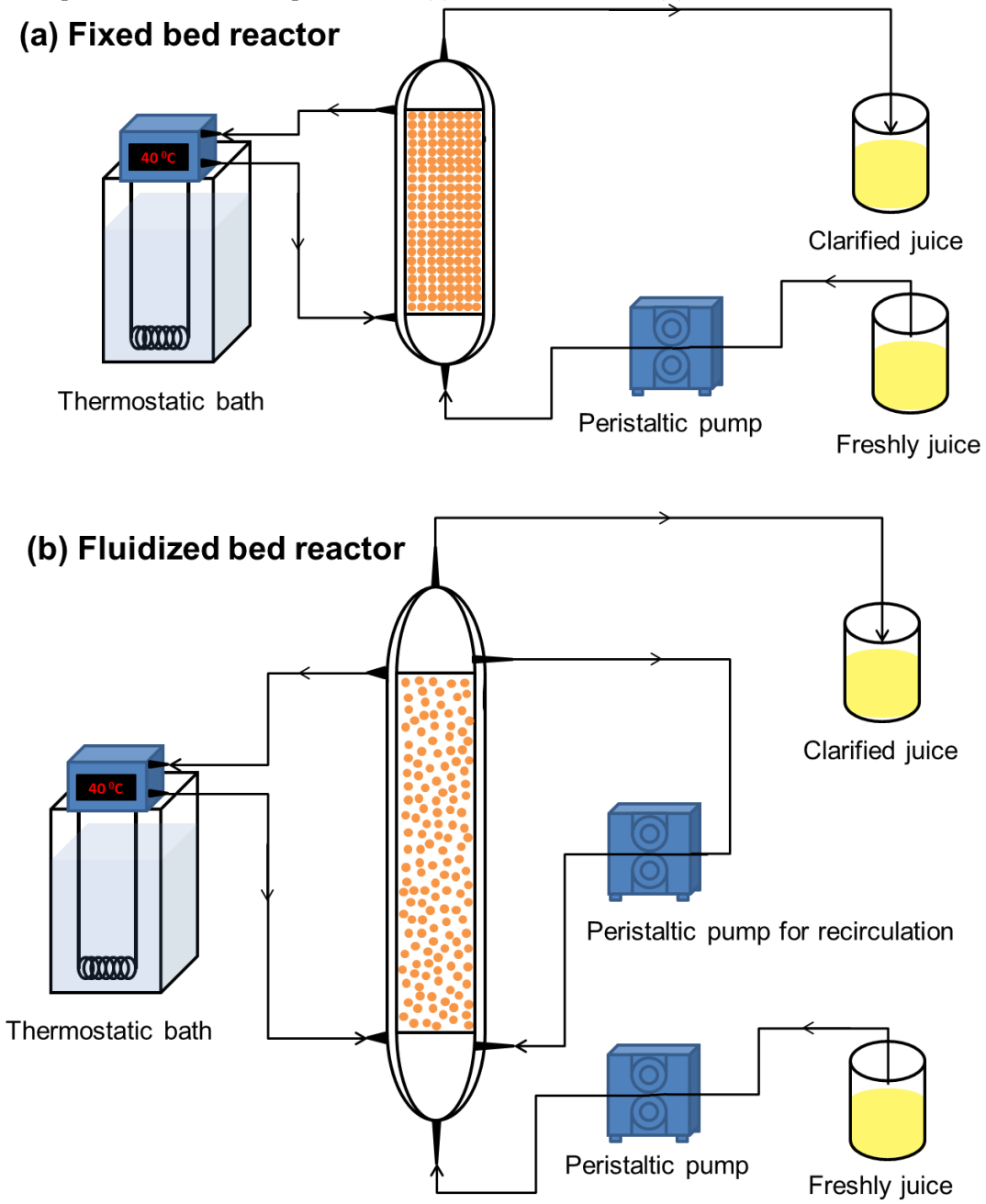
The continuous clarification of the orange juice was evaluated in flow rates varying from 0.5 $mL.min^{-1}$ to 3 $mL.min^{-1}$ for 2 h, for both bioreactors.

2.10. Operational stability

The operational stability of the biocatalyst, using both reactor configurations, was evaluated by the continuous clarification of orange juice. Periodically, samples were collected from the top of the reactors and the clarification percentage of the orange juice was calculated using Equation 2, which takes into consideration the initial and final juice turbidity. The turbidity was determined spectrophotometrically by detection of the scattered light at 860 nm, as described by Anderson (2005). Before measurements, all samples were centrifuged at 2700 × *g* for 3 min.

$$\text{Clarification percentage (\%)} = \frac{\text{Initial turbidity} - \text{Final turbidity}}{\text{Initial turbidity}} \text{ x } 100 \qquad (2)$$

Figure 1. Operational scheme of packed-bed (a) and fluidized-bed (b) reactors.



3. Results and discussion

3.1. Effect of enzyme concentration in the recovered activity in the immobilized enzyme biocatalyst

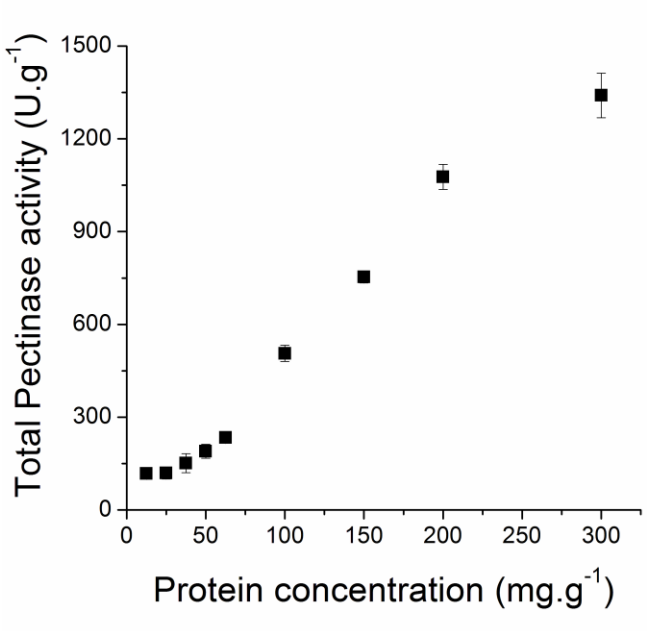
Firstly, in order to prepare a highly active biocatalyst, different enzyme cocktail concentrations, ranging from 12.5 to 300 mg of protein per gram of support were used to

prepare the immobilized biocatalyst. As it can be seen in Figure 2, the mass activity of the immobilized enzymes biocatalyst showed a positive correlation with the increase in enzyme concentration.

The highest activity of the biocatalyst was achieved when 300 $mg.g^{-1}$ protein were used, resulting in a recovery activity of 1340 $U.g^{-1}$. Further increasing enzyme loading did not result in the increase of biocatalyst activity and resulted in a lower recovered activity (less than 1 %) (data not reported).

Some enzymes eventually lose some enzymatic activity after their immobilization, either by some modification in their structure, or by diffusional limitations (Liu et al., 2011; Sojitra et al., 2016). Several studies reported problems regarding low recovered activities after pectinases immobilization. This was mainly associated with the difficulty of the substrate accessing the enzymes active sites (Kmínková and Kučera, 1983; Lozano et al., 1990; Dal Magro et al., 2016c; Dal Magro et al., 2018; Dal Magro et al., 2019c).

Figure 2. Influence of enzymatic protein concentration on the total pectinase activity of the biocatalyst. Immobilization was performed as described in item 2.5.



3.2. Characterization of enzymatic activities

As shown in Table 1, this commercial enzyme preparation has an outstanding pectinolytic activity, particularly of polygalacturonase and pectin methyl esterase. This combination of enzymes is important for juice clarification, since most fruit juices have a high degree of esterified pectin in their composition (Kashyap et al., 2001). Polygalacturonase (PG) and pectin methyl esterase (PME) act together to hydrolyze pectin. First, PME acts by removing the methyl group attached to the galacturonic acid of the chain, converting pectin to pectate (non-esterified polymer), and then PG catalyzes the hydrolysis of the α-1,4 bond between two non-esterified galacturonic acid (Alkorta et al., 1998; Jayani et al., 2005; Tapre and Jain, 2014).

Extracts with multiple enzymes allow highly cooperative catalytic mechanisms, in which one enzyme assists another by synergistic action, generating a cascade reaction (Keighron and Keating, 2010). Thus, the spatial arrangement of the enzymes on solid supports can facilitate this type of reaction, since approximation of the different immobilized enzymes on the same support allows intermediate products to be transported rapidly from one active site to the next (Pescador et al., 2008; Keighron and Keating, 2010; Jia et al., 2015).

In this work, the commercial enzymatic cocktail preparation Novozym 33095 presented low recovered activity after immobilization, for all enzymes analyzed (Table 1). Although this is not desirable, this reduction in the activity was expected and was generally associated with several factors. Firstly, it may be related to steric impediments and diffusional limitations, since the large size of the substrate hinders the access of enzymes, especially those immobilized inside the porous support (Lozano et al., 1990; Sojitra et al., 2016; Dal Magro et al., 2018). In addition, the excessive reaction between enzyme and support contributes to the increase of the rigidity of the enzymatic structure and it may also cause some protein distortion, causing loss of activity (Mateo et al., 2004; Soozanipour et al., 2015;

Nadar et al., 2016; Sojitra et al., 2016; Talekar et al., 2017). Moreover, the crowding effects may also negatively influence enzyme activity by increasing the steric effects (Pastor et al., 2014; Fernandez-Lopez et al., 2017; Zaak et al., 2017; Siar et al., 2018).

This decrease in enzyme activity may be compensated by several facts: now it is possible to use the enzyme in continuous reactor configurations, the enzyme may become more rigid and that way more stable, enabling its use under more drastic conditions, etc. (de Oliveira et al., 2018).

Table 1. Enzymatic activities of total pectinase (PE), polygalacturonase (PG), pectinlyase (PL), pectin methyl esterase (PME) and total cellulase (CE) of the free and immobilized enzymes.

	Total enzymatic activity (U)				
	PE	**PG**	**PL**	**PME**	**CE**
Free enzymes	67341.14	83754.30	6283.33	62941.66	2290.36
Immobilized biocatalyst	477.72	639.77	29.04	460.32	32.27

3.3. Optimal pH and temperature

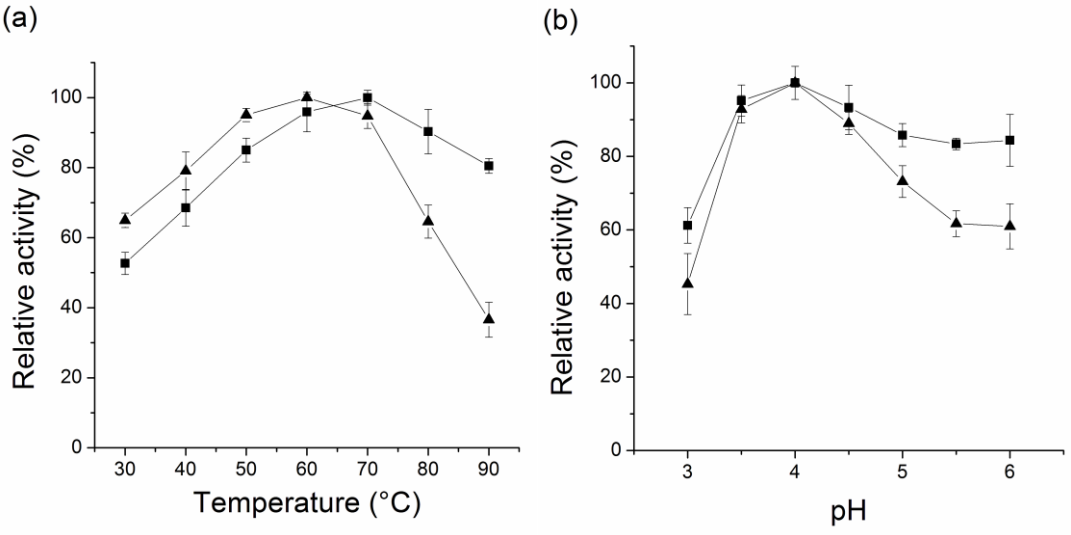
The enzyme activity is directly linked to reaction conditions. Therefore, to analyze the influence of these factors, the activities of free and immobilized enzymes were assayed under different conditions of pH and temperature.

Figure 3a shows that at pH 4.8, an increase in the activity for both enzyme preparations was observed when increasing the temperature up to a maximum activity at 60 ºC for the free enzyme and at 70 ºC for the immobilized enzyme, followed by a decline in the activities for both enzymes. At 90 °C the immobilized enzyme still presented 80 % of its activity at 70 ºC while the free enzyme presented around 35 % of the activity at 60 ºC. The improved relative activity of the immobilized biocatalysts may be related to the multipoint covalent attachment between the enzymes and the support, which protects the enzyme active

conformation from distortion caused by heat (Garcia-Galan et al., 2011; Rodrigues et al., 2013; Guo et al., 2016; Talekar et al., 2017; Dal Magro et al., 2019c; Dal Magro et al., 2020). In addition, high temperatures may cause a decrease in the pectin solution viscosity, increasing substrate mobility inside the support pores, favoring the interaction with the enzymes located inside.

Figure 3b shows the effect of pH on enzyme activity at 37 ° C. The highest activity was achieved at pH 4 for both enzymes. However a higher relative activity was observed for the immobilized enzyme at other pH values. For example, at pH 3 the immobilized enzyme showed 61 % of the maximum activity while the free enzyme presented 45 %. On the other hand, at pH 6, the immobilized enzyme retained 84 % of its activity at pH 4 while the free enzyme retained only 61 %. Variations in pH may result in damage to the enzyme structure through changes in the ionization state of the microenvironment around the active site, preventing substrate binding and affecting its activity (Karimpil et al., 2011; Guo et al., 2016; Talekar et al., 2017). Thus, this increased residual activity under higher pH, observed for the immobilized biocatalyst, may be due to improved stability (Rodrigues et al., 2013; Dal Magro et al., 2019c; Dal Magro et al., 2020).

Figure 3. Relative activity of free enzyme (▲) and immobilized enzyme (■) under different conditions of temperature (a) and pH (b).

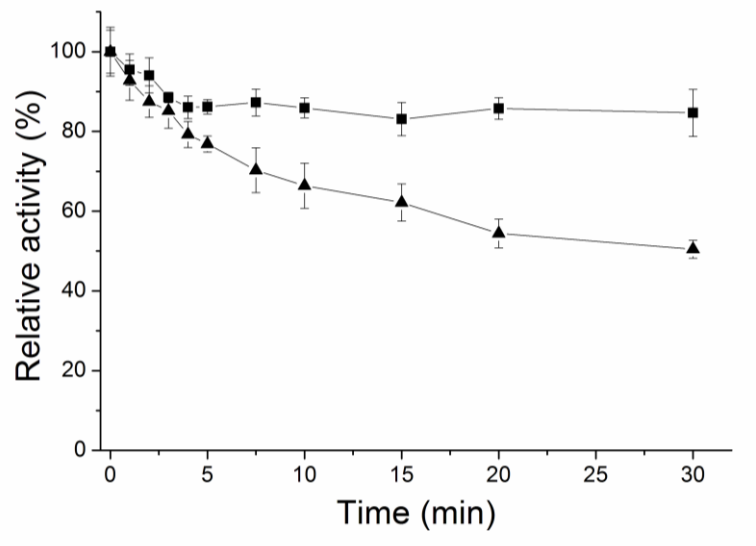


3.4. Thermal stability

One of the main objectives of enzymatic immobilization is to obtain a stable biocatalyst for industrial application, thus the thermal stability of the free and immobilized enzymes have to be determined. With this aim, the biocatalysts were incubated at 60 °C, and the residual activities were determined after different time intervals up to 30 min.

As it can be seen in Figure 4, both enzyme preparations presented an initial decrease in enzyme activity; it can be observed that the free enzyme presented only 50 % of its initial activity after 30 min of incubation at 60 ºC. The residual activity of the immobilized enzyme decreased in the first 4 min to 15 % and they remained unaltered and this biocatalyst maintained 85 % of the activity up to 30 min. This initial inactivation may be due to heterogeneity in the immobilized enzyme populations, as different enzymes are involved, together with the fact that some enzyme molecules may have more or less intense multipoint covalent attachment.

Figure 4. Kinetic of thermal inactivation of the free (▲) and immobilized (■) enzyme at 60 ºC during 30 min.



3.5. Effect of flow rate in the continuous reactors

The prepared immobilized biocatalyst was used for the setting up of two continuous reactors, a packed-bed and a fluidized-bed reactor for the clarification of orange juice. Initially, different flow rates were tested (0.5 to 3.0 mL min^{-1}), and the juice turbidity was evaluated after 2 h of operation in the tested condition to ensure steady-state operation. The residence times for each flow rate for each reactor is presented in Table 2.

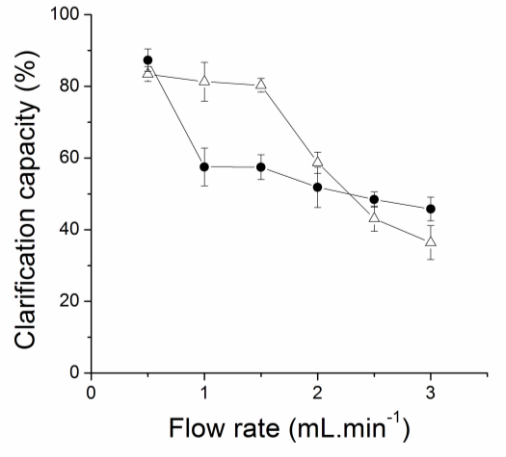
As it can be seen in Figure 5, there was an outstanding decrease in the clarification capacity (from 87 % to 57 %), for the fluidized-bed reactor, when the flow was increased from 0.5 $mL.min^{-1}$ to 1.0 $mL.min^{-1}$. Increasing the juice flow rate to 3 $mL.min^{-1}$ caused a decrease in the clarification capacity to 45 %. These results must be related to the shorter residence time of the juice inside the reactor when higher flow were used, reducing the contact time of the juice with the biocatalyst. The use of low flow rates allows an adequate diffusion of the pectin to the inner areas of the immobilized biocatalysts and permits the substrate to reach the enzyme catalytic site, providing a good clarification. When using higher flow rates, the substrate is not able to penetrate inside the biocatalyst particle, thus clarification capacity of the biocatalyst was lower (Diano et al., 2008).

Table 2. Residence times for continuous reactor at each flow rate.

Flow rate ($mL.min^{-1}$)	**Residence time (min)**	
	Packed-bed reactor	*Fluidized-bed reactor*
0.5	14.0	48.0
1.0	7.0	24.0
1.5	4.7	16.0
2.0	3.5	12.0
2.5	2.8	9.6
3.0	2.3	8.0

For the packed-bed reactor, the clarification capacity decreased from 83 % to 80 %, when the flow rate increased from 0.5 to 1.5 mL.min^{-1}. These results may be related to the larger amount of biocatalyst per reaction volume compared to fluidized-bed reactor. Using higher flow rate, there was a high reduction in clarification capacity, becoming only 36 % clarification when a flow rate of 3 mL.min^{-1} was used. In addition to the shorter residence time, these results for the packed-bed may also be related to the fact that this reactor may promote an initial reduction of turbidity by retention of insoluble juice particles inside the reactor just by a filtering effect. However, the accumulation of these particles inside the reactor caused diffusional problems, making the access of the substrate to the biocatalysts particles coated with these insoluble substrate particles difficult. Consequently, dead zones and preferential paths of the substrate inside the reactor can be formed, reducing the efficiency of this reactor type due to decrease in the actually useful volume, requiring longer residence times for proper performance (Poppe et al., 2015).

Figure 5. Influence of different orange juice flow rates on the clarification capacity in the packed-bed (Δ) and fluidized-bed reactors (●).

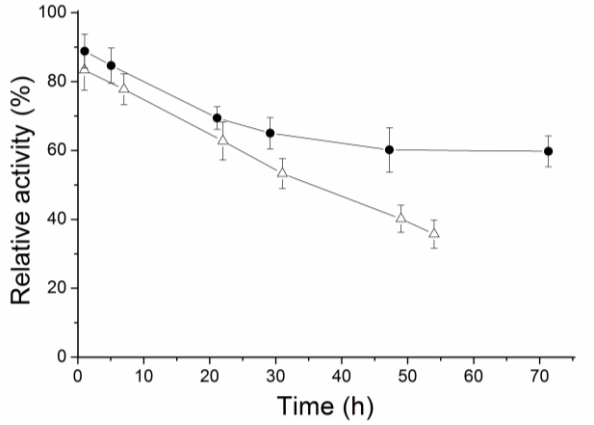


3.6. Operational stability

Based on the results obtained, the flow rate of 0.5 mL.min^{-1} was chosen to test the operational stability for both reactors. The results for continuous use were presented in Figure

6. It can be observed that after an initial reduction for both reactors, the fluidized-bed maintained the juice clarification of 60 % after 71 h of operation, while the packed-bed reactor showed a linear decrease of its catalytic capacity during 54 h of operation. At this time, an increase of the juice particles accumulation inside the reactor over time could be visualized. These results agreed with Diano et al. (2008), who verified a 25 % increase in the clarification efficiency of apple juice when the fluidized-bed reactor was compared to the packed-bed reactor, possibly due to the better mass transfer of this reactor.

Figure 6. Reduction of orange juice turbidity in continuous packed (Δ) and fluidized (●) bed reactors.



In the fluidized-bed reactor, 0.22 g of biocatalyst were used to clarify more than 2 L of juice and, even after 3 operation days, these biocatalysts still presented 60 % of their clarification capacity. Despite the low clarification capacity of the packed-bed reactor, it can be properly used if it is periodically washed for internal particle removal. Considering the flow rate of 0.5 $mL.min^{-1}$ and 0.22 g of biocatalyst in each reactor, the productivity per gram of biocatalyst per day is 3.2 $L.g^{-1}.d^{-1}$.

4. Conclusions

The results presented in this study demonstrated that immobilized enzyme biocatalysts may be an appropriate strategy for their application in reactors for the continuous clarification

of orange juice. As expected, the immobilization of pectinases and cellulases on a porous support caused a decrease in enzymatic activity related to diffusional problems due to characteristics of size and viscosity of the substrate. Despite this fact, the biocatalyst stability was greatly improved, with a marked activity even under extreme conditions of temperature and pH, suggesting a wide variety of possible applications of this biocatalyst in different processes.

In addition, the setting-up of reactors containing immobilized enzymes for the continuous clarification of orange juice proved to be a technology with great potential for industrial application, contributing to the productive cost reduction. Thus, this work contributes to give new perspectives for the development of a promising technology for industrial juice clarification process.

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DISCUSSÃO GERAL

A presente Tese de Doutorado teve como objetivo principal desenvolver biocatalisadores compostos por pectinases e celulases imobilizadas para a clarificação de sucos de frutas. Dentro do tema proposto, investigou-se a utilização de partículas magnéticas para a preparação de dois biocatalisadores. Duas metodologias distintas de imobilização foram utilizadas, obtendo-se os CLEAs magnéticos (CLEA-MP*), através do entrecruzamento das enzimas com as partículas magnéticas, e a imobilização das enzimas na superfície da partícula magnética ativada com glutaraldeído (Enz-Glu-MP*). Posteriormente, estudou-se diferentes metodologias de recobrimento das partículas magnéticas com quitosana, alcançando 3 diferentes biocatalisadores com distintos tamanhos: Nano (Nano-CMag), Micro (Micro-CMag) e Macro (Macro-CMag). A caracterização dos biocatalisadores preparados nos dois trabalhos, em relação às atividades recuperadas, parâmetros cinéticos, estabilidades, reutilização na clarificação dos sucos, bem como as propriedades morfológicas, estruturais, magnéticas e texturais dos biocatalisadores, foram estudadas.

Durante o período sanduíche no Laboratório de Ingeniería de Biocatalizadores y Biotransformaciones do Instituto de Catálisis y Petroleoquímica do CSIC na Espanha, procurou-se estudar cada enzima do preparado comercial em separado, buscando entender como cada enzima reage às diferentes condições de imobilização, investigando possíveis favorecimentos e direcionamentos na imobilização das enzimas PL e PG em diferentes suporte a base de agarose. Inicialmente, as enzimas foram incubadas em diferentes condições de pHs e temperaturas, obtendo as estabilidades das mesmas nessas condições. Visto que a grande maioria dos protocolos de imobilização utilizam pHs elevados, diferentes agentes estabilizantes foram utilizados, buscando alcançar a estabilidade das diferentes frações enzimáticas nessas condições. O efeito de glicerol, dextrana e polietilenoglicol como

estabilizantes foram testados. Em seguida, a imobilização de duas das principais enzimas para a hidrólise dos polissacarídeos pécticos, PL e PG, foram estudadas variando a condição de imobilização em suportes glioxil-agarose, vinilsulfona-agarose, MANAE e MANAE-glutaraldeído. A caracterização dos biocatalisadores, em relação atividade recuperada, estabilidades e potencial de reutilização foram estudados.

Por fim, avaliando todas as metodologias de imobilização testadas, bem como as características dos biocatalisadores obtidos, pectinases e celulases foram imobilizadas em macro-esferas de quitosanas e aplicadas em reatores de leito empacotado e leito fluidizado, no quais a clarificação contínua do suco de laranja foi avaliada.

Inicialmente, a partir dos resultados de trabalhos anteriores da preparação de combi-CLEAs do preparado Rohapect® 10L foi verificada perda do biocatalisador devido a difícil recuperação deste do meio reacional, visto que o pequeno tamanho do biocatalisador era facilmente misturado com os sedimentos da clarificação do suco. Desta forma, as partículas magnéticas foram introduzidas a esse protocolo de imobilização buscando superar esse problema. Assim, os CLEAs-magnéticos (CLEA-MP*) e a imobilização na superfície da partícula magnética ativada com glutaraldeído (Enz-Glu-MP*) foram preparados. Os resultados alcançados permitiram concluir que os CLEAs-magnéticos apresentaram as maiores atividades e estabilidades, proporcionando uma maior eficiência, o qual pode ser utilizado por 8 ciclos de clarificação de suco de uva. Entretanto, a menor atração magnética do biocatalisador dificultou a separação deste do suco, ocasionando perdas ao longo dos ciclos de clarificação. Por outro lado, as enzimas imobilizadas na superfície das partículas magnéticas apresentaram boas propriedades magnéticas, porém com baixa atividade e estabilidade, obtendo um menor potencial de reutilização para a clarificação do suco de uva.

A partir destes resultados, o recobrimento das partículas magnéticas com quitosana surgiu como uma possibilidade de alcançar biocatalisadores que apresentassem ambas as

características, alta atividade e estabilidade, aliado a boas propriedades magnéticas para a fácil separação e reutilização. Assim, diferentes metodologias de recobrimento das partículas magnéticas foram realizadas, as quais possibilitaram a obtenção de 3 biocatalisadores com diferentes tamanhos. As nanopartículas magnéticas de quitosana (Nano-CMag) foram preparadas por gelificação ionotrópica, as micropartículas magnéticas de quitosana (Micro-CMag) foram preparadas usando a técnica de reticulação em emulsão e as macropartículas magnéticas de quitosana (Macro-CMag) foram preparadas pelo método de precipitação. Através dos resultados obtidos pode-se verificar que o biocatalisador Macro-CMag apresentou a maior estabilidade térmica entre os biocatalisadores preparados, com um fator de estabilização de 18,4 vezes maior que a enzima solúvel, enquanto que a Nano-CMag apresentou somente 2,2. Esses resultados podem estar diretamente ligados a maior área interna disponível para imobilização e a um aumento de ligações da enzima com esse suporte, criando um biocatalisador mais rígido e compacto, favorecendo, desta forma, uma maior proteção da estrutura tridimensional da enzima. Ao contrário, a Nano-CMag, que se mostrou um biocatalisador mais aberto, favorece uma maior exposição das enzimas às condições reacionais.

Entretanto, essa mesma característica pode ter contribuído para o biocatalisador Nano-CMag alcançar os melhores parâmetros cinéticos e maior recuperação de atividade enzimática, visto a maior facilidade de acesso dessas enzimas imobilizadas ao substrato. Além disso, algumas variações estruturais das enzimas, após sua imobilização, podem ter sido geradas, bem como diferenças na sua orientação e no seu arranjo espacial nos suportes, características que podem dificultar ou facilitar os mecanismos catalíticos cooperativos.

Em relação à estabilidade operacional, o biocatalisador Macro-CMag apresentou as maiores atividades relativas após os 25 ciclos de clarificação para todos os sucos de frutas, destacando, principalmente, o suco de laranja, onde a atividade residual final foi de 85 %. No

entanto, para os sucos de uva e maçã, devido a maior atividade catalítica do biocatalisador Nano-CMag, menores quantidades deste são necessários para alcançar resultados semelhantes ao Macro-CMag na clarificação desses tipos de sucos. Essa diferença pode estar relacionada à composição de cada suco, visto que alguns componentes do suco de uva e maçã podem ter se ligado ao biocatalisador, aumentando os problemas difusionais ou até mesmo inativando as enzimas. Além disso, apesar do menor magnetismo apresentado pelos biocatalisadores Macro-CMag e Micro-CMag, suas separações dos sucos foram facilitadas quando comparada ao Nano-CMag, devido as maiores dimensões desses biocatalisadores.

O seguinte passo realizado foi desenvolvido no Laboratório de Ingeniería de Biocatalizadores y Biotransformaciones do ICP-CSIC, durante o período de doutorado sanduíche. Primeiramente, as enzimas presentes no preparado Rohapect® 10L foram submetidas a diferentes condições de pHs e temperaturas, buscando verificar como cada enzima reage às distintas condições de imobilização. Com esse teste prévio, pode-se verificar que todas as enzimas têm atividades e estabilidade máximas em pH 4, condições próximas às encontradas na maioria dos sucos de frutas. No entanto, quando as enzimas são submetidas a condições alcalinas, necessárias para algumas metodologias de imobilização, suas estabilidades se tornam extremamente baixas. Por exemplo, em pH 10, apenas a CE e a PME são razoavelmente estável a 25 °C, condição necessária para uma imobilização multipontual, enquanto outras enzimas foram rapidamente inativadas mesmo a 4 °C. Desta forma, alguns agentes estabilizantes, como glicerol, dextrana e polietilenoglicol, foram testados, buscando alcançar a estabilidade de todas as frações enzimáticas em pH 10 a 25 ºC.

A partir desse teste foi verificado que 20 % de polietilenoglicol (PEG) apresentou os melhores resultados de estabilização para todas as enzimas do preparado, mantendo atividades próximas a 100 %, quando estas foram incubados em pH 10 a 25 °C. Além disso, o glicerol apresentou bons resultados para PG, PME e CE com atividades próximas a 100 %, e

proporcionou estabilidades razoáveis para PE e PL com atividades acima de 50 %, enquanto que a dextrana foi o estabilizante ideal para PL, porém para as outras enzimas do preparado, esta apresentou uma pequena melhora na estabilidade enzimática (PE, PG e CE) e até tornou-se negativo para a estabilidade da PME.

Além disso, o efeito da concentração do preparado na estabilidade das enzimas também foi avaliado. Os resultados mostraram que nenhuma diferença na cinética de inativação foi encontrada. Desta forma, pode-se sugerir que a instabilidade enzimática não está ligada a dissociação de subunidade, metais e outros íons, e também não há interações enzima-enzima com efeito estabilizante. Além disso, o extrato parece não conter nenhum estabilizador, pois nesse caso, a diluição da amostra deveria apresentar uma diminuição nas estabilidades enzimáticas. Assim, os resultados obtidos nessa etapa foram utilizados para os seguintes passos.

Em seguida, a imobilização das principais enzimas do preparado Rohapect® 10L foram estudadas em separados, buscando verificar se as mesmas apresentam diferentes condições ideias de imobilização, as quais poderiam proporcionar uma imobilização direcionada, visualizando um possível arranjo espacial para o sistema multi-enzimático, onde as enzimas que atuam primeiro na hidrólise do substrato poderiam ser imobilizadas por último, melhorando as reações sequenciais. No entanto, devido a pouca disponibilidade de tempo, a purificação do preparado enzimático não foi realizada, sendo a imobilização conduzida com o extrato bruto, porém com foco nas enzimas PL e PG.

Assim, primeiramente, a imobilização da PL em diferentes suportes a base de agarose foi estudada. Devido aos resultados pouco satisfatórios com a imobilização da PL em glioxil-agarose e vinilsulfona-agarose, estes não foram apresentados. Já em relação ao MANAE, inicialmente, acreditou-se que altas atividades recuperadas tinham sido alcançadas, porém foi verificado que essa alta atividade ocorreu devido à dessorção da enzima do suporte durante a

medição da sua atividade em pH 4,8. Para evitar esta dessorção, as enzimas imobilizadas em MANAE foram incubadas com 1 % de glutaraldeído para a formação ligações covalentes, mas a atividade desapareceu quase completamente. Desta forma, somente a imobilização de PL em MANAE, previamente ativado com glutaraldeído, foi estudada em mais detalhes.

A imobilização da PL em MANAE-glutaraldeído foi realizada em pH 5, 6,5 e 8 e posteriormente incubada em pH 8 por 48 h para aumentar as ligações entre enzima e suporte. Além disso, uma amostra foi imobilizada em pH 8 com alta força iônica (300 mM NaCl). A imobilização em baixa força iônica foi mais rápida, independente do pH. Este resultado deve estar relacionado ao efeito cooperativo da adsorção das enzimas por forças iônicas, seguido pela ligação covalente.

No entanto, as atividades recuperadas dos biocatalisadores ficaram abaixo de 10 %, sendo os melhores resultados obtido com pH 8 (9 %) e os piores em pH 5 (4 %). Estes resultados que já era esperado, visto que problemas difusionais são relatados na imobilização enzimática, principalmente, quando se trabalha com substratos grandes, como é o caso da pectina. A maior queda de atividade no pH 5, também pode estar relacionada a uma pior orientação da enzima no suporte proporcionada por esse pH, seguido de uma interação multipontual gerada pela incubação posterior a pH 8, o qual também pode ter conferido maior estabilidade a esse biocatalisador.

Além disso, outro resultado interessante do trabalho, é a medição da atividade em diferentes condições de temperaturas e pHs. Enquanto a enzima livre perde atividade acima de 60 ºC, sendo quase completamente inativada a 90 ºC, os biocatalisadores aumentaram quase que exponencialmente suas atividades até 90 ºC. Esses resultados podem estar relacionados à diminuição da viscosidade do substrato em elevadas temperaturas, facilitando a mobilidade deste dentro dos poros do suporte e, consequentemente, diminuindo as limitações difusionais. Também em pHs elevados, as enzimas imobilizadas apresentaram maiores

atividades enzimáticas quando comparadas a enzima livre. Por fim, o biocatalisador mais estável (imobilização a pH 5) foi submetido a 5 ciclos de hidrólise de pectina, mantendo mais de 90 % da sua atividade inicial.

Desta mesma forma, o seguinte passo, foi avaliar a imobilização da PG em diferentes condições pH, utilizando suportes glioxil-agarose, vinilsulfona-agarose, MANAE e MANAE-glutaraldeído. Assim, como verificado na imobilização da PL, o suporte MANAE-glutaraldeído apresentou os melhores resultados, sendo a imobilização neste suporte estudada em detalhes. As imobilizações em MANAE-glutaraldeído foram conduzidas em pH 5, 6,5 e 8, durante 3 h. Após esse tempo, as amostras foram incubadas em pH 8 até 24 h. Uma amostra foi mantida em pH 5 até o final da imobilização e outra amostra foi imobilizada em pH 8 com 300 mM de NaCl.

Diferentes taxas de imobilização foram encontradas para os distintos pHs. A maior taxa foi observada em pH 8, enquanto que a menor taxa foi verificada para o pH 5. Na amostra que foi imobilizada durante todo tempo em pH 5, após 24 h, uma parcela proteica ainda não havia sido imobilizada. Da mesma forma, na amostra com alta força iônica foi detectada uma grande quantidade de enzima no sobrenadante da solução de imobilização. No entanto, atividade recuperada dos biocatalisadores parece se beneficiar da imobilização lenta, visto que amostra com alta força iônica (pH 8 e 300 mM de NaCl) alcançou a maior atividade, em torno de 15 %, sendo seguido pela amostra que foi imobilizada durante todo o período em pH 5, apresentando 10 % de atividade recuperada.

Novamente, como no trabalho anterior, os biocatalisadores de PG também apresentaram maiores atividades em condições extremas de reação, altas temperaturas e pHs. Como se pode verificar, acima de 60 ºC ou de pH 6, os biocatalisadores apresentaram maiores atividades que a enzima livre, destacando a PG imobilizada em pH 5 por 24 h. Os biocatalisadores também apresentaram maiores estabilidades térmicas quando comparados à

enzima livre. Apesar da proximidade dos resultados de estabilidade para os diferentes biocatalisadores, houve uma variação conforme as condições de inativação térmica. Por exemplo, em pHs menores a PG imobilizada em pH 8 com 300 mM de NaCl, apresentou as menores estabilidades. Enquanto que no maior pH de inativação, a enzima imobilizada em pH 5 (24 h) apresentou a menor estabilidade. Entretanto, como era esperado, a PG imobilizada em pH 5 e, posteriormente, incubada a pH 8, alcançou uma maior estabilidade quando comparada a enzima imobilizada somente em pH 5, mostrando que a incubação em pH alcalino permitiu uma ligação covalente multipontual mais intensa. Esse fator também pode ter contribuído para a melhor estabilidade operacional dos biocatalisadores que foram incubados, posteriormente, a pH 8 em baixa força iônica, o quais apresentaram as menores perdas de atividade ao longo dos 10 ciclos de reusos.

Por fim, analisando as metodologias de imobilização testadas, bem como as características dos biocatalisadores preparados, visto a elevada estabilidade em conjunto a uma fácil separação do meio reacional, as pectinases e celulases foram imobilizadas em macroesferas de quitosanas e aplicadas em reatores de leito empacotado e leito fluidizado, nos quais a clarificação contínua do suco de laranja foi avaliada. Devido as maiores dimensões desse suporte, as partículas magnéticas não foram necessárias para a separação do biocatalisador do meio reacional. Assim, estas foram retiradas do protocolo de preparação do suporte, proporcionando um maior número de grupos amino livres da quitosana, onde uma elevada carga de enzima pode ser imobilizada.

Nesse trabalho foi utilizado o preparado enzimático Novozym® 33095 devido a grande necessidade de enzimas para a construção dos reatores. Entretanto, esse preparado, previamente, já havia apresentado resultados semelhantes ao preparado Rohapect® 10L para a clarificação de sucos. Em relação a imobilização enzimática, apesar da baixa atividade recuperada, uma boa atividade por grama de biocatalisador foi alcançada (1340 $U.g^{-1}$). Além

disso, as enzimas imobilizadas também apresentaram maiores atividades em condições extremas de reação. Por exemplo, quando a atividade foi medida a 90 °C, as enzimas imobilizadas apresentaram 80 % de sua atividade inicial, enquanto a enzima livre reteve apenas 35 %. Outro ponto positivo foi a menor inativação térmica encontrada para as enzimas imobilizadas a 60 ºC.

Após a caracterização do biocatalisador, este foi utilizado para a construção de reatores de leito empacotado e leito fluidizado, onde a clarificação contínua do suco de laranja foi avaliada. Inicialmente, diferentes vazões foram testadas (0,5 a 3,0 $mL.min^{-1}$), no qual foi verificado uma queda inicial mais acentuada no rendimento do reator de leito fluidizado em comparação ao reator de leito empacotado, com o aumento da vazão de suco. Esse resultado pode estar relacionado ao menor tempo de residência do suco nesse reator, em conjunto a uma menor quantidade de biocatalisador por volume de reação, quando comparado ao reator de leito empacotado. No entanto, nas maiores vazões testadas, essa redução no rendimento também foi observado para o reator de leito empacotado, o qual pode estar relacionado ao fato desse reator promover uma redução inicial da turbidez pela retenção de partículas insolúvel do suco, promovendo, de certa forma, uma filtração. No entanto, o acúmulo dessas partículas no seu interior pode causar problemas de difusão, dificultando o acesso do substrato aos biocatalisadores. Assim, o menor fluxo testado (0,5 $mL.min^{-1}$) foi escolhido para a clarificação contínua do suco de laranja em ambos reatores.

Para o reator de leito fluidizado, após uma redução inicial do potencial de clarificação, o mesmo apresentou uma estabilização em torno de 60 % da sua capacidade com 72 h de uso contínuo. Enquanto no reator de leito empacotado, foi observada uma diminuição quase linear da capacidade de clarificação durante as 54 h de trabalho contínuo, apresentando uma atividade relativa próxima a 38 % após esse tempo. Esse resultado também pode estar relacionado ao acúmulo de partículas no interior desse reator, o qual ocasionou a formação de

zonas mortas e caminhos preferenciais ao longo do leito, reduzindo a eficiência desse reator devido à diminuição do volume realmente útil. Apesar desse fato, este reator pode ser corretamente utilizado, mediante periódicas etapas de lavagem para remoção interna das partículas.

CONCLUSÃO E PERSPECTIVAS

Como conclusão geral, pode-se afirmar que o trabalho atingiu os objetivos propostos, desenvolvendo biocatalisadores de pectinases e celulases com elevadas estabilidades, podendo ser utilizados por vários ciclos de clarificação de sucos, bem como na clarificação contínua por meio da construção de reatores enzimáticos. Essa tecnologia se mostra bastante promissora com grande potencial para aplicação industrial, trazendo contribuições para a eficiência do processo produtivo e para o aumento da qualidade dos sucos de frutas. As principais conclusões obtidas foram:

- A preparação de biocatalisadores magnéticos de pectinases e celulases pode ser um método adequado de imobilização enzimática para aplicação na clarificação de suco de frutas, visto sua maior facilidade para a separação dos biocatalisadores das partículas insolúveis dos sucos.
- O uso combinado de quitosana e partículas magnéticas como suporte para imobilização enzimática mostrou-se uma estratégia interessante para a clarificação do suco de frutas, visto que o revestimento permitiu melhorar a área superficial do suporte, mantendo as propriedades magnéticas que facilitam a separação dos biocatalisadores do meio reacional.
- As propriedades morfológicas, estruturais e texturais dos suportes têm influencia direta nas características dos biocatalisadores. Enquanto suportes grandes e porosos conferem uma maior estabilidade às enzimas, suportes pequenos com maior área superficial proporcionam maiores atividades recuperadas e melhoras nos parâmetros cinéticos.
- Durante a clarificação dos sucos de frutas, componentes presentes na composição dos mesmos, como polifenóis, proteínas, amido, entre outros, podem se ligar aos

biocatalisadores, contribuindo para o aumento dos problemas de difusão e reduzindo a eficiência catalítica.

- O uso de 20 % polietilenoglicol mostrou ter efeitos positivos para a estabilização em pH elevado de todas as enzimas analisadas presentes no preparado Rohapect® 10L. Desta forma, aumentando as condições de manuseio, importante para o desenvolvimento de algumas estratégias de imobilização, visto que as mesmas usam valores de pH neutro ou alcalino. Condição que promoveria a perda de atividade do extrato.
- As baixas atividades recuperadas após a imobilização de pectinases e celulases podem estar relacionadas à distorção e rigidez da estrutura enzimática, combinado ao grande tamanho do substrato. No entanto, esses problemas são parcialmente superados quando temperaturas mais elevadas de reação são utilizadas, as quais reduzem a viscosidade do substrato, diminuindo os problemas difusionais e, consequentemente, facilitando o acesso do substrato as enzimas imobilizadas, que apresentam maior resistência frente às condições adversas de reação quando comparado às enzimas livres.
- Os diferentes pHs utilizados para a imobilização de PL e PG influenciaram a troca iônica das enzimas com os suportes, as quais podem ter proporcionado distintas orientações enzimáticas, resultando em diferentes atividades recuperadas. Além disso, a posterior incubação das amostras em pH alcalino (pH 8) favoreceu a formação das ligações covalentes multipontuais, aumentando a estabilidade do biocatalisador.
- A construção de biorreatores de leito empacotado e leito fluidizado, contendo enzimas imobilizadas para a clarificação contínua de suco de laranja apresentou bons resultados, onde o reator de leito fluidizado apresentou os melhores rendimentos produtivos ao longo do tempo, possivelmente devido à melhor transferência de massa

desse reator. Por outro lado, o reator de leito empacotado apresentou zonas mortas e caminhos preferenciais, diminuindo a eficiência do reator.

Este trabalho traz uma nova visão para o desenvolvimento de uma tecnologia promissora na clarificação de sucos de frutas industriais. Desta forma, abrem-se perspectivas para trabalhos futuros, buscando a inovação e as melhorias tecnológicas.

Assim, uma das ideias sugeridas é a purificação desse preparado enzimático comercial seguido pela imobilização em multicamada das enzimas isoladas. Através dessa metodologia de imobilização pode-se organizar o arranjo espacial do sistema multi-enzimático no biocatalisador, onde as enzimas que atuam primeiro na hidrólise do substrato serão imobilizada na parte externa do suporte, diminuindo o tamanho do substrato e facilitando sua infiltração pelos poros até alcançar as demais camadas de enzimas, melhorando as reações sequenciais.

Além disso, outros reagentes bifuncionais poderiam ser utilizados para ligar as enzimas covalentemente ao MANAE e a quitosana, como, por exemplo, a genipina que tem demonstrado bons resultados na imobilização de enzimas, dando origem a biocatalisadores ativos e estáveis, além de apresentar baixa citotoxicidade.

Outra sugestão para a clarificação contínua dos sucos de frutas é o desenvolvimento de um biocatalisador com boas propriedades magnéticas para a construção de um reator enzimático envolvido por um campo magnético, o qual irá promover a dispersão dos biocatalisadores através da força de repulsão, mantendo os mesmos dispersos no interior do reator, facilitando a transferência de massa que pode conferir aos sucos uma eficiente clarificação.

Por fim, a avaliação da composição físico-química, bem como das propriedades sensoriais surgem como uma necessidade para atestar a qualidade dos sucos preparados pelos métodos proposto no trabalho.

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ANEXO 1 – Ficha técnica Rohapect 10L

ROHAPECT 10L

COATEC

DESCRIÇÃO

ROHAPECT 10L é um complexo enzimático de aplicação no processamento de sucos de frutas.
Ela tem as seguintes características:

- produto líquido
- coloração marrom com aroma característico
- peso específico: ~1,18 g/mL
- atividade enzimática: > 70.000 ADJU/mL

APLICAÇÃO

ROHAPECT 10L utilizada universalmente e quebra a pectina solúvel e insolúvel, bem como arabanos. No processamento da fruta é qualificada por redução da viscosidade, maior extração de suco, melhor prensagem – e tratamento do suco, clarificação e despectinização total. ROHAPECT 10L é recomendada para despectinização de sucos de baixo pH, como de limão e lima.

APLICAÇÃO	CONDIÇÕES DE REAÇÃO	DOSAGEM
Maça, Pêra Tratamento do Suco Tratamento do Mosto	50ºC (30 – 60min) 20 – 30ºC (60min)	20 – 30g/1000L 5 – 10g/hL
Framboesa, Amora Silvestre, Morango, Amora Preta, Groselha, Cereja, etc.	Prensagem: 50ºC (1 – 3h) Suco: 20 – 45ºC (1 – 6h)	80 – 200g/t 4 – 8g/hL
Suco de Lima, Limão	20-35ºC (1 – 3h)	6 – 10g/hL
Frutas tropicais: Goiaba, Banana, etc.	Conforme processo 20–50ºC (1 – 3h)	30 – 60g/t

PRODUÇÃO

A pectinase é produzida a partir do microorganismo Aspergillus Niger. Estes são cultivados com a ajuda de substratos naturais. As enzimas são extraídas com água, purificadas, concentradas e padronizadas.

- IUB-No.: 3.2.1.15
- CAS-No.: 9032-75-1

ESPECIFICAÇÕES

Requerimentos correspondentes as recomendações estabelecidas pela FAO/WHO´s Joint Expert Comittee for Food Additives (JECFA) e Food Chemicals Codex (FCC).

- arsênico: <3ppm
- chumbo: <2ppm
- metais pesados: <30ppm
- micotoxinas: Negativo
- atividade antibact.: Negativo
- pureza microbiológica: <30 por g
- coliformes: negativo em 25
- E. coli: negativo em 25g
- Salmonela: negativo em 25g
- Viabilidade total: g $<5x10^4$ por g

CONSERVAÇÃO

Em lugar seco e fresco, a perda de atividade em um ano será inferior a 10%.

EMBALAGEM

Bombonas de 25Kg
Frascos de 1Kg (produto fracionado denomina-se COAPECT 10L)
Um produto com a qualidade AB Enzymes.

AMAZON GROUP

AMAZON GROUP PRODUTOS PARA BEBIDAS LTDA
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ANEXO 1 – Ficha técnica Novozym 33095

Novozym® 33095

Valid from 2010-02-16

Product Characteristics:

Declared enzyme	Pectin lyase
Declared activity	10000 PECTU/ml
Other activities	Polygalacturonase
Colour	Brown Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity.
Physical form	Liquid
Approximate density (g/ml)	1.16
Stabilisers	Glycerol Potassium chloride
Preservatives comment	No preservatives added
Odour	Slight fermentation odour
Solubility	Active component is readily soluble in water at all concentrations that occur in normal usage. Standardisation components can cause turbidity in solution.
Production organism	Aspergillus aculeatus Aspergillus niger
Production method	Produced by fermentation of micro organisms. The enzyme protein is separated and purified from the production organisms.

Product Specification:

	Lower Limit	Upper Limit	Unit
Pectinase Unit PECTU	10000		/ml
Total Viable Count		50000	/g
Coliform Bacteria		30	/g
Enteropathogenic E.Coli	Not Detected		/25 g
Salmonella	Not Detected		/25 g

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Packaging: See the standard packaging list for more information.

Recommended Storage:

Best before	When stored as recommended, the product is best used within 18 months from date of delivery.
Storage at customer's warehouse	0-10°C (32°F-50°F)
Storage Conditions	In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.