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NICOLE SOUZA OKURA

PREPARAÇÃO E CARACTERIZAÇÃO DE UM SUPORTE HETEROFUNCIONAL À BASE DE QUITOSANA E SEU USO NA IMOBILIZAÇÃO DE LIPASE

Alfenas/MG
2021

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“Não tenho medo de errar, só medo de desistir
Mas tenho vinte e poucos e não vou parar aqui.”

(Lagum, 2020)

RESUMO

Neste estudo, um novo suporte heterofuncional (Qui-Glu-Gli) foi preparado pela ativação sequencial do hidrogel de quitosana (Qui) com glutaraldeído (Glu) e posterior funcionalização com glicina (Gli). A imobilização da lipase de *Thermomyces lanuginosus* (TLL) neste suporte foi comparada com o hidrogel de quitosana ativado com glutaraldeído (Qui-Glu) e hidrogel sem modificação química (Qui). Os suportes foram caracterizados por análises de espectroscopia no infravermelho (FT-IR), potencial zeta (ZP) e de termogravimetria (TGA) para confirmar a introdução de grupos funcionais na superfície do biopolímero. Inicialmente, foi avaliado o efeito do pH no processo de imobilização nos diferentes suportes preparados empregando baixo carregamento de proteína (5 mg/g). Estes testes foram conduzidos na razão suporte:solução enzimática de 1:20, baixa força iônica (5 mM), 25ºC e 18 h de contato sob agitação mecânica em shaker orbital (200 rpm). Completa adsorção da lipase no suporte Qui-Glu-Gli foi observada em meio ácido (pH entre 3,0 e 6,0), enquanto no suporte Qui-Glu não foi observada influência do pH de imobilização (completa imobilizada na faixa de pH entre 4,0 e 10,0). Por outro lado, o suporte sem modificação química (Qui) foi parcialmente solubilizado em meio ácido (pH 4,0) e máxima adsorção de 1,5 mg proteína/g de hidrogel foi obtida em pH 7,0. Com relação ao efeito do carregamento de proteína variando de 5 a 70 mg/g, máxima concentração de lipase imobilizada de 53-55 mg por grama de suporte foi obtida para ambos os suportes Qui-Glu e Qui-Glu-Gli. A adsorção da lipase neste novo suporte foi explicada pelo modelo de isoterma de Langmuir (R^2 = 0,9545). Este modelo assume que a adsorção de moléculas de TLL na superfície do suporte ocorre na forma de monocamadas e que este material possui um número finito de sítios de adsorção. Os biocatalisadores preparados empregando Qui-Glu-Gli como suporte foram 4 vezes mais ativos na hidrólise da emulsão de azeite de oliva do que aqueles preparados com Qui-Glu – 1000 U/g e 265,1 ± 14,4 U/g respectivamente. Estes dois biocatalisadores mantiveram ≈40% de sua atividade inicial após 48 h de incubação a 50°C em solventes orgânicos apolares (heptano, tolueno ou isooctano). Testes de dessorção realizados em solução de NaCl e Triton X-100 com diferentes concentrações a 50ºC, revelaram que a imobilização da TLL em Qui-Glu ocorreu preferencialmente por ligação covalente (95%). No entanto, a imobilização em Qui-Glu-Gli ocorreu preferencialmente via adsorção por interação iônica – 65% (catiônica e aniônica), interação hidrofóbica (20%) e ligação covalente (15%). A atividade catalítica dos biocatalisadores preparados foi também avaliada em reações de esterificação de ácido palmítico em meio de isooctano, solvente selecionado nos testes de estabilidade em solventes, a 50ºC empregando razão equimolar de ácido e álcoois (500 mM de cada reagente) em 70 min de reação e agitação contínua em shaker orbital de 240 rpm. Estes testes foram conduzidos empregando diferentes álcoois como isoamílico, hexanol, 2-etil-1-hexanol e decanol. TLL imobilizada em Qui-Glu-Gli foi também ≈4 vezes mais ativa na produção de ésteres de ácido palmítico. Máxima conversão do ácido de 80,3 ± 0,6% e 23,7 ± 2,5% foi obtida para TLL imobilizada em Qui-Glu-Gli e Qui-Glu, respectivamente, empregando álcool isoamílico. Em seguida, foi avaliado o efeito do tempo de reação na produção do éster empregando o álcool selecionado e a máxima conversão do ácido da ordem de 85% após 90 min de reação foi obtida para o sistema de reação conduzido com o novo biocatalisador preparado neste estudo (TLL imobilizada em Qui-Glu-Gli). Após nove sucessivas bateladas de esterificação, o biocatalisador reteve cerca de 40% de sua atividade inicial. Estes resultados mostram claramente a relevância do novo suporte para a imobilização de TLL para catalisar reações em meio aquoso (hidrólise da emulsão de azeite de oliva) e em orgânico (produção de ésteres lubrificantes).

Palavras-chave: Suportes heterofuncionais, Hidrogéis, Quitosana, Imobilização, Lipase, Enzima, Propriedades catalíticas.

ABSTRACT

In this study, a new mixed heterofunctional support (Chit–GA–Gly) has been prepared by sequential activation of chitosan hydrogel (Chit) with glutaraldehyde (GA) and further functionalization with glycine (Gly). The immobilization of the lipase from *Thermomyces lanuginosus* (TLL) on this support was compared with that on GA-activated Chit hydrogel (Chit–GA). The supports have been characterized by FT–IR, zeta potential and TG analyses to confirm the introduction of functional groups on the support surfaces. Initially, the effect of immobilization pH on TLL immobilization process has been evaluated for the different prepared supports using a low protein loading (5 mg/g). These tests have been performed at low ionic strength (5 mM) using a volume ratio support:lipase solution of 1:20, 18 h of contact and mechanical agitation in an orbital shaker at 200 rpm. Complete immobilization of TLL on the new support (Ghit-GA-Gly) has been observed at acidic medium (pH range from 3.0 to 6.0). On the other hand, TLL was fully immobilized on Chit–GA under a broad pH range varying from 4.0 to 10.0. The immobilization of TLL on non-chemically modified chiotsan hydrogel (Chit) was not possible at acidic medium (pH 4.0) due to a partial solubilization of the support and maximum immobilized protein concentration of 1.5 mg/g of hydrogel was obtained at pH 7.0. The effect of offered protein loading varying from 5 to 70 mg/g was also studied and a similar maximum lipase loading of 53-55 mg per gram of support has been obtained for both supports (Chit-GA and Chit-GA-Gly). Langmuir isotherm model obtained better fit to the experimental data obtained by both prepared supports The adsorption process of TLL on the new prepared support was better fitted to the Langmuir isotherm model on account of achieving higher correlation coefficient ($R^2 = 0.9545$). This model assumes monolayer adsorption of TLL molecules on a support surface and the adsorption of each lipase molecule on the adsorbent surface has the same adsorption activation energy. The catalytic activity (olive oil emulsion hydrolysis) of the prepared heterogeneous biocatalyst using Chit-GA-Gly as support was 4-times higher than TLL immobilized on classical Chit-GA – 1000 U/g and 265.1 ± 14.4 U/g, respectively. Both biocatalysts retained ≈40% of their initial activity after 48 h of incubation at 50°C in apolar organic solvents (heptane, toluene or isooctane). Desorption tests performed under different conditions (NaCl + Triton X-100 at different concentrations) revealed that TLL was preferentially immobilized on Chit-GA via covalent attachment (95%). On the other hand, TLL was immobilized on Chit-GA-Gly via ionic interactions (65%), followed by interfacial activation (hydrophobic interactions (20%) and covalent attchment (15%). The catalytic activity of the prepared biocatalysts on the esterification of palmitic acid with several alcohols (isoamyl alcohol, hexanol, 2-ethyl-1-hexanol and decanol) in isooctane medium, apolar organic solvent chosen in stability tests, was also performed. The esterification reactions were conducted under fixed experimental conditions: 50 ºC, stoichiometric acid:alcohol molar ratio (500 mM of each reactant) at 70 min of reaction under mechanical agitation in an orbital shaker at 240 rpm. TLL immobilized on Chit–GA–Gly was ≈4-times more active than when immobilized on Chit–GA in both olive oil emulsion hydrolysis and alkyl palmitate synthesis via esterification. Maximum acid conversion of 80.3 ± 0.6% and 23.7 ± 2.5% using TLL immobilized on Chit-GA-Gly and Chit-GA, respectively, was achieved for isoamyl alcohol. In That way, isoamyl palmitate synthesis in iso-octane at 50 ºC using this new biocatalyst gave a maximum acid conversion of 85% after 90 min of reaction. After nine consecutive esterification batches, the biocatalyst retained around 40% of its initial activity. These results show clearly that the new biocatalyst prepared in this study is promising to catalyze reactions of industrial interest in both aqueous (olive oil emulsion hydrolysis) and organic (lubricant ester production) media.

Keywords: Heterofunctional supports, Hydrogels, Chitosan, Immobilization, Lipase, Enzyme, Catalytic properties.

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LISTA DE ABREVIATURAS

AGL	Ácidos graxos livres
CLEAs	Ligação cruzada de agregados enzimáticos
DD	Grau de desacetilação
FT-IR	Espectroscopia no infravermelho com transformada de Fourier
GlcN	Glucosamina
GlcNAc	N-acetilglucosamina
Gli	Glicina
Glu	Glutaraldeído
Qui	Quitosana
Qui-Glu	Suporte de Quitosana-Glutaraldeído
Qui-Glu-Gli	Suporte preparado de Quitosana-Glutaraldeído-Glicina
TGA	Análise Termogravimétrica
TLL	Lipase de *Thermomyces lanuginosus*
ZP	Potencial Zeta

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1 INTRODUÇÃO

As lipases (triacilglicerol acilhidrolases - EC 3.1.1.3) são uma classe importante de enzimas que catalisam a hidrólise de diversos triacilgliceróis (óleos vegetais e microbianos, gorduras animais e óleos residuais) em mono-, diacilglicerois e ácidos graxos livres (AGL) na interface óleo/água. Em sistemas não aquosos (meios orgânicos), elas também catalisam reações de esterificação, transesterificação e interesterificação para a produção de ésteres de interesse industrial como biodiesel, biolubrificantes, ésteres de aroma, lipídios estruturados, polímeros, emolientes, surfactantes, dentre outros (FERNANDEZ-LAFUENTE, 2010; ADLERCREUTZ, 2013; BASSO; SERBAN, 2019; BOLINA *et al*., 2021). O mercado global de lipases foi de 590,5 milhões de dólares até o ano de 2020, com uma taxa de crescimento anual de 6,5% entre 2015 e 2020 (MARKETS AND MARKETS, 2020). Atualmente, presume-se que esse mercado atingirá 1,5 bilhão de dólares em 2026 (RESEARCH AND MARKETS, 2020). Essas enzimas têm sido amplamente utilizadas em diversas indústrias como alimentícia, biocombustível, têxtil e couro, celulose e papel, tratamento de águas residuais, química fina, farmacêutica e cosmética (GLOBE NEWS WIRE, 2020).

O uso de lipases nas formas solúveis ou em pó (livres) em alguns processos industriais é dificultado devido à sua baixa estabilidade e redução de sua atividade durante o armazenamento, recuperação cara e/ou ineficiente para subsequente reuso no processo (ADLERCREUTZ, 2013; BOLINA *et al*., 2021). Porém, essas limitações podem ser superadas por aplicação de protocolos de imobilização com o intuito de aumentar a sua estabilidade contra agentes desnaturantes como temperatura de reação, pH e solventes orgânicos e proteólise (hidrólise de enzimas por ação de proteases); facilitar sua recuperação do meio de reação no final dos processos que possibilita posterior reuso em múltiplos ciclos catalíticos, com redução concomitante dos custos nos processos *downstream* (etapas de separação e purificação); evitar a agregação enzimática, na qual melhora sua atividade catalítica e especificidade em várias reações); e aumentar a flexibilidade de configurações de reatores – modos batelada e contínuos como reatores de leito fixo e fluidizado, reatores contínuos agitados e fluxo pistonado (ADLERCREUTZ, 2013; BASSO; SERBAN, 2019). As lipases têm sido imobilizadas por diferentes métodos tais como: (i) adsorção física por interações hidrofóbicas, (ii) adsorção em suportes de troca iônica (adsorção iônica), (iii) aprisionamento em suportes inertes, (iv) ligação covalente em suportes ativados e (v) *crosslinking* via formação de lipases insolúveis - enzimas

agregadas (CLEAs) (MATEO *et al.*, 2007; FERNANDEZ-LAFUENTE, 2010; ADLERCREUTZ, 2013; BEZERRA *et al.*, 2015).

O protocolo de imobilização mais popular na imobilização de lipases é baseado na sua adsorção física em suportes hidrofóbicos via "ativação interfacial" (MATEO *et al.*, 2007; ADLERCREUTZ, 2013; RODRIGUES *et. al.*, 2019). A maioria das lipases possui uma cadeia polipeptídica (tampa ou lid) com uma face hidrofílica e outra hidrofóbica. Na conformação fechada das lipases, a área hidrofóbica desta tampa interage com as áreas hidrofóbicas de seus sítios ativos, tornando-se inacessível às moléculas de substrato como gotas de óleo. Na presença de uma interface hidrofóbica, como alguns tipos de suportes ou gotas de óleo, essa tampa sofre uma mudança conformacional expondo o sítio ativo e a sua região hidrofóbica para o meio – "conformação aberta". Em meios com alta concentração de íons, estas enzimas encontram-se preferencialmente na conformação fechada devido à sua forte agregação – formação de agregados bimoleculares. Neste sentido, a imobilização de lipases por adsorção física via ativação interfacial tem sido realizada em meios com baixa força iônica para evitar a formação destes agregados e para deslocar o equilíbrio para a conformação aberta (MANOEL *et al.*, 2015; RODRIGUES *et al.*, 2019).

No entanto, a imobilização de lipases em suportes iônicos e a ligação covalente em suportes ativados também se mostram estratégias promissoras na preparação de biocatalisadores ativos e estáveis para posterior utilização em processos industriais (MATEO *et al.*, 2007; ADLERCREUTZ, 2013; Bezerra *et al.*, 2015). Com relação à adsorção iônica, este protocolo tem sido amplamente utilizado não somente na preparação de biocatalisadores, como também em processos de separação e purificação de diferentes enzimas (FUENTES *et al.*, 2004; FUENTES *et al.*, 2007a,b; GARCIA-GALÁN *et al.*, 2011). Este processo de imobilização também é conduzido em baixa força iônica para evitar possível competição entre os íons da solução enzimática com grupos iônicos da estrutura da enzima e/ou grupos iônicos pré-existentes na superfície dos suportes (FUENTES *et al.*, 2004; FUENTES *et al.*, 2007a,b; GARCIA-GALÁN *et al.*, 2011; RUEDA *et al.*, 2016; VIRGEN-ORTIZ *et al.*, 2016). A imobilização de enzimas por adsorção iônica é um processo multipontual e requer suportes heterofuncionais, grupos carregados positivamente (trocadores âniônicos) e negativamente (trocadores catiônicos), para obter elevada interação entre enzimas e estes grupos iônicos existentes nos suportes. Estudos na literatura mostram que a aplicação de suportes monofuncionais para a adsorção iônica de enzimas apresenta baixa eficiência de imobilização devido à complexa natureza das enzimas que possui uma ampla e diversificada densidade de

grupos carregados positivamente e negativamente em suas superfícies (FUENTES *et al.*, 2004; FUENTES *et al.*, 2007a,b; RUEDA *et al.*, 2016; VIRGEN-ORTIZ *et al.*, 2016).

Uma variedade de suportes orgânicos e inorgânicos naturais ou sintéticos têm sido geralmente aplicada para esse fim (BEZERRA *et al*., 2015; BILAL; IQBAL, 2019; ZHONG *et al*., 2020). Neste sentido, o uso de polímeros naturais como polissacarídeos (celulose, agarose, amido, quitina, quitosana, pectina, alginato e carragenina) e proteínas (colágeno, e gelatina) desperta grande atenção devido às suas características promissoras, como biodegradabilidade, biocompatibilidade, ausência de toxicidade e por conta da possibilidade de serem modificados quimicamente usando uma variedade de agentes ativadores como glicidol, glutaraldeído, epicloridrina dentre outros (BEZERRA *et al*., 2015; BILAL; IQBAL, 2019).

A quitosana é um polissacarídeo linear policatiônico obtido por desacetilação parcial da quitina, o segundo biopolímero mais abundante após a celulose (MONTEIRO; AIROLDI, 1999). Estes materiais têm sido preparados a partir de resíduos de animais marinhos, principalmente caranguejos, mariscos e camarão (VERMA *et al*., 2020). É um produto natural, de baixo custo, renovável e biodegradável e de grande importância econômica e ambiental (QIN *et al*., 2020; VERMA *et al*., 2020). As carapaças de crustáceos são resíduos abundantes e rejeitados pela indústria pesqueira que, em muitos casos, as consideram poluentes. Sua utilização reduz o impacto ambiental causado pelo acúmulo nos locais onde é gerado ou estocado (MONTEIRO; AIROLDI, 1999). Esse biopolímero tem sido amplamente utilizado em sistemas de liberação controlada de fármacos, desenvolvimento de engenharia têxtil, cultivo sustentável de plantas, agente antimicrobiano, adsorvente na remoção de compostos poluentes (íons de metais pesados, corantes e outros materiais orgânicos recalcitrantes) de águas residuais industriais e como suporte na imobilização de enzimas industriais, incluindo lipases (PAKDEL; PEIGHAMBARDOUST, 2018; AHMED *et al*., 2020; QIN *et al*., 2020; SAHRANAVARD *et al*., 2020; VERMA *et al*., 2020; WEI *et al*., 2020). A quitosana tem sido considerada um suporte promissor devido à presença de grupos reativos (grupos hidroxila e amino) em sua estrutura química, capaz de reagir com um grande número de agentes ativadores, como divinil-sulfona e agentes epóxi (epicloridrina e glicidol) e aldeídos (glutaraldeído e outros). A modificação química permite introduzir grupos funcionais para acoplamento com aminoácidos das enzimas e melhorar suas propriedades mecânicas – maior rigidez na estrutura dos biopolímeros (MONTEIRO; AIROLDI, 1999; MENDES *et al*., 2011, MENDES *et al*., 2013; MONTEIRO *et al*., 2019; PINHEIRO *et al*., 2019; RAVISHANKAR; DHAMODHARAN, 2020).

Dentre eles, o glutaraldeído tem sido o agente mais usado na ativação de uma variedade de suportes, incluindo a quitosana, para a imobilização de enzimas (BARBOSA *et al.*, 2014). Os grupos aldeído livres introduzidos nas superfícies de suporte reagem com grupos amino livres de suportes e proteínas com a formação de ligações imina (bases de Schiff, –C = N–), embora também possam eventualmente reagir com outros grupos nucleofílicos das enzimas como imidazol, fenol e tiol (MIGNEAULT *et a*l., 2004; BARBOSA *et al.*, 2014). Esses grupos iminas também podem ser formados entre grupos amino de diferentes cadeias poliméricas de suportes (*crosslinking* intermolecular) ou entre grupos amino na mesma estrutura (*crosslinking* intramolecular), resultando em uma rigidez global de sua estrutura (MONTEIRO; AIROLDI, 1999; BARBOSA; *et al.*, 2014; RAVISHANKAR; DHAMODHARAN, 2020). Os mecanismos envolvidos na reação do glutaraldeído com suportes aminados ou estruturas proteicas são altamente complexos devido à forte influência de alguns parâmetros como concentração, pH da solução, área superficial do suporte e tempo de reação em sua reatividade e estrutura química (MONSAN, 1978; MIGNEAULT *et al.*, 2004; BARBOSA *et al.*, 2014). De fato, estudos anteriores demonstraram diferentes formas de glutaraldeído obtidas em soluções aquosas (MIGNEAULT *et al.*, 2004; BARBOSA *et al.*, 2014). A ativação dos suportes tem sido preferencialmente realizada em condições moderadas (pH ≤ 7,0) devido à sua baixa estabilidade química (reações de polimerização) em condições alcalinas (MONSAN, 1978; BARBOSA *et al.*, 2014). Nestas condições, o glutaraldeído está em equilíbrio entre suas formas monomérica (por exemplo, aldeído livre e formas hemiacetais cíclicas) e polimérica (por exemplo, oligômeros hemiacetais cíclicos) (MIGNEAULT *et al.*, 2004; BARBOSA *et al.*, 2014; VESCOVI *et al.*, 2016). No entanto, alguns estudos anteriores demonstraram que a ativação de suportes amino em pH 7,0 e a concentração de ativação acima de 10% v/v ocorre via ligação covalente de duas moléculas de glutaraldeído (forma dimérica) por grupo amino do suporte, preferencialmente sintetizado via condensação aldólica (MONSAN, 1978; BETANCOR *et al.*, 2006; CAI *et al.*, 2016). A ativação de suportes amino, incluindo quitosana, com glutaraldeído possibilita a preparação de suportes heterofuncionais com diferentes grupos funcionais em suas superfícies capazes de interagir com enzimas via: i) ligação covalente de grupos amino de enzimas com grupos aldeídos livres na superfície de suporte para formar ligações imina; ii) interação iônica de grupos negativos das enzimas com grupos imina protonados (trocadores aniônicos) introduzidos no processo de ativação; e iii) adsorção física via ligações de hidrogênio e interações hidrofóbicas com cadeias carbônicas introduzidas via ativação com dímeros de glutaraldeído (BARBOSA *et al.*, 2014). Estes diferentes mecanismos de interação entre enzimas e superfícies de suporte heterofuncionais são importantes ferramentas para fornecer

aos biocatalisadores industriais alta estabilidade, atividade catalítica e seletividade (BARBOSA *et al*., 2012; VESCOVI *et al*., 2016, RUEDA *et al*., 2016; PINHEIRO *et al*., 2019).

No presente estudo, um novo suporte heterofuncional à base de quitosana (denominado Qui-Glu-Gli) foi preparado por ativação sequencial com glutaraldeído em pH 7,0 e funcionalização com glicina. Esse suporte foi utilizado na imobilização por adsorção de lipase de *Thermomyces lanuginosus* (TLL). O critério de seleção desta lipase foi a sua alta atividade catalítica e estabilidade operacional (reuso) na produção de diferentes ésteres industriais como biolubrificantes, biodiesel e emolientes (FERNANDEZ-LAFUENTE, 2010; ALVES *et al.*, 2016; MIGUEZ *et al.*, 2018; MACHADO *et al.*, 2019). Atualmente, a funcionalização de superfícies de suporte com aminoácidos tem sido aplicada para a preparação de suportes heterofuncionais para melhorar a estabilização, seletividade e atividade catalítica de enzimas industriais como lipases, fosfolipase, glicose 6-fosfato desidrogenase e L-asparaginase (RUEDA *et al*., 2016; BOLINA *et al*., 2018; MONAJATI *et al*., 2018; BRIONES *et al*., 2019; ZHOU *et al*., 2019). O desempenho do novo biocatalisador foi comparado com o da enzima imobilizada no hidrogel de quitosana ativada com glutaraldeído (Qui-Glu) e hidrogel de quitosana não modificado quimicamente (Qui), empregado como controle neste estudo.

Os suportes preparados foram caracterizados por diferentes técnicas como espectroscopia no infravermelho (FT–IR), potencial zeta e análise de termogravimetria (TGA) para confirmar a introdução de grupos funcionais na superfície do biopolímero. Avaliou-se o efeito de fatores relevantes (pH da solução, carga inicial de proteínas e tempo de contato) no processo de adsorção e atividade catalítica dos biocatalisadores preparados. As propriedades catalíticas dos biocatalisadores foram determinadas na hidrólise da emulsão de azeite de oliva e na síntese de ésteres biolubrificantes por reação de esterificação. Testes de reuso do biocatalisador foram realizados após nove ciclos sucessivos de síntese de palmitato de isoamila, um éster de grande relevância industrial devido às suas propriedades lubrificantes (BARBOSA *et al*., 2021). O éster foi produzido por esterificação direta de ácido palmítico, um ácido graxo representativo amplamente encontrado em diferentes óleos vegetais como azeite de dendê, polpa de macaúba, dentre outros, e álcool isoamílico, um álcool proveniente do óleo fúsel (≈70% mol/mol) gerado na destilação do etanol e de fácil obtenção no país (CERÓN *et al.*, 2018). Estas matérias-primas são uma opção atrativa para o setor industrial brasileiro devido à ampla disponibilidade e baixo custo.

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo do presente trabalho consistiu na preparação e caracterização de um novo suporte heterofuncional a partir do hidrogel de quitosana ativado com glutaraldeído e funcionalizado com glicina para a adsorção de lipase de *Thermomyces lanuginosus* (TLL) e posterior aplicação na síntese de éster de interesse industrial.

Levando em consideração este aspecto, o objetivo geral do projeto foi alcançado mediante a execução dos seguintes objetivos específicos:

2.2 OBJETIVOS ESPECÍFICOS

- Preparação do suporte via sequencial preparação dos hidrogeis de quitosana, modificação química com agente bifuncional (glutaraldeído) e funcionalização com glicina (Qui-Glu-Gli);
- Caracterização dos suportes preparados (Qui-Glu-Gli) utilizando diferentes técnicas (FT-IR, ZP, TGA);
- Preparação do biocatalisador heterogêneo;
- Determinação do efeito do pH no processo de imobilização;
- Realização de estudos isotérmicos de carga e de adsorção de proteínas;
- Realização de testes de estabilidade e de dessorção;
- Aplicação do biocatalisador preparado na síntese de éster de interesse industrial e em testes de reutilização.

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3 ARTIGO PUBLICADO EM PERIÓDICO INDEXADO

As atividades desenvolvidas no mestrado foram publicadas no periódico indexado *International Journal of Biological Macromolecules* (https://doi.org/10.1016/j.ijbiomac.2020.07.021). Essas atividades consistiram na preparação e caracterização de um suporte heterofuncional a partir do hidrogel de quitosana ativado com glutaraldeído e funcionalizado com glicina para imobilização da lipase de *Thermomyces lanuginosus* (TLL). Neste artigo, foram determinados os parâmetros de imobilização e posterior aplicação deste biocatalisador em reações de esterificação e verificação da sua reuso após sucessivos ciclos de reações de esterificação e estudos de dessorção para elucidar o processo de imobilização da lipase no novo suporte preparado em comparação com o suporte quitosana ativado com glutaraldeído. De acordo com os resultados obtidos, foi possível preparar um biocatalisador eficiente devido à sua alta capacidade de adsorção de lipase e alta atividade catalítica.

Improved immobilization of lipase from *Thermomyces lanuginosus* on a new chitosan-based heterofunctional support: mixed ion exchange plus hydrophobic interactions

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Abstract

In this study, a new mixed heterofunctional support (Chit–GA–Gly) has been prepared by sequential activation of chitosan hydrogel (Chit) with glutaraldehyde (GA) and further functionalization with glycine (Gly). The immobilization of the lipase from *Thermomyces lanuginosus* (TLL) on this support was compared with that on GA-activated Chit hydrogel (Chit–GA). The supports have been characterized by FT–IR, zeta potential and TG analyses. A similar maximum lipase loading of 53-55 mg per gram of support has been obtained for both supports. Both biocatalysts retained ≈40% of their initial activity after 48 h of incubation at 50°C in heptane, toluene or iso-octane. The immobilization of TLL on Chit–GA proceeded via preferential covalent attachment (95%) and a combined ion exchange (cationic and anionic) and hydrophobic adsorption was observed using Chit–GA–Gly. TLL immobilized on Chit–GA–Gly was ≈4-times more active than when immobilized on Chit–GA in both olive oil emulsion hydrolysis and alkyl palmitate synthesis via esterification. Isoamyl palmitate synthesis in iso-octane at 50 ºC using this new biocatalyst gave a maximum acid conversion of 85% after 90 min of reaction. After nine consecutive esterification batches, the biocatalyst retained around 40% of its initial activity.

Keywords: Heterofunctional supports, Chitosan-based hydrogels, Lipase, Catalytic properties.

1. Introduction

Lipases (triacylglycerol acylhydrolases – EC 3.1.1.3) are an important class of versatile enzymes that naturally catalyze the hydrolysis of triacylglycerols (oils and fats) to glycerol, mono-, di-, and free fatty acids (FFA) at an oil/water interface [1,2]. In non-aqueous systems, they also catalyze esterification [3], alcohohysis [4,5], acidolysis [6], and interesterification [7] reactions. They have been broadly applied in several industries such as food, biofuel, textile and leather, pulp and paper, wastewater treatment, fine chemical, pharmaceutical, and cosmetic [8–10]. The use of free lipases (in liquid or powder forms) in industrial processes is often hindered due to their low long-term stability and gradual decrease of activity during storage, expensive and/or inefficient recovery and subsequent reuse [11]. However, these limitations can be overcome by applying appropriate immobilization techniques to improve their stability against denaturing agents and proteolysis, facilitate their recovery from the reaction media at the end of the processes for subsequent reuse with concomitant reduction of costs in downstream processes, avoid enzyme aggregation, develop processes with multienzymatic cascade reactions and increase the flexibility of reactor designs [11–17].

In the case of lipases, the most popular immobilization protocol is based on their physical adsorption on hydrophobic supports via "interfacial activation", a peculiar mechanism involving their open conformation on hydrophobic surfaces [11,15,18,19]. However, the immobilization of lipases on ion-exchangers and their covalent attachment on pre-existing functionalized supports have also proved to be promising strategies in the preparation of active and stable biocatalysts for further use in industrial processes [11,12,20]. A variety of natural or synthetic organic and inorganic supports has been usually applied for such a purpose [20–22]. In this field, the use of natural polymers such as polysaccharides (cellulose, agarose, starch, chitin, chitosan, pectin, alginate, and carrageenan) and proteins (collagen, hydrophobins and gelatin) has gained increasing attention due to their promising features such as biodegradability,

biocompatibility, non-toxicity and their ability to be chemically modified using a variety of activating agents [20,21,23]).

Chitosan is a polycationic linear polysaccharide obtained by partial deacetylation of chitin, the second most abundant biopolymer in nature after cellulose [24]. This biopolymer has been widely used in drug delivery systems [25], in the development of tissues engineering [26], sustainable plant crops [27], as antimicrobial agent [28], as adsorbent in the removal of pollutant compounds (heavy metal ions, dyes and other recalcitrant organic materials) from industrial wastewaters [29], and as support in the immobilization of industrial enzymes, including lipases [30]. Chitosan has been considered a promising support due to the presence of different reactive groups (hydroxyl and amino groups) in its structure that are able to react with a great number of activating agents such as divinyl sulfone, epoxy- and aldehyde-based agents, etc. This chemical modification allows introducing functional groups in the biopolymer for coupling with amino acids and proteins and to improve its mechanical properties [24,31–35].

Glutaraldehyde is the most used bifunctional agent in the activation of a variety of supports, including chitosan, for the immobilization of enzymes via covalent attachment [24,36,37]. The glutaraldehyde groups introduced on the support surfaces react with other free amino groups of supports and proteins or with amino groups that have already reacted with one glutaraldehyde molecule, although it can also eventually react with other nucleophilic groups such as imidazole, phenol and thiol [36,37]. These reactions can also occur between groups of different polymeric chains of the support (intermolecular crosslinking) or between groups in the same chain (intramolecular crosslinking), resulting in a rigidification of the support structure [24,35,37]. The mechanisms involved in the reaction of glutaraldehyde with amino supports or protein structures are highly complex due to the strong influence of some parameters such as glutaraldehyde concentration, medium pH, density of groups in the support and reaction time on its reactivity and chemical structure [36–38]. In fact, previous reports have

demonstrated the different forms of glutaraldehyde obtained in aqueous solutions [36,37]. In an overview published by Migneault *et al.* [36], thirteen possible molecular forms of glutaraldehyde in aqueous solution have been identified based on previous reports. The activation of several supports has been preferentially performed under moderate conditions (pH≤ 7.0) due to the low chemical glutaraldehyde stability and the occurrence of glutaraldehyde polymerization reactions under alkaline conditions [37,38]. Some previous reports have demonstrated that the activation of amino supports at pH 7.0 and glutaraldehyde concentration of 10% (v/v) proceeds via covalent attachment of two molecules of glutaraldehyde per amino group of the support [37–40], which has been synthesized via aldolic condensation [41], a chemical reaction between glutaraldehyde molecules to form α,β-unsaturated aldehydes [36]. The activation of pre-existing amino supports, including chitosan, with glutaraldehyde promotes the preparation of heterofunctional supports with different functional groups on their surfaces able to interact with enzymes via chemisorption (covalent attachment) and physisorption (ionic interactions, hydrogen bonds and interfacial activation) [37]. These different mechanisms of interaction between enzymes and heterofunctional support surfaces are an important tool for providing industrial biocatalysts with high stability, catalytic activity and selectivity [34,42–44].

In the present study, a new chitosan-based heterofunctional support (labelled Chit–GA–Gly) was prepared via sequential activation with glutaraldehyde at pH 7.0 and further functionalization with glycine. The final composite will present amino groups on the support that are glutaraldehyde modified but still with cationic properties, the moderate hydrophobicity of the glutaraldehyde moiety (two glutaraldehyde molecules per amino group), and a carboxylate and an amino group attached to the glutaraldehyde groups. The most exposed moieties, that is, those which will more easily interact with the proteins, are the mixed ionic groups from glycine. It has been previously shown that mixed ionic supports, bearing cationic

and anionic residues, may become interesting to ionically immobilize proteins by a mixed anion/cation exchange [45,46]. This, further reinforced by the hydrophobic interaction with the glutaraldehyde moieties, may permit a strong immobilization of enzymes.

This new heterofunctional support was used in the immobilization of a very interesting lipase, that from *Thermomyces lanuginosus* – TLL [47]. The performance of the new biocatalyst was compared with that of the enzyme immobilized on chitosan hydrogel activated with glutaraldehyde (Chit–GA) and non-activated chitosan hydrogel (Chit). These supports were characterized by several techniques (FT–IR, Zeta potential and TG analyses) to confirm the introduction of the different functional groups on the chitosan structure. The functionalization of the support surface with amino acids has been applied for the preparation of heterofunctional supports, and in other cases to improve the stabilization, selectivity or catalytic activity of industrial enzymes such as lipases [48–50], phospholipase [48], glucose 6-phosphate dehydrogenase [51], and *L*-asparaginase [52]. The effects of immobilization pH and protein loading on the adsorption process and catalytic activity of the obtained biocatalysts were evaluated. The stability of the biocatalysts was assessed by incubating them in different organic solvents. Reusability tests were performed using the esterification reaction to produce isoamyl palmitate as model reaction, a compound with lubricant [53,54], and good cold flowing [55] properties.

2. Materials and methods

2.1. Materials

TLL, a liquid lipase preparation solution with 17 mg protein/mL and specific activity of 1300 U/mg protein, was purchased from Sigma-Aldrich (St. Louis, MO, USA), and used without further treatment. Chitosan powder from shrimp shells (50–190 kDa and 75–85% deacetylation degree) was also acquired from Sigma-Aldrich. Glutaraldehyde solution (25%

v/v) and palmitic acid were purchased from Vetec Química Ltda (São Paulo, SP, Brazil). Isoamyl alcohol, glycine and gum Arabic were acquired from Synth® (São Paulo, SP, Brazil). Hexanol, 2-ethyl-hexanol, decanol and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Olive oil (Carbonell) was acquired in a local market (Alfenas, MG, Brazil). All other chemicals, including buffer salts and organic solvents, were of analytical grade acquired from Synth®.

2.2. Preparation of the heterofunctional supports

The preparation of glutaraldehyde-activated chitosan hydrogel (Chit–GA) was based on a previous study [31], with some slight modifications. Five grams of chitosan powder was added to 100 mL of a glacial acetic acid solution (5% v/v) at 25°C for 4 h under mechanical stirring (1000 rpm). This homogeneous solution was dropped via a syringe to a 0.1 M NaOH solution (volume ratio Chit:NaOH solutions 1:10) under low mechanical stirring (100 rpm) overnight at 25°C to prepare chitosan hydrogel (Chit). Then, the suspension was filtered in a Buchner funnel under vacuum using Whatman n° 41 filter paper, thoroughly washed with distilled water until pH 7.0 and stored in ethanol solution (70% v/v) at 4°C. Following this, 10 g of chitosan hydrogel was incubated in a freshly prepared glutaraldehyde solution by mixing 11.2 mL of 0.2 M sodium phosphate solution pH 7.0 with 16.8 mL of glutaraldehyde solution at 25% (v/v). The suspension was maintained for 1 h under continuous mechanical stirring (200 rpm) at 25°C to obtain glutaraldehyde-activated chitosan hydrogel (Chit–GA). The activated support was also filtered in a Buchner funnel under vacuum using Whatman n° 41 filter paper and thoroughly washed with distilled water. The new heterofunctional support (Chit–GA–Gly) was prepared by mixing 10 g of Chit–GA hydrogel with 90 mL of 0.5 M glycine solution at pH 8.0 and 25°C in an orbital shaker (200 rpm) for 48 h. Finally, the support was filtered in a Buchner funnel under vacuum using Whatman n° 41 filter paper, thoroughly washed with distilled water

to remove unreacted glycine molecules. The support was stored in 70% (v/v) ethanol solution at 4°C prior to use.

2.3. Supports characterization techniques

The support samples were submitted to sequential lyophilization in a freeze-dryer and overnight drying in an oven at 50°C. The structural characterization of the dried supports was performed by Fourier transform infrared spectroscopy – FT–IR (Nicolet iS50 FTIR – Thermo Scientific, Madison, WI, USA) in Attenuated Total Reflectance (ATR) mode in the range of 400–4000 cm^{-1} with a spectral resolution of 4 cm^{-1}. Zeta potentials were determined in a Zetasizer Nano ZS90 model ZEN 3690 by Malvern Instrument (Malvern, Worcestershire, UK). For this purpose, 0.1 g of the samples was added to 100 mL of deionized water under mild stirring. The pH adjustment was performed using 0.1 M NaOH or 0.1 M HCl solutions. Thermogravimetric analyses (TGA) in a synthetic air atmosphere were performed using a TA Instruments STD Q600 unit (New Castle, DE, USA) with a temperature program from 30 to 900°C at a rate of 10°C/min.

2.4. Lipase immobilization procedure

The immobilization of TLL via physical adsorption on non-activated chitosan hydrogel (Chit) and new heterofunctional support (Chit–GA–Gly) was performed at low ionic strength (5 mM) using 1 g of supports and 19 mL of lipase solution [50,56,57]. The preparation of biocatalysts using activated chitosan hydrogel with glutaraldehyde (Chit–GA) as support was conducted by adding 1 g of support to 9 mL of lipase solution at 100 mM sodium phosphate, according to the methodology described in previous reports [31,58]. The immobilization suspensions were incubated in a water thermostatized bath under continuous mechanical stirring (200 rpm) at 25°C for 18 h, followed by filtration in a Buchner funnel under vacuum

using Whatman n° 41 filter paper and washing with distilled water to remove unbound lipase molecules. The biocatalysts were stored at 4°C for 24 h prior to use. The biocatalyst hydrolytic activity was assayed by olive oil emulsion hydrolysis [56,57]. One international activity unit (U) was defined as the amount of biocatalyst necessary to liberate 1 µmol of free fatty acids per minute at pH 8.0 (100 mM buffer sodium phosphate) and 37°C. The immobilization process was monitored by determining the residual protein concentration in the supernatant according to Bradford's method [59], using BSA as the standard protein. The adsorption capacity of TLL on the different supports was determined according to Eq. (1):

$$q_e = \frac{V_{enz} \times (C_0 - C_e)}{m} \tag{1}$$

where q_e is the concentration of immobilized lipase at equilibrium (mg/g); V_{enz} is the volume of enzyme solution (mL); C_0 and C_e are respectively the initial and residual (at equilibrium) protein concentration in the immobilization supernatant (mg/mL or mg/g of solution); and m is the mass of support (g).

The equilibrium constant (K_e) was calculated according to Eq. (2):

$$K_e = \frac{q_e}{C_e} \tag{2}$$

where K_e is the adsorption equilibrium constant (dimensionless); q_e is the concentration of immobilized lipase at equilibrium (mg/g); and C_e is the residual protein concentration in the immobilization supernatant at equilibrium (mg/mL or mg/g of solution).

The average specific activity of the biocatalysts was determined as follows (Eq. (3)):

$$SA = \frac{HA}{q_e} \tag{3}$$

where SA is the average specific activity (U/mg_{IP}); HA is the hydrolytic activity (U/g); and q_e is the concentration of immobilized lipase at equilibrium (mg/g).

2.4.1. Effect of pH on the immobilization process

The effect of medium pH on the immobilization parameters was examined in the interval between pH 3.0 and pH 10.0. The used buffers were sodium acetate at pH 3.0-5.0, sodium phosphate at pH 6.0-8.0 and sodium carbonate at pH 9.0-10.0. The assays were performed overnight under stirring at 200 rpm at 25°C, initial protein loading of 5 mg/g of support and buffer concentration of 5 mM using Chit and Chit–GA–Gly as supports and 100 mM for Chit–GA, as described in Section 2.4.

2.4.2. Effect of protein loading and adsorption isotherm studies on the parameters of the immobilization

The effect of protein loading on the immobilization parameters was evaluated by varying it from 5 to 70 mg protein/g of support (Chit–GA–Gly). The assays were performed under fixed experimental conditions – overnight, 5 mM sodium acetate pH 4.0 at 25°C and continuous mechanical stirring at 200 rpm.

In this set of experiments, Langmuir (Eq. (4)) and Freundlich (Eq. (5)) isotherm models were used to design a model where the experimental data of lipase adsorption using Chit–GA–Gly as support were fitted [60].

$$q_e = \frac{q_{\max} \times C_e}{K_L + C_e} \tag{4}$$

$$q_e = K_F \times C_e^{\frac{1}{n}} \tag{5}$$

where q_e is the experimental adsorption capacity at equilibrium (mg protein/g support); C_e is the residual protein concentration in the immobilization supernatant at equilibrium (mg protein/mL); q_{max} is the theoretical maximum adsorption capacity (mg protein/g support); K_L is the Langmuir constant related to the energy of adsorption (mL/mg protein); K_F is the Freundlich isotherm constant (mL/mg support); and n is the Freundlich exponent (dimensionless).

An assay was also conducted for Chit–GA using an initial protein loading of 65 mg/g of support, overnight, and 100 mM sodium phosphate at pH 7.0.

2.5. Biocatalysts solvent stability tests

0.1 g of each biocatalyst were incubated with 1.0 mL of organic solvent (heptane, toluene or iso-octane) in screw cap vials. The suspensions were maintained at 50°C under continuous mechanical stirring at 200 rpm in a horizontal water bath shaker for 48 h. Samples were periodically withdrawn from the inactivating suspensions, followed by filtration to remove organic solvents and determination of the residual activity in the hydrolysis of olive oil emulsion, as aforementioned (Section 2.4). The biocatalysts not exposed to the organic solvents were used as reference – relative activity of 100%. In this set of experiments, the heterogeneous biocatalysts were prepared using 5 mg protein/g of support, pH 4.0 at 5 mM sodium acetate for TLL immobilized on Chit–GA–Gly (HA = 607.7 ± 17.2 U/g) and pH 7.0 at 100 mM sodium phosphate for TLL immobilized on Chit–GA (HA = 43.1 ± 5.1 U/g), as shown in Table 3.1. The assays were performed by duplicate.

2.6. Biocatalysts desorption tests

In this study, 0.1 g of the biocatalysts prepared using a protein loading of 65 mg/g were suspended in distilled water or 1 M NaCl or 1 M NaCl + 0.2% (v/v) Triton X-100 at pH 7.0 under different concentrations varying from 5 g/L to 100 g/L (that corresponds to volume ratio biocatalyst:desorption solution from 1:10 to 1:200). The suspensions were incubated at 50°C under continuous mechanical stirring at 200 rpm by 24 h in a horizontal water bath shaker. The desorbed protein concentration in the supernatant was determined according to Bradford's method, as described in Section 2.4.

2.7. Esterification reaction and reusability tests

The enzymatic synthesis of alkyl esters via direct esterification reaction was conducted in screw-capped glass bottles with a capacity of 100 mL containing 6 mL of reaction mixture (0.5 M of alcohol and palmitic acid in iso-octane). The reaction mixtures were incubated under continuous mechanical stirring (240 rpm) in an orbital shaker at 50°C for 15 min, followed by adding 0.6 g of TLL immobilized on Chit–GA or Chit–GA–Gly using a protein loading of 65 mg/g support. The effect of alcohol nature (isoamyl alcohol, hexanol, 2-ethyl-hexanol, and decanol) on the palmitic acid conversion was firstly examined at a fixed reaction time of 70 min. The reaction system with the highest acid conversion was then selected to evaluate the reaction course necessary to achieve the chemical equilibrium. Samples (100 µL) of the reaction mixture were periodically withdraw from the reaction system and titrated with 30 mM NaOH solution using phenolphthalein as the indicator to determine the acid conversion percentage, according to previous studies [56,57].

Reusability tests were also performed by the employment of the biocatalyst in nine successive reactions cycles of isoamyl palmitate synthesis of 90 min each. After each batch, the heterogeneous biocatalyst was recovered by filtration in a Buchner funnel under vacuum using Whatman n° 41 filter paper and washed with cold hexane to remove product and unreacted substrate molecules from the biocatalyst particle. Then, the biocatalyst was added into a fresh reaction mixture to perform a new reaction batch [56–58].

3. Results and discussion

3.1. Supports characterization

FT–IR analyses were performed to obtain structural information of the different chitosan-based hydrogels prepared in this study (Fig. 3.1). The spectrum of chitosan hydrogel (Chit) reveals the presence of the typical bands in its structure, 3420 cm^{-1} (O–H and N–H

stretching vibrations), 2900 cm^{-1} (C–H stretching vibration), 1640 cm^{-1} (N–H bending vibration), 1420 cm^{-1} (C–N stretching vibration), 1380 cm^{-1} (C–O stretching vibration), and 1025 cm^{-1} (C–O–C stretching vibration). The band at 1320 cm^{-1} shows the presence of some remaining *N*-acetylglucosamine units (GlcNAc) in its structure [61]. These results fit with those reported in previous studies [62–66]. When studying the FT-IR spectrum of Chit–GA hydrogel, a decrease of the intensity of the band assigned to stretching vibration of O–H and N–H bonds at 3420 cm^{-1} and the disappearance of the band associated with the stretching vibration of C–N bond at 1420 cm^{-1} in the chitosan structure after activation can be observed. These changes are due to modification of the amino groups in Chit structure by GA molecules [62,64,65]. In fact, an increase of the band intensity at 1640 cm^{-1} for Chit–GA due to the formation of the glutaraldehyde-amino bonds can be observed. Moreover, the appearance of a yellow color after activation of chitosan hydrogel with GA can be observed in Fig. S1-(B).

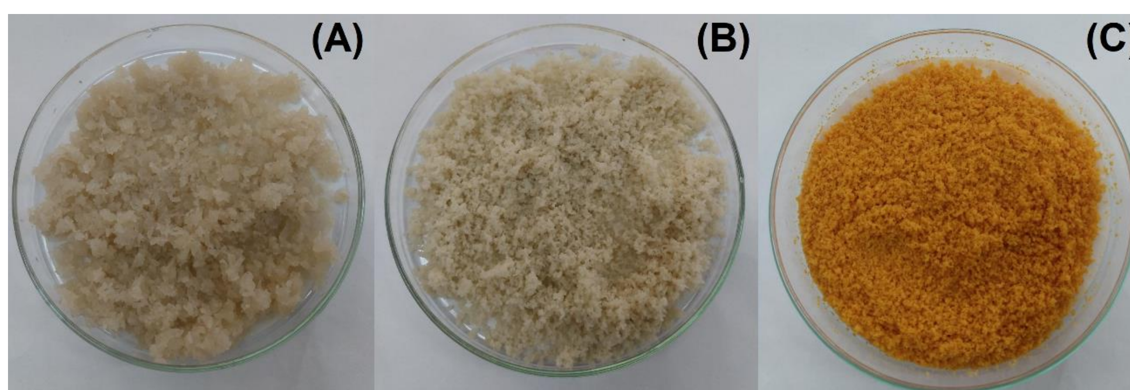

Figure S1 - Chitosan-based supports prepared in this study: Chit hydrogel – Chit (A), glutaraldehyde-activated chitosan hydrogel – Chit–GA (B) and glutaraldehyde-activated chitosan hydrogel and functionalized with glycine – Chit–GA–Gly (C).

Next, we analyzed the modification of the previous support with glycine. The spectrum of Chit–GA–Gly reveals an increase in the band intensity in the region between 1500–1700 cm^{-1} compared with Chit–GA hydrogel due to overlapping of the C=O introduced via

functionalization with glycine and formation of new amino-glutaraldehyde bonds [52,67]. This high reactivity of aldehyde groups of the glutaraldehyde with the amino group of the glycine under moderate conditions (pH values between 7.0 and 9.0) has been demonstrated in a previous study [67]. In fact, the prepared Chit–GA–Gly support exhibited more intense yellow color than Chit–GA hydrogel (Fig. S1-(C)). FT–IR spectra confirmed the introduction of the different functional groups on the chitosan hydrogel surface after sequential activation with GA and functionalization with Gly.

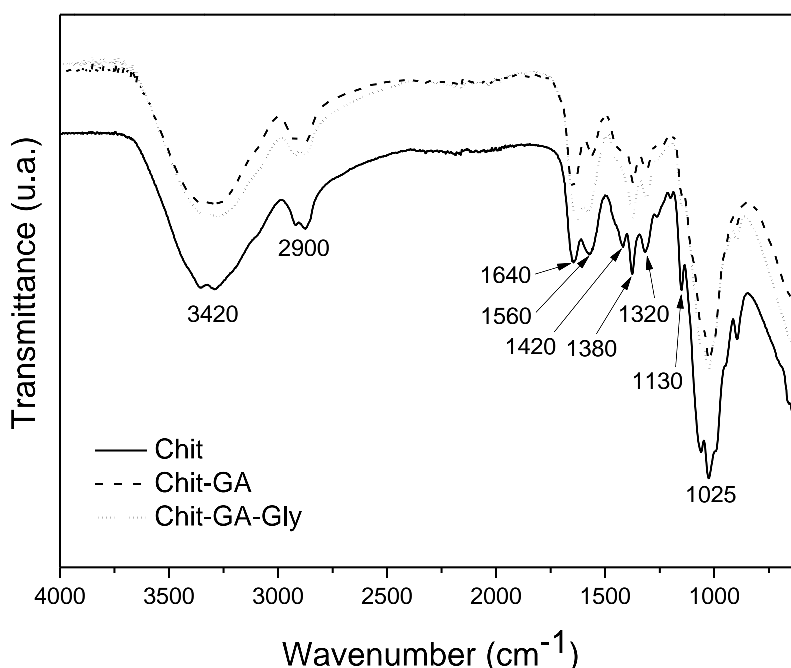

Figure 3.1 - FT–IR spectra of chitosan hydrogel (Chit), glutaraldehyde-activated chitosan hydrogel (Chit–GA) and glutaraldehyde-activated chitosan hydrogel functionalized with glycine (Chit–GA–Gly). Experiments were performed as described in Materials and Methods.

Zeta potential (ZP) analyses of the different supports were also performed to determine the possible changes of ionic groups nature on the chitosan surface. Fig. 3.2 shows the effect of

the medium pH on the ZP values of the three prepared supports. The chitosan hydrogel presents a global cationic nature under acidic conditions due to protonation of free amino groups (NH_3^+) in its structure. At pH 7.0, the support has a similar density of anionic and cationic groups (isoelectric point of Chit). Under alkaline conditions, this hydrogel possesses an anionic nature. This result is consistent with a previous study performed by Silva *et al.* [68], which also observed an anionic nature of chitosan at pH > 6.5. After activation and functionalization, the ionic profiles changed, as expected. At a pH ≤ 5.5, both Chit–GA and Chit–GA–Gly supports are mainly of cationic nature. At pH 4.5, both supports exhibited similar ZP values. However, the ZP values of Chit–GA–Gly were lower than Chit–GA in the pH range between 4.7 and 5.7 due to presence of carboxylate groups introduced after functionalization with glycine molecules. The isoelectric points of Chit–GA and Chit–GA–Gly were very similar, pH 5.6 and 5.8, respectively. This suggests a slight increase of the density of amine-glutaraldehyde groups in Chit–GA–Gly, as expected. When the pH increase (pH ≥ 6.0), both support surfaces become negatively charged, very likely as a consequence of the carboxylate group of the glycine and, perhaps some anionic groups such as carboxylate groups derived from the glutaraldehyde cyclization, oxidation, etc. [36,37].

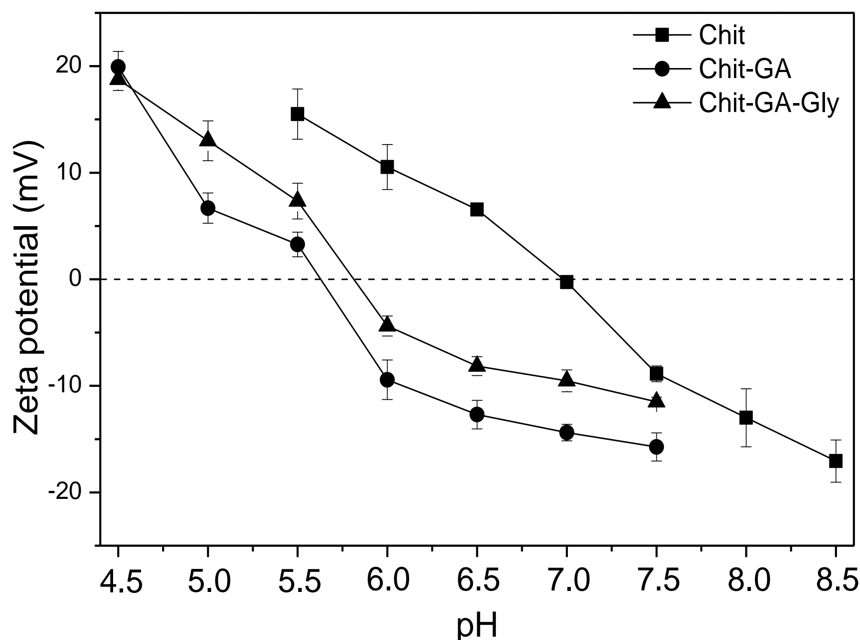

Figure 3.2 - Zeta potential of chitosan hydrogel (Chit), glutaraldehyde-activated chitosan hydrogel (Chit–GA), and glutaraldehyde-activated chitosan hydrogel and functionalized with glycine (Chit–GA–Gly) as a function of the pH value in the medium. Experiments were performed as described in Materials and Methods.

TGA was also performed to evaluate the effect of the chemical modification on the thermal stability of chitosan-based hydrogels. According to Fig. 3.3, both supports presented three thermal events of mass losses in the temperature intervals between 30 and 900°C. The first event between 30 and 120°C is explained by the evaporation of water molecules physically adsorbed on their surfaces. In this range of temperature, the mass losses of Chit, Chit–GA and Chit–GA–Gly were around of 8, 9 and 11%, respectively. The second mass loss event in the temperature range from 150 to 320°C is due to the removal of structurally bound water molecules and/or decomposition of some shorter chains [57,64]. In this second thermal event, it is also possible to note that the chitosan hydrogel was slightly more thermally stable than the chemically modified supports and a mass loss plateau between 150 and 230°C can be observed.

A similar loss mass plateau in this range of temperature for non-activated chitosan hydrogel has been observed in previous reports [64,69]. These results could be due to different internal geometries generated in the drying processes required to perform the analyses (see section "3.1. Supports characterization"). Under such conditions, an ordered compaction of adjacent polymeric chains in the original chitosan structure could promote the formation of a more rigid structure that can be stabilized by intense hydrogen bonds that increases its thermal stability. The introduction of functional groups via activation and functionalization could reduce this polymer compaction effect, thus providing more porous structures and less thermally stable structures in this range of temperature [69]. The increase of the resistance to compression after chemical modification of chitosan-based scaffolds with glutaraldehyde has been proved in a recent study [70], which agrees with these results. In fact, the mass loss in the second stage for Chit, Chit–GA and Chit–GA–Gly were 49%, 54% and 56%, respectively. The third event between 360°C and 600°C is due to their oxidative decomposition. The supports chemically modified were thermally more resistant than chitosan hydrogel, showing that the introduction of functional groups on the chitosan structure increased its resistance to thermal degradation in this range of temperature. It is also possible to observe that both Chit–GA and Chit–GA–Gly exhibited similar thermal degradation profiles. However, Chit–GA was slightly more stable, thus suggesting that glycine moieties could be firstly thermally degraded. These results agree with those obtained in previous reports that also observed increase of the thermal stability of chemically modified chitosan-based matrices at high temperature [63,64].

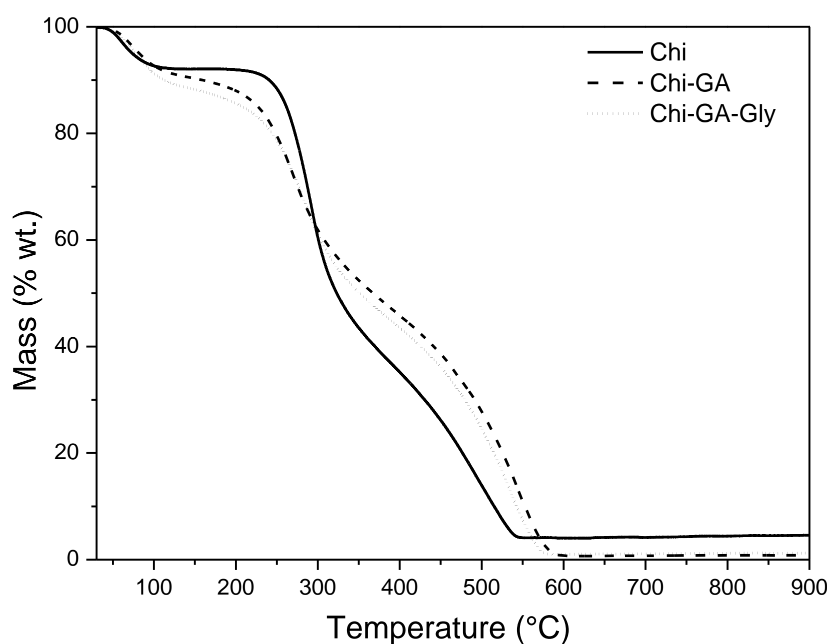

Figure 3.3 - TG curves of chitosan hydrogel (Chit), glutaraldehyde-activated chitosan hydrogel (Chit–GA), and glutaraldehyde-activated chitosan hydrogel and functionalized with glycine (Chit–GA–Gly). Experiments were performed as described in Materials and Methods.

According to these results and properties of glutaraldehyde in aqueous medium, based on previous reports [21,24,36–38,41,61], the possible mechanism of preparation of the heterogeneous support was proposed. The steps of activation of chitosan hydrogel with glutaraldehyde dimers obtained via aldolic condensation [41], and functionalization with glycine are shown in Fig. 3.4. In this proposed mechanism, it is possible to observe that the lipase can be immobilized by different strategies such as: *(i)* via covalent attachment of functional groups of the enzyme with free aldehyde groups from glutaraldehyde and its dimer, *(ii)* ionic adsorption of carboxylate groups ($–COO^-$) of the enzyme with protonated imine groups ($–C{=}NH^+–$) of the support and carboxylate groups of the support with protonated amino groups of TLL – amino terminal and amino groups of lysine and guanidine residues of arginine

, *(iii)* physical adsorption via hydrophobic interactions – mechanism of “interfacial activation” [18,19] with introduced carbon-chain moieties, and *(iv)* hydrogen bonds between enzyme and free hydroxyl and amino groups of the glucosamine units of chitosan. In addition, the intra- and intermolecular crosslinking reactions formed in the activation of chitosan with glutaraldehyde that are responsible by the improvement of its structural stabilization against shear stress and pH changes are also shown [21,24].

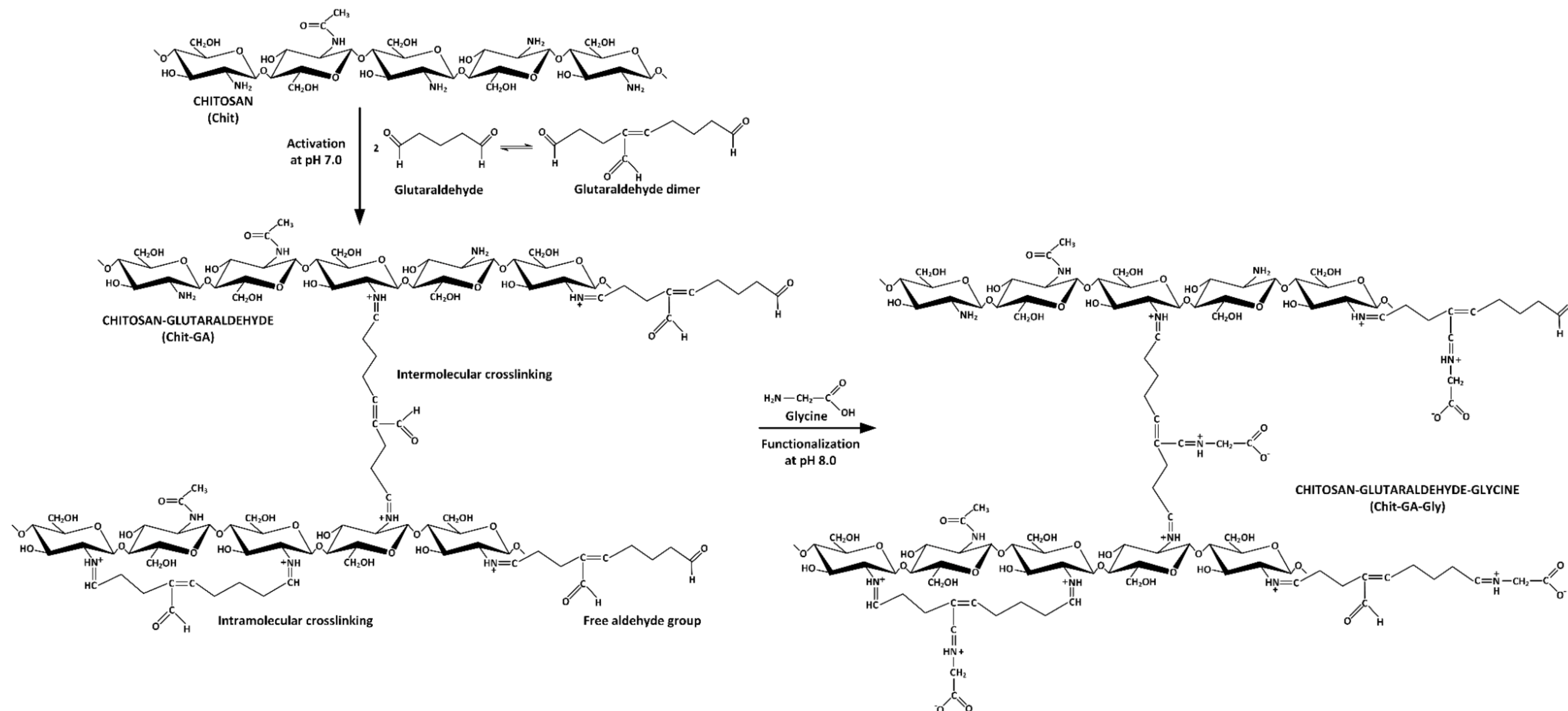

Figure 3.4 - Representative scheme of preparation of the heterofunctional support (Chit–GA–Gly) and immobilization of TLL via adsorption.

3.2. Effect of immobilization pH on the TLL immobilization process

The immobilization pH is an important parameter in the preparation of heterogeneous biocatalysts via ionic exchange because it influences the surface ionization of the support (see ZP analysis in Section 3.1), and enzymes [50,57,71]. In this study, the effect of immobilization pH on the TLL immobilization process and the final hydrolytic activity of the TLL biocatalysts was examined. The results, summarized in Table 3.1, show that the maximum immobilized protein loading on the new support (Chit–GA–Gly) was obtained in the pH range from 3.0 to 6.0, with equilibrium constant values (K_e) varying from 1,063.8 to 1,250.0 that indicates high adsorption capacity. In the pH range from 3.0 to 5.0, the support surface becomes cationic (see ZP study) and interacts with the anionic groups of the enzyme surface such as carboxylate groups of aspartate and glutamate residues and/or carboxyl terminal group. At pH 6.0, both TLL and support surfaces become negatively charged, since isoelectric points are at pH 4.4 [47,72], and 5.8 (see Fig. 3.2), respectively. It should remarked that ionic exchange is a multipoint process involving different groups of the enzyme and support surfaces, and the immobilization may be produced even when support and enzyme have the same ionic nature, as long as a net of ionic interactions should be produced to fix the enzyme in the support [73].

Table 3.1 - Influence of solution pH on the immobilization parameters of TLL adsorbed on Chit–GA–Gly. Experiments were performed as described in Materials and Methods.

pH	q_e[a] (mg/g)	C_e[b] (mg/mL)	K_e[c]	HA[d] (U/g)	SA[e] (U/mg_{IP})
3.0	≈5.0	4.2×10^{-3}	1,190.5	562.3 ± 14.2	112.5
4.0	≈5.0	4.0×10^{-3}	1,250.0	607.7 ± 17.2	121.5
5.0	≈5.0	4.5×10^{-3}	1,111.1	555.7 ± 9.7	111.1
6.0	≈5.0	4.7×10^{-3}	1,063.8	414.1 ± 10.4	82.8
7.0	4.4 ± 0.1	3.2×10^{-2}	137.5	328.3 ± 37.5	74.6
8.0	1.7 ± 0.2	1.7×10^{-1}	10.0	261.8 ± 18.0	154.0
9.0	1.2 ± 0.1	2.0×10^{-1}	6.0	163.2 ± 1.0	136.0
10.0	0.8 ± 0.2	2.2×10^{-1}	3.6	126.5 ± 16.6	158.1
Chit[f]					
4.0	ND[g]	ND[g]	ND[g]	ND[g]	ND[g]
7.0	1.5 ± 0.1	1.8×10^{-1}	8.3	394.8 ± 10.8	263.2
10.0	0	2.6×10^{-1}	0	0	0
Chit–GA[h]					
4.0	≈5.0	4.6×10^{-3}	1,087.0	74.1 ± 1.5	14.8
7.0	≈5.0	4.3×10^{-3}	1,162.8	43.1 ± 5.1	8.6
10.0	≈5.0	4.5×10^{-3}	1,111.1	82.1 ± 4.2	16.4

a – Immobilized protein at equilibrium.
b – Residual protein concentration in the immobilization supernatant at equilibrium.
c – Equilibrium constant
d – Hydrolytic activity
e – Specific activity
f – Immobilization at 5 mM (ratio support:enzyme solution of 1:20)
g – Not determined
h – Immobilization at 100 mM (ratio support:enzyme solution of 1:10)

In this way, it has been shown that a high percentage of proteins were immobilized on a support containing similar amounts of anionic and cationic groups [45,46], or almost all proteins in a crude extract could also be immobilized on supports coated with anionic or cationic polymers under the same conditions [74–76], indicating that many proteins were immobilized on both supports. That way the lipase molecules could be fully adsorbed on the support surface due to some possible mixed ionic interactions of: *i)* cationic groups of the support with anionic groups of TLL, and *ii)* cationic residues of the enzyme with anionic groups of the support. Additionally, some hydrogen bonds of the lipase with free hydroxyl groups of the support and hydrophobic interactions of the lipase with the carbon chains introduced on the support surface via activation with GA could also be related to the adsorption process. An increase in the immobilization pH strongly decreased the adsorption of the enzyme on the support surface; both enzyme and support anionic nature value produced even some repulsion forces. At pH 9.0 and 10.0, where the cationic groups on the support were negligible, only 20% of the offered protein was immobilized on the support ($q_e \approx 1$ mg/g support), thus suggesting that under these conditions the immobilization process could be mainly due to hydrophobic interactions, as discussed above. Under these conditions, K_e values were of 6.0 (pH 9.0) and 3.6 (pH 10.0), thus showing low adsorption capacity. These results agree with a previous study performed on the immobilization of lipase B from *Candida antarctica* on kaolin particles via physical adsorption [72]. The immobilization of the enzyme on Chit or Chit–GA was also studied, as shown in Table 3.1. The immobilization of TLL on Chit was not possible at pH 4.0 due to the partial solubilization of the support. At pH 10, both enzyme and support possess an anionic character, resulting in a strong electrostatic repulsion. However, partial immobilization of TLL was obtained at pH 7.0 ($q_e = 1.5 \pm 0.1$ mg/g). This result clearly pointed out the advantages that the new heterofunctional support presented when compared to the unmodified support as ionic exchanger support to immobilize enzymes.

On the other hand, TLL was fully immobilized on Chit–GA under a broad pH range varying from 4.0 to 10.0, very likely via different mechanisms. Interfacial activation of the lipase versus the moderately hydrophobic layer of glutaraldehyde moieties may be relevant under all pH conditions [42]. Moreover, the role of covalent reactions and ionic exchange cannot be discarded in all the pH range, with different relevance of each one depending on the studied pH (e.g., at acidic pH value the covalent reaction will be very slow because the amino groups will be protonated).

As it can be seen in Table 3.1, the highest hydrolytic activity values were observed in the pH range from 3.0 to 5.0 for TLL immobilized on Chit–GA–Gly (above 550 U/g support). The maximum value was obtained at pH 4.0 (607.7 ± 17.2 U/g), followed by immobilizing at pH 7.0 TLL on Chit hydrogel (394.8 ± 10.8 U/g). This very different optimal pH when immobilizing TLL on both supports reinforces the very different mechanisms of ion exchange on both supports: using Chit only anion exchange is relevant to immobilize TLL. In fact, a cationic support and an anionic enzyme is preferred. Using Chit–GA–Gly, the mixed ion exchange is favored under pH values where both enzyme and support offer cationic and anionic groups, that is, near the isoelectric point of the enzyme [45,46,75,76]. The biocatalysts prepared using Chit–GA exhibited the lowest hydrolytic activity values (from 43.1 ± 5.1 to 82.1 ± 4.2 U/g), very likely because of the distortion of the enzyme promoted by the covalent reaction of the enzyme with the support.

With respect to specific activity (SA) values of TLL (Table 3.1), the maximum value was obtained by immobilizing TLL on Chit hydrogel at pH 7.0 (263.2 U/mg_{IP}), followed by the biocatalysts prepared above pH 8.0 using Chit–GA–Gly – from 136.0 to 158.1 U/mg_{IP}. This may be due to the lower amount of immobilized protein when using Chit, which reduces the diffusional problems (both, substrate limitations and pH gradients) that can raise using higher enzyme loadings [15,77]. Therefore, the immobilization pH value was fixed at pH 4.0 for

subsequent studies using Chit–GA–Gly as support. Although the lowest hydrolytic activity for TLL immobilized on Chit–GA has been observed at pH 7.0, this was selected as a comparison to ensure a maximum of covalently immobilized enzyme molecules in the support [37]. Chit was discarded for further studies due to poor physical resistance of the hydrogel.

3.3. Effect of offered protein amount on the immobilization process

The effect of initial protein loading on the adsorption process and hydrolytic activity of the biocatalysts prepared using Chit–GA–Gly as support was conducted in 5 mM sodium acetate at pH 4.0 and 25°C. According to results in Table 3.2, almost complete adsorption of TLL on the support surface was obtained for assays conducted until 50 mg protein/g support, thus indicating that this support has high surface area and/or suitable pore sizes to accommodate protein molecules in both the external and internal surfaces. Under such conditions, K_e values varied from 1,250.0 (for protein loading of 5 mg/g) to 365.4 (for protein loading of 50 mg/g). Following this, a slight decrease of immobilization yield [78] and K_e values was observed if the amount of enzyme increased, suggesting that the support has a maximum adsorption capacity of 53 mg/g support. This maximum TLL loading was achieved by offering 65 mg of protein/g of support. These results clearly showed that this support exhibited higher TLL immobilization capacity than those supports, including chitosan-based matrices, reported in Table 3.3.

Table 3.2 - Effect of initial protein loading on the immobilization parameters of TLL adsorbed on Chit–GA–Gly. Experiments were performed as described in Materials and Methods.

Protein loading (mg/g)	q_e[a] (mg/g)	C_e[b] (mg/mL)	K_e[c]	HA[d] (U/g)	SA[e] (U/mg_{IP})
5	≈5.0	4.0×10^{-3}	1,250.0	607.1 ± 21.1	121.5
7.5	7.4 ± 0.1	8.0×10^{-3}	925.0	602.7 ± 8.0	81.4
10	9.9 ± 0.1	1.1×10^{-2}	900.0	653.3 ± 16.7	66.0
12.5	12.3 ± 0.2	2.8×10^{-2}	439.3	657.5 ± 1.4	53.4
15	14.4 ± 0.1	3.7×10^{-2}	389.2	747.8 ± 16.7	51.9
20	19.1 ± 0.4	5.5×10^{-2}	347.3	1,024.7 ± 39.4	56.3
25	23.6 ± 0.2	7.5×10^{-2}	314.7	1,031.0 ± 47.9	43.7
40	38.4 ± 0.2	8.2×10^{-2}	468.3	1,047.8 ± 15.1	27.3
50	47.5 ± 0.5	1.3×10^{-1}	365.4	1,001.3 ± 27.8	21.1
55	50.8 ± 0.2	2.2×10^{-1}	230.9	1,016.1 ± 48.9	20.0
65	53.0 ± 0.6	6.3×10^{-1}	84.1	1,042.8 ± 63.1	19.7
70	53.4 ± 0.6	9.0×10^{-1}	59.3	1,031.5 ± 14.9	19.3
Chit–GA[f]					
65	55.3 ± 3.3	1.08	51.2	265.1 ± 14.4	4.8

a – Immobilized protein at equilibrium
b – Residual protein concentration in the immobilization supernatant at equilibrium
c – Equilibrium constant
d – Hydrolytic activity
e – Specific activity
f – Immobilization at pH 7.0 (100 mM buffer sodium phosphate).

The effect of initial protein loading on the catalytic activity of the biocatalysts was also studied using the hydrolysis of olive oil emulsion (Table 3.2). As the initial protein loading was

raised from 5 to 20 mg/g, the hydrolytic activity of the prepared biocatalysts only increased almost twice (from 607.1 ± 21.1 U/g to 1,024.7 ± 39.4 U/g), thus suggesting strong mass transfer limitations. Further increase of offered protein above 20 mg/g support did not increase the volumetric hydrolytic activity (see results in Table 3.2), suggesting that the diffusional limitations become more relevant than the gain in enzyme molecules number. In fact, a decrease of specific activity from 121.5 U/mg_{IP} to 19.3 U/mg_{IP} was observed by increasing the initial protein loading from 5 to 70 mg/g. These results are consistent with previous reports in literature [50,56,57].

The analysis of the adsorption equilibrium (physisorption and/or chemisorption) is a crucial tool in the development of a model that can be used for biocatalyst design purposes [60]. In this context, several adsorption isotherm models have been successfully used to correlate equilibrium experimental data that describe the relation between the adsorption capacity at equilibrium (q_e) and residual adsorbate concentration at equilibrium (C_e). In the present study, the most reported models used for describing the adsorption of lipases from several sources on a variety of supports were used: Langmuir and Freundlich isotherm models [50,56,57]. Fig. 3.5 shows the experimental adsorption data and isotherm models, including correlation coefficients (R^2) and adsorption rate constants. As it can be observed, the R^2 value for the Freundlich isotherm model was lower than that for Langmuir model, thus indicating that this model is more suitable for describing the adsorption process on the new support. The Langmuir isotherm model has been successfully applied to many lipase adsorption processes on a variety of supports [50,56,57]. This model is based on the assumption that the adsorption process is forming a monolayer and takes place at specific homogeneous sites on the support surfaces [60]. According to Fig. 3.5, the theoretical value for maximum adsorption capacity (q_{max}) of this support was 59.3 mg protein/g, which was slightly higher than the experimental values (q_e = 53 mg/g) reported in Table 3.2.

Table 3.3 - Literature survey for the preparation of heterogeneous biocatalysts via immobilization of TLL on several supports.

Support	Supplier	Immobilization protocol	Immobilized TLL load (mg/g)	Application	References
Glutaraldehyde-activated chitosan/alginate-TNBS[a]	Non-commercial	Covalent attachment	17.5	Hydrolysis of olive oil emulsion and biodiesel synthesis from palm oil	[31]
Epoxy-chitosan/alginate hydrogel	Non-commercial	Covalent attachment	25.4	Hydrolysis of olive oil emulsion and butyl butyrate synthesis	[32]
Glutaraldehyde-activated Toyopearl AF-amino-650M	Tosoh Bioscience	Covalent attachment	21.9	Hydrolysis of olive oil emulsion and biodiesel synthesis from palm and babassu oils	[79]
Activated magnetic Fe_3O_4@chitosan nanoparticles with EDC[b]	Non-commercial	Covalent attachment	16.8	Hydrolysis of *p*-nitrophenyl palmitate and ascorbyl palmitate synthesis	[80]
Poly-hydroxybutyrate	PHB Industrial	Physical adsorption	26.5	Hydrolysis of olive oil emulsion and methyl/ethyl oleate synthesis	[81]
Glyoxyl-agarose	GE Healthcare	Covalent attachment	19.4–21.2	Hydrolysis of olive oil emulsion and biodiesel synthesis from palm and babassu oils	[82]
Octyl-agarose	GE Healthcare	Physical adsorption	5	Hydrolysis of *S*-methyl mandelate, triacetin and methyl phenylacetate	[83]
Macroporous styrene	Purolite®		12		
Styrene methacrylate			6		
Octadecyl methacylate			17		
DVB[c] methacrylate			13		
Octadecyl methacrylate			10		
Activated porous carbon material with EDC[b]	Non-commercial	Covalent attachment	45	Hydrolysis of *p*-nitrophenyl palmitate	[84]
Chit–GA–Gly	Non-commercial	Physical adsorption	53	Hydrolysis of olive oil emulsion and isoamyl palmitate synthesis	This study
Chit–GA	Non-commercial	Covalent attachment	55		

a – 2,4,6-trinitrobenzene sulfonic acid
b – 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
c – Divinylbenzene

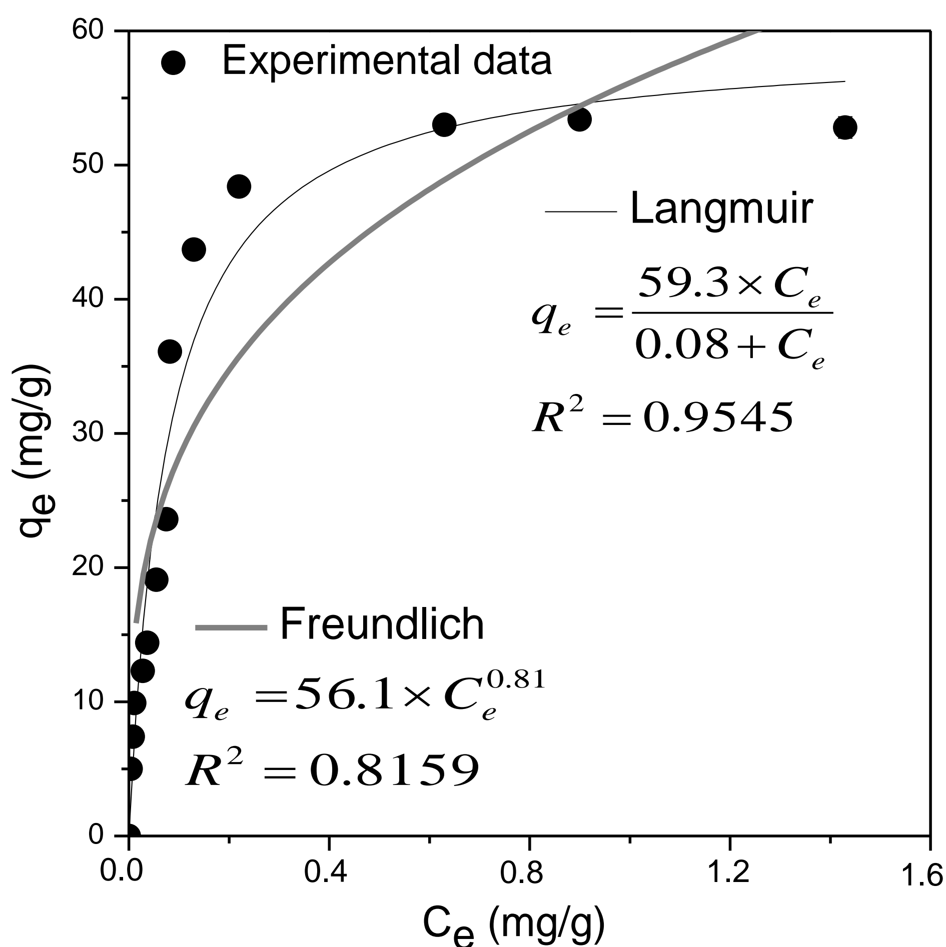

Figure 3.5 - Equilibrium isotherm curves for TLL adsorption on the heterofunctional support (Chit–GA–Gly) in 5 mM buffer sodium acetate at pH 4 and 25°C. Experiments were performed as described in Materials and Methods.

The immobilization of TLL on Chit–GA was also performed at 65 mg protein/g of support. The maximum enzyme loading was similar (55.3 ± 3.3 mg/g) to that found for Chit–GA–Gly. However, its hydrolytic activity was 4-times lower than TLL immobilized on the new heterofunctional support, in accordance with the experimental data summarized in Table 3.2. These results suggest that the immobilization using Chit–GA–Gly could result in the hyperactivation of some lipase molecules – immobilization in open conformation on

hydrophobic moieties introduced on the chitosan surface [15,18,19], thus producing a more active biocatalyst in the hydrolysis of olive oil emulsion.

3.4. Biocatalysts stability in organic solvents

The stabilities of the biocatalysts prepared using Chit–GA and Chit–GA–Gly were determined using three organic solvents commonly used as reaction media in enzymatic synthesis of esters such as heptane (Hep), toluene (Tol) and iso-octane (Iso) [85–87]. These tests were conducted at 50°C for 48 h of incubation under mechanical stirring (200 rpm). As shown in Fig. 3.6, both biocatalysts retained the initial activity after 4 h of incubation in all assayed solvents. Then, a similar inactivation profile for both heterogeneous biocatalysts in the three different solvents can be observed. In fact, both biocatalysts retained between 35-45% of their initial activity at 48 h of incubation in the different assayed solvents. That is, the covalent immobilization of the enzyme on the support seemed not to have a relevant positive effect on TLL stability in the presence of these organic solvents compared to the physically immobilized lipase. It should be considered that in a medium composed of organic solvents, the ionic adsorption of the enzyme will be reinforced, and that way enzyme release may be ruled out.

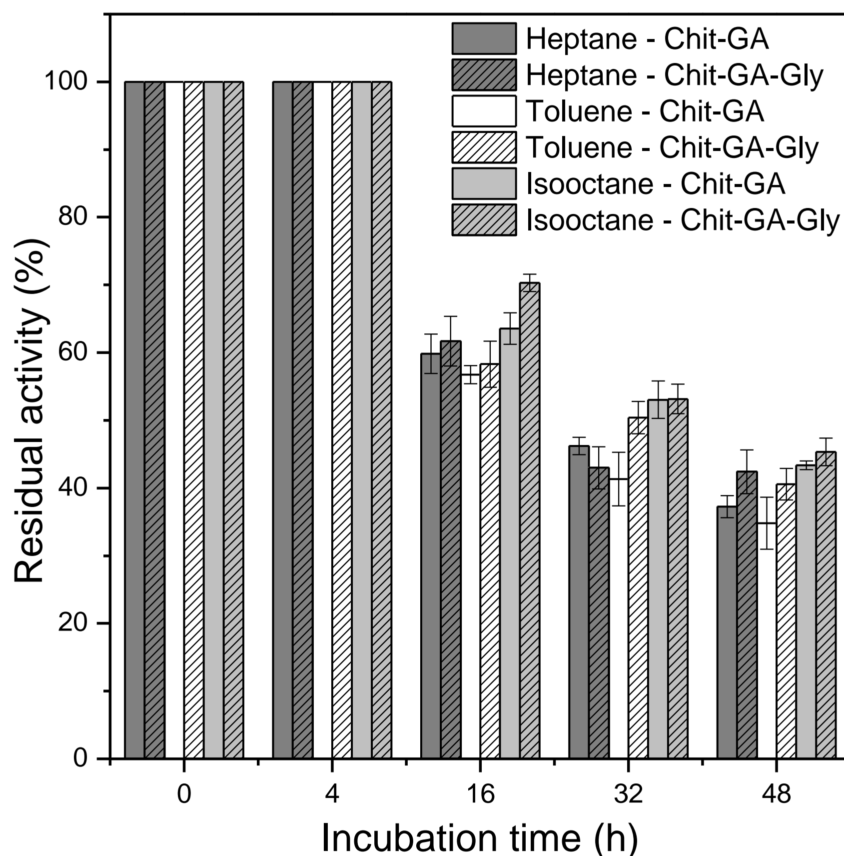

Figure 3.6 - Inactivation courses of TLL immobilized on the glutaraldehyde-activated chitosan hydrogel (Chit–GA), and glutaraldehyde-activated chitosan hydrogel and functionalized with glycine (Chit–GA–Gly) in organic solvents at 50°C. The used heterogeneous biocatalysts were prepared at 5 mg protein/g support. Experiments were performed as described in Materials and Methods.

3.5. Release of the enzyme from the different biocatalysts under different conditions

To better understand the mechanism of interaction of TLL in each heterofunctional support prepared in this paper, desorption tests were performed under fixed experimental conditions: 24 h of incubation at pH 7.0, 50°C, and continuous mechanical stirring of 200 rpm (see Methods section). As it can be noted in Table 3.4, the enzyme was not desorbed by incubating any of the biocatalysts in distilled water (Assay 1). The incubation in 1 M NaCl

under different biocatalysts concentration from 10 g/L to 100 g/L (Assays 2, 3, and 4) desorbed ≈65% of TLL activity immobilized on Chit–GA–Gly, which shows the great importance of ion exchange in the enzyme molecules immobilized on this support, and it also suggests a very strong enzyme adsorption as under these conditions most enzyme should be released. Under such conditions, lipase desorption from the Chit–GA surface was negligible suggesting that ionic exchange was not relevant to maintain the enzyme molecules on the support, even if the first immobilization step may be the ionic exchange or the interfacial activation of the enzyme [42]. Next, a desorption study incubating the biocatalysts in 1 M NaCl + 0.20% (v/v) Triton X-100 under different concentrations (from 5 g/L to 100 g/L) was performed. Triton X-100, a non-ionic detergent, was chosen as detergent due to its wide used in desorption tests for several lipases immobilized on a variety of hydrophobic supports [88]. At high biocatalyst concentration (100 g/L and 50 g/L – Assays 5 and 6), it was possible to observe that 5% of TLL immobilized on Chit–GA was released from the support. This confirms the preferential immobilization of the enzyme on this support via covalent attachment (95%) and only 5% is just due to physical adsorption (via hydrophobic interactions or a mixed ionic/hydrophobic adsorption).

When studying the biocatalyst prepared using Chit–GA–Gly, at low biocatalyst concentration (5 g/L to 10 g/L), a maximum desorption percentage of 85% was observed. This suggests that the adsorption on this support is quite complex, including ionic and hydrophobic interactions. This means that this new biocatalyst may be used under a wide variety of conditions with moderate to low risk of enzyme release. It is not possible to fully rule out that some TLL molecules can be covalently immobilized in some remaining glutaraldehyde groups (accounting for a maximum of 15% of the total activity of the biocatalyst), but it is more likely that some enzyme molecules may be so strongly adsorbed on the support that the enzyme

release may require much more drastic conditions. The incubation in 0.5 M Gly at pH 8.0 should be enough to fully block the glutaraldehyde groups.

Table 3.4 - Desorption of TLL immobilized on Chit–GA and Chit–GA–Gly at 50°C, pH 7.0, 24 h of incubation and mechanical stirring of 200 rpm. The biocatalysts were prepared using an initial protein loading of 65 mg/g. Experiments were performed as described in Materials and Methods.

Assay	Experimental conditions	Biocatalyst concentration (g/L)	Desorption (%)	
			Chit–GA	Chit–GA–Gly
1	Distilled water[a]	100	0	0
2	NaCl 1 M	100	0	21.4 ± 0.8
3	NaCl 1 M	20	ND[b]	65.2 ± 2.9
4	NaCl 1 M	10	ND[b]	64.2 ± 1.9
5	NaCl 1 M + 0.2% (v/v) Triton X-100	100	5.1 ± 0.3	29.8 ± 1.6
6	NaCl 1 M + 0.2% (v/v) Triton X-100	50	5.4 ± 0.2	32.2 ± 1.8
7	NaCl 1 M + 0.2% (v/v) Triton X-100	20	ND[b]	78.5 ± 2.9
8	NaCl 1 M + 0.2% (v/v) Triton X-100	10	ND[b]	85.9 ± 3.4
9	NaCl 1 M + 0.2% (v/v) Triton X-100	5	ND[b]	85.4 ± 2.1

a – Assay performed without pH adjustment.

b – Not detected (high dilution factor).

3.6. Performance of the heterogeneous biocatalyst in esterification reactions

The catalytic activity of the biocatalysts was also evaluated in the synthesis of esters with lubricant properties via esterification reactions in iso-octane medium. Initially, the effect of the alcohol on the ester synthesis at fixed reaction time – 70 min was evaluated (Table 3.5).

Table 3.5 **-** Esterification of palmitic acid with several alcohols catalyzed by TLL immobilized on Chit–GA or Chit–GA–Gly. Experiments were performed as described in Materials and Methods.

Alcohol	Acid conversion percentage[a] (%)	
	Chit–GA	Chit–GA–Gly
Isoamyl alcohol	23.7 ± 2.5	80.3 ± 0.6
Hexanol	20.5 ± 0.7	74.7 ± 1.3%
2-ethyl-hexyl alcohol	15.8 ± 1.9	70.3 ± 3.2%
Decanol	17.5 ± 1.1	73.2 ± 2.3%

a – The reactions were performed at 50°C, equimolar ratio acid:alcohol (0.5 M of each reactant in iso-octane), biocatalyst concentration of 10% m/v of medium, 240 rpm and 70 min.

TLL immobilized on Chit–GA–Gly was more active in ester synthesis – between 3.4- and 4.4-times- than the one prepared using Chit–GA (maintaining the activity differences between both preparations in the hydrolysis of olive oil). According to Table 3.5, and using the new TLL biocatalyst, a maximum palmitic acid conversion of 80.3 ± 0.6% was obtained using isoamyl alcohol after 70 min of reaction, followed by the results obtained hexanol (74.7 ± 1.3%), decanol (73.2 ± 2.3%), and 2-ethyl-hexanol (70.3 ± 3.2%). From these results, isoamyl alcohol was selected for further studies. Fig. 3.7A shows that a maximum acid conversion of 85% was obtained after 90 min of reaction. The initial reaction rate (determined from the slope

of the palmitic acid consumption *versus* reaction time – Fig. 3.7A inset) was 8.3 mM/min of reaction, which corresponds to an esterification activity of 13,833.3 µmoles/min.g_{biocat}, 13.3-times higher than its hydrolytic activity (1,042.8 ± 63.1 U/g – see Table 3.2) due to diffusional problems using olive oil emulsion as substrate. These results clearly show the excellent diffusion of both palmitic acid and isoamyl alcohol from the reaction mixture to the biocatalyst microenvironment. This very high activity in esterification reactions makes this biocatalyst a very promising one for this kind of reaction.

The operational stability of the new biocatalyst was studied under the fixed experimental conditions described in Section 2.6, this study can be considered a stress operational stability determination (high temperature and high concentrations of starting materials – *iso*amyl alcohol and palmitic acid). According to Fig. 3.7B, the prepared biocatalyst maintained the original conversion yields in the first 3 cycles. In the 4th and 5th reuses, the acid conversion decreased to 65%, yield was 34% in the 6th reuse and this value remained unaltered until the 9th cycle. This decrease in enzyme activity in the first cycles may be due to diverse reasons. One is the possible distortion of the 3D structure of some TLL molecules due to experimental conditions – 50 ºC in iso-octane medium (see Fig. 3.6). Another possibility is that the hexane washing may not be efficient enough to avoid the accumulation of unconverted substrates and/or products in the biocatalyst microenvironment during successive cycles of reaction, using just hydrophobic supports this acid elimination is simple [89,90], but using this support, it could retain some acid molecules by ionic interactions. Finally, even if the adsorption is very strong and based on different enzyme interactions, palmitic acid may behave as a detergent [91], breaking the hydrophobic interactions, while also is an anionic compound, that could break ion interactions.

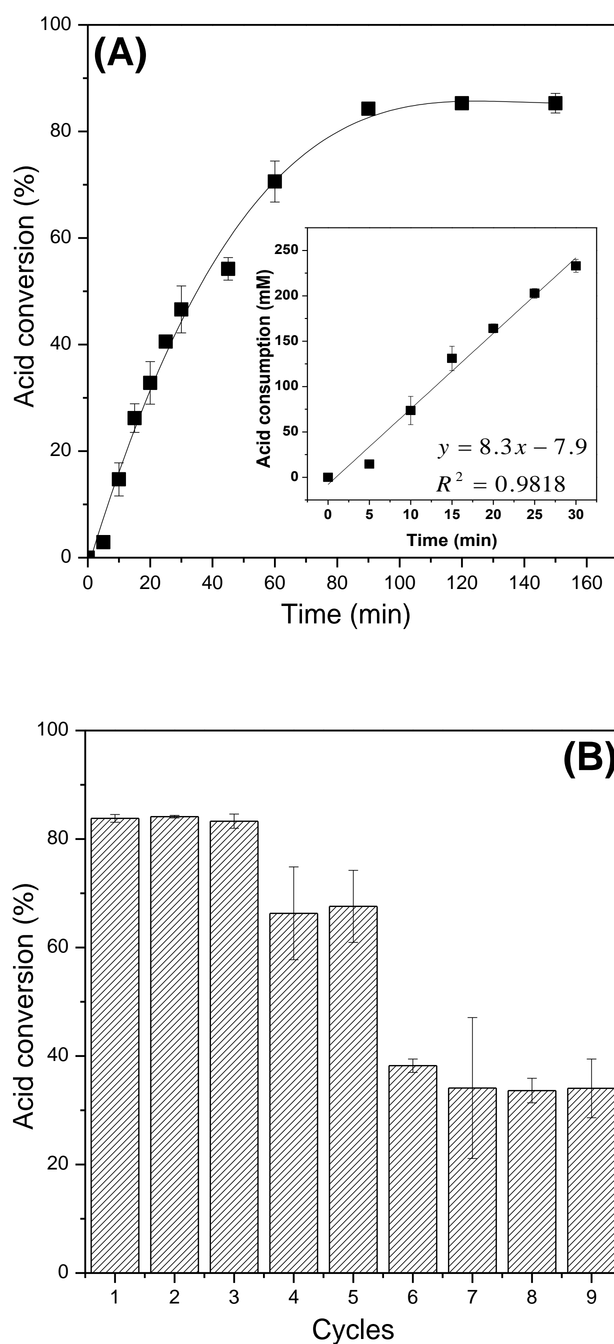

Figure 3.7 - Effect of reaction time on the isoamyl palmitate synthesis via esterification reaction in an iso-octane system at equimolar ratio acid:alcohol, at 50°C and 240 rpm – Inset is the determination of initial reaction rate (A), and operational stability after successive batches of 90 min each (B). Experiments were performed as described in Materials and Methods.

4. Conclusion

In this study, a chitosan-based heterofunctional support (Chit–GA–Gly) was prepared via activation with glutaraldehyde and glycine and its performance to immobilize TLL has been compared with a classical immobilization support (Chit–GA). The introduction of the different functional groups on the chitosan surface was confirmed by different techniques (FT–IR, ZP and TGA). According to the results, both supports exhibited similar lipase immobilization capacity (between 53 and 55 mg/g) and stability when incubated at 50°C in organic solvents. On the other hand, the functionalization of the support with glycine allowed preparing biocatalysts around 4-times more active than TLL covalently immobilized on Chit–GA in olive oil hydrolysis and alkyl palmitate synthesis. TLL was preferentially immobilized via physical adsorption (ionic and hydrophobic interactions) on this new support, while covalent attachment (95%) was the main immobilization final cause for TLL using classical Chit–GA support. The present study demonstrates that this support can be successfully used in the preparation of industrial biocatalysts due to its high protein capacity and catalytic activity.

Author contributions

Nicole S. Okura, Guilherme J. Sabi, Marcela C. Crivellenti and Raphael A. B. Gomes performed the experimental work, original draft preparation and final editing of the manuscript. Roberto Fernandez-Lafuente and Adriano A. Mendes took care of the conceptualization, supervision, funding acquisition, and paper writing. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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4 CONCLUSÃO

Os resultados obtidos no desenvolvimento das atividades propostas nesta dissertação de mestrado mostraram que a preparação de suportes heterofuncionais para a preparação de biocatalisadores heterogêneos podem garantir um bom ou melhor desempenho de enzimas em processos industriais. Foi demonstrado que a aplicação deste novo suporte possibilitou melhorar a atividade catalítica de uma lipase amplamente empregada em reações de biotransformação quando comparado ao suporte convencional preparado por ativação com glutaraldeído. A preparação deste novo suporte a partir de um biomaterial orindo do setor pesqueiro, comumente encontrdo em nosso país, abrirá novas oportnidades no campo da biocatálise para a peparação de novos biocatalisadores heterogêneos por imobilização de outras enzimas industriais como lipases, carboidrases e proteases, as enzimas mais empregadas em processos industriais. Além disso, o projeto executado possibilitará ampliar as aplicações deste suporte para outras importantes finalidades como a separação/parcial purificação de biomoléculas obtidas em processos biotecnológicos e em processos de tratamento de águas residuárias por remoção de poluentes como substâncias orgânicas, por exemplo medicamentos, metais pesados e corantes devido à sua promissora aplicação como adsorvente.

5 PERSPECTIVAS PARA TRABALHOS FUTUROS

Diante das conclusões obtidas, propõem-se algumas sugestões para a continuidade desse trabalho:

i) Produção de biolubrificantes empregando lipase de *Thermomyces lanuginosus* imobilizada, a partir de outros óleos vegetais comumente encontrados em nosso país tais como: óleo de macaúba, óleo de milho, borra de refino de óleos vegetais, óleos microbianos e de algas;

ii) Produção de biolubrificantes empregando outros álcoois como o óleo fúsel;

iii) Aplicação do biocatalisador preparado em outras reações de interesse industrial;

iv) Funcionalização das partículas de quitosana com outros aminoácidos para avaliação do efeito no processo de imobilização e desempenho catalítico da enzima;

v) Imobilização de outras lipases como Eversa Transform 2.0 no suporte preparado, uma lipase de baixo custo obtida de *Thermomyces lanuginosus* e fornecida pela Novozymes