UNIVERSIDADE FEDERAL DO PARANÁ

GLAUCO SILVA DIAS

IMOBILIZAÇÃO DA LIPASE DE *Pseudomonas cepacia* EM COMPOSTOS LAMELARES PARA UTILIZAÇÃO EM BIOCATÁLISE

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Ciências-Bioquímica do Setor de Ciências Biológicas da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências (Bioquímica).

Orientadora: Prof.ª Dr.ª Nadia Krieger
Coorientador: Prof. Dr. Fernando Wypych

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Vencer a si próprio é a maior de todas as vitórias”
Platão

RESUMO

Este trabalho teve como objetivo geral estudar o uso dos compostos lamelares sintéticos, hidróxidos duplos lamelares (HDLs) e hidróxidos sais lamelares (HSLs), como suportes para imobilização de lipases para aplicação em meios orgânicos, utilizando a lipase de *Pseudomonas cepacia*. Inicialmente, foram utilizados como suportes HDLs de Zn/Al-Cl com diferentes razões molares Zn:Al. Os melhores valores de retenção de atividade (R) e eficiência de imobilização (EI) (188 e 96%, respectivamente) e a maior atividade de hidrólise em meio orgânico (279 U g^{-1}) foram obtidos para o HDL com a maior razão molar (4:1 Zn:Al), carga de proteína de 162 mg g^{-1} e tampão Tris-HCl (10 mmol L^{-1}, pH 7,5) como solvente de preparo da solução enzimática. A lipase imobilizada apresentou 50% de conversão na resolução do álcool (*rac*)-1-feniletanol, com excessos enantioméricos de substratos e produtos (*ee* > 99%) e enantiosseletividade (*E* > 200). Em estudos de reutilização, foi possível usar o derivado imobilizado em 30 ciclos sucessivos de síntese, com 1 h de duração cada ciclo. Dois HSLs diferentes também foram utilizados como suportes para LipPS. Hidroxinitrato de zinco (HNZ) e hidroxicloreto de zinco (HCZ) foram testados para diferentes cargas de proteína sendo a melhor de 162,5 mg g^{-1} para ambos suportes, que gerou derivados imobilizados com altos valores de atividade de hidrólise contra trioleína em meio orgânico (103 e 105 U g^{-1}, respectivamente), com alta EI (acima de 90%) e R (acima de 170%). Esses derivados imobilizados foram avaliados quanto à estabilidade em diferentes solventes orgânicos e armazenamento em diferentes temperaturas e também foram avaliados quanto à dessorção das lipases quando incubados em soluções com diferentes forças iônicas. A performance de LipPS imobilizada em HNZ e HCZ foi avaliada também quanto à resolução cinética do álcool (*rac*)-1-feniletanol, onde obteve-se conversões de 50 e 40% em 2 horas e ee_s de 99 e 73%, respectivamente. Para ambos preparados imobilizados, o ee_p foi superior a 99% e *E* superior a 200. No estudo de reutilização, foram realizados 5 ciclos consecutivos de síntese, de 2 h cada, onde os valores de conversão e ee_s foram mantidos para ambos preparados enzimáticos. Os resultados obtidos neste trabalho servem de base para o desenvolvimento de aplicações em biocatálise usando compostos lamelares tipo HDLs e HSLs como suporte para lipases.

Palavras-chave: *Pseudomonas cepacia* lipase, imobilização, compostos lamelares, hidróxidos duplos lamelares, hidróxidos sais lamelares, resolução cinética enzimática.

ABSTRACT

This work aimed to study the use of synthetic layered double hydroxides (LDHs) and layered hydroxide salts (LHSs), as supports for lipases immobilization with application in organic media, using the *Pseudomonas cepacia* lipase. Initially, LDHs of Zn/Al-Cl were used with different molar ratios Zn:Al. The best values of activity retention (R) and immobilization efficiency (IE) (188 and 96%, respectively) and the highest hydrolysis activity in organic medium (279 U g^{-1}) were obtained for LDH with the highest ratio molar (4:1 Zn:Al), 162.5 mg g^{-1} of protein loading and Tris-HCl buffer (10 mmol L^{-1}, pH 7.5) as solvent for preparing the enzyme solution. The immobilized lipase showed 50% of conversion in the resolution of alcohol (*rac*)-1-phenylethanol, with enantiomeric excesses of substrates and products (*ee*> 99%) and enantioselectivity (*E*> 200). In reuse studies, it was possible to use the immobilized derivative in 30 successive synthesis cycles, each of 1 h. Two different LHSs were also used as supports for LipPS. Zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC) were tested for different protein loading, the best being 162.5 mg g^{-1} for both supports, which produced immobilized derivatives with high values of triolein-hydrolyzing activity in medium organic (103 and 105 U g^{-1}, respectively), with high IE (above 90%) and R (above 170%). These immobilized derivatives were evaluated for stability in different organic solvents and storage at different temperatures and were also evaluated for lipase desorption when incubated in solutions with different ionic forces. The performance of LipPS immobilized on HNZ and HCZ was also evaluated for the kinetic resolution of alcohol (*rac*)-1-phenylethanol, where 50 and 40% conversions were obtained in 2 hours and 99 and 73% ee_s, respectively. For both immobilized preparations, the ee_p was greater than 99% and *E* greater than 200. In the reuse study, 5 consecutive cycles of synthesis were carried out, of 2 h each, where the conversion and ee_s values were maintained for both enzymatic preparations. The results obtained in this work serve as a basis for the development of applications in biocatalysis using layer compounds as LDHs and LHSs as support for lipases.

Keywords: *Pseudomonas cepacia* lipase, immobilization, layered compounds, layered double hydroxides, layered hydroxide salts, enzymatic kinetic resolution.

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CAPÍTULO I – REVISÃO BIBLIOGRÁFICA

1.1 INTRODUÇÃO

Lipases (E.C. 3.1.1.3 - triacilglicerol hidrolases) são enzimas que atuam em uma ampla gama de reações incluindo esterificação, transesterificação e interesterificação (HASAN; SHAH; HAMEED, 2006; JOSEPH; RAMTEKE; THOMAS, 2008). Uma das mais nobres aplicações de lipases é a resolução de misturas racêmicas, o que as colocam em posição de destaque na área de biocatálise (JAEGER; REETZ, 1998; KAPOOR; GUPTA, 2012).

Em processos industriais, recomenda-se a utilização de lipases imobilizadas pois estas apresentam muitas vantagens em relação às lipases livres. O uso de lipases livres é restrito principalmente devido à dificuldade na recuperação e consequente reutilização desses biocatalisadores. Assim, a imobilização de lipases em materiais inertes tem sido aplicada para resolver esse problema e ao mesmo tempo favorecer a redução do custo dos processos (MATEO *et al.*, 2007).

Dentro desse contexto, a imobilização de lipases é um tema atual, muito explorado por diferentes grupos de pesquisa que utilizam diversos materiais para este fim, tais como sílica gel, óxido metálico, resinas poliméricas, nanopartículas magnéticas e muitos outros (BELLEZZA; CIPICIANI; QUOTADAMO, 2005; KANG *et al.*, 2007; LIU *et al.*, 2011; MAHMOOD *et al.*, 2008; SHYLESH; SCHÜNEMANN; THIEL, 2010; WANG *et al.*, 2011). Essa variedade de materiais permite, além da recuperação da enzima, obter biocatalisadores com diferentes propriedades em termos de atividade, estabilidade e seletividade (SANTOS *et al.*, 2015).

Na área de química de materiais, compostos lamelares como os hidróxidos duplos lamelares (HDLs) e hidróxidos sais lamelares (HSLs) têm se destacado tanto para uso como nanocompósitos quanto na composição de materiais nanohíbridos funcionais (ARIZAGA; SATYANARAYANA; WYPYCH, 2007; TAVIOT-GUÉHO *et al.*, 2018). Especificamente para imobilização de enzimas, HDLs de diferentes composições já foram utilizados como suporte para imobilização de lipases, ureases, quinases, transcetolases, penicilina G acilase, frutose-6-fosfato aldolase, triosefosfato isomerase e acetilcolinesterase (FORANO; VIAL; MOUSTY, 2006; TAVIOT-GUÉHO *et al.*, 2018), enquanto que HSLs ainda não foram explorados com essa mesma finalidade.

As publicações que mostram a viabilidade do uso de HDLs para imobilização de lipases são direcionadas para a síntese e caracterização do HDL e posterior comparação entre as lipases imobilizadas e livres em relação à parâmetros como estabilidade térmica, estabilidade em

solventes orgânicos, atividade e estabilidade em ampla faixa de pH e estabilidade ao armazenamento (DONG *et al.*, 2014; RAHMAN *et al.*, 2008; RAHMAN *et al.*, 2004a; RAHMAN *et al.*, 2004b). Assim sendo, são necessários estudos que demonstrem não só a viabilidade da imobilização, mas também as vantagens da aplicação desses derivados imobilizados em processos biocatalisados. Além disso, mostrar o potencial de HSLs para imobilização de enzimas abre novas perspectivas de aplicações para esses materiais.

Portanto, considerando-se o grande potencial biocatalítico das lipases, a demanda por novas metodologias e materiais para imobilização desses biocatalisadores, e que os compostos lamelares são uma classe de materiais facilmente sintetizados em laboratório e que são compatíveis com a adsorção de enzimas, este trabalho justifica-se na necessidade de estudos de imobilização de lipases em compostos lamelares (HDL e HSL), de modo a explorar sua potencialidade visando aplicação em biocatálise.

1.2 OBJETIVOS

1.2.1 Geral

O objetivo deste trabalho foi estudar o uso dos compostos lamelares sintéticos, hidróxidos duplos lamelares (HDLs) e hidróxidos sais lamelares (HSLs), como suportes para imobilização de lipases para aplicação em meios orgânicos, utilizando a lipase de *Pseudomonas cepacia* como modelo enzimático.

1.2.2 Específicos

- Sintetizar e caracterizar hidróxidos duplos lamelares de Zn/Al em diferentes razões molares intercalados com ânions cloreto;
- Sintetizar e caracterizar o hidróxido sal lamelar hidróxido cloreto de zinco e hidróxido nitrato de zinco;
- Imobilizar, por adsorção, a lipase comercial de *Pseudomonas cepacia* nos compostos lamelares sintetizados e avaliar os principais parâmetros do processo de imobilização: efeito da densidade de carga das lamelas dos HDLs; efeito do solvente no preparo da solução enzimática e efeito da carga de proteína no suporte;
- Avaliar os diferentes suportes de imobilização e selecionar os melhores derivados imobilizados em relação aos parâmetros de retenção de atividade (R), eficiência de imobilização (EI);
- Caracterizar os melhores derivados imobilizados em meio orgânico como atividade de hidrólise e estabilidade em solventes orgânicos;
- Avaliar o efeito da incubação do derivado imobilizado em soluções com diferentes forças iônicas na dessorção das lipases;
- Avaliar a estabilidade de armazenamento dos derivados imobilizados em diferentes temperaturas;
- Determinar o efeito do tipo de suporte no coeficiente de enantiosseletividade (E) da lipase através da utilização dos derivados imobilizados em reações-modelo de resolução cinética do álcool racêmico 1-feniletanol.

1.3 FUNDAMENTAÇÃO TEÓRICA

1.3.1 Lipases

1.3.1.1 Definição, características e aplicações

Lipases são enzimas que têm como função natural a hidrólise de ligações éster de triacilgliceróis de cadeia longa (acima de dez átomos de carbono) produzindo di- e monoacilgliceróis, glicerol e ácidos graxos (JAEGER; REETZ, 1998; KAPOOR; GUPTA, 2012). Lipases também são capazes de catalisar reações em sistemas não-aquosos, o que confere a elas um grande potencial de uso em biocatálise, especialmente em reações de esterificação, transesterificação e interesterificação (KLIBANOV, 2001; KRIEGER *et al.*, 2004).

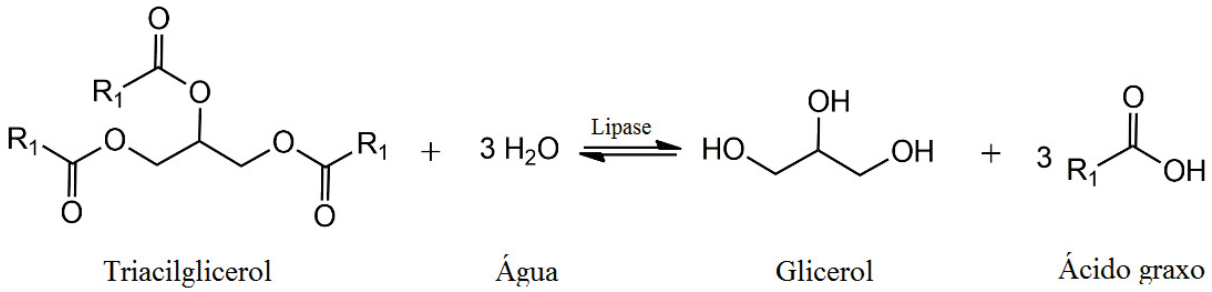

FIGURA 1.1 – REAÇÃO DE HIDRÓLISE DE UM TRIACILGLICEROL CATALISADA POR LIPASES
FONTE: O autor (2019).

Lipases possuem uma estrutura de dobramento α/β hidrolase, que apresenta um núcleo central composto por folhas β pregueadas, consistindo de oito diferentes fitas β antiparalelas (β1-β8), conectadas, interligadas e envolvidas por até seis α-hélices (α1-α6) (FIGURA 1.2) (BORNSCHEUER, 2002; JAEGER; DIJKSTRA; REETZ, 1999; POUDEROYEN *et al.*, 2001). O sítio ativo das lipases é composto por uma tríade catalítica, formado pelas cadeias laterais dos resíduos dos aminoácidos serina (Ser), histidina (His) e aspartato (Asp) ou glutamato (Glu). O resíduo de serina, que atua como nucleófilo, encontra-se ligado por ligações de hidrogênio à cadeia lateral do resíduo de histidina, que, por sua vez, encontra-se ligado por ligações de hidrogênio a um resíduo de aspartato ou glutamato (JAEGER *et al.*, 1994; JAEGER; REETZ, 1998).

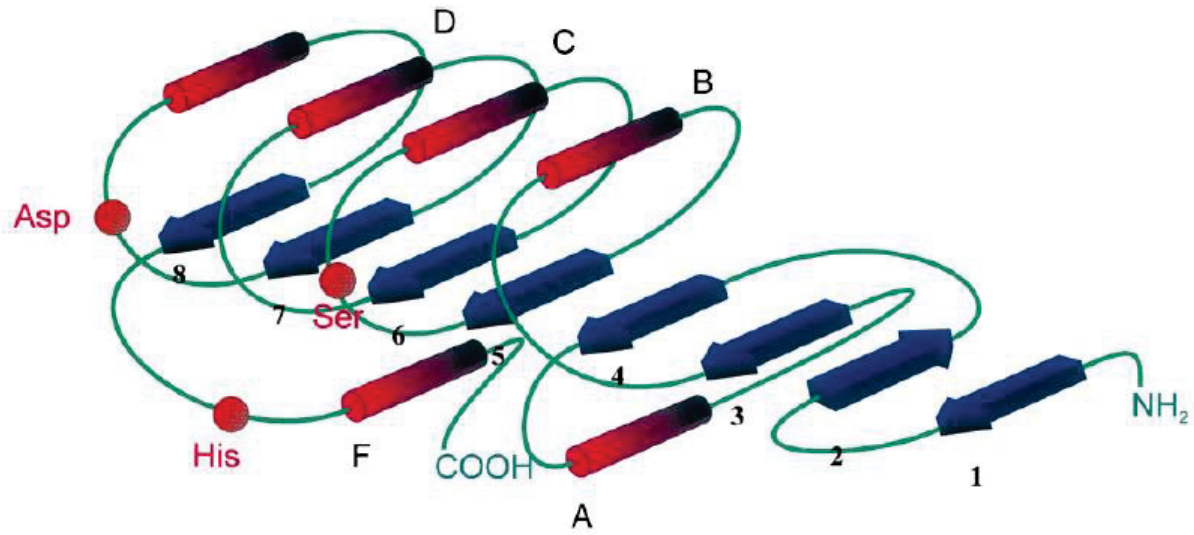

FIGURA 1.2 – MODELO ESTRUTURAL DE α/β HIDROLASES

FONTE: BORNSCHEUER (2002)
LEGENDA: As α-hélices são representadas pelos cilindros (A-F) e as folhas β pelas setas planas (1-8). Os círculos sólidos em vermelho representam as posições topológicas dos resíduos que compõem o sítio ativo.

Na maioria das lipases, o sítio ativo é coberto por uma "tampa" (*lid*) com características hidrofóbicas. Esta tampa é uma estrutura helicoidal que, em meios aquosos na ausência de um substrato hidrofóbico, deixa o sítio ativo inacessível ao substrato. Na presença de substratos hidrofóbicos, ocorre a adsorção da enzima na interface hidrofóbica e, então, a tampa move-se, e altera a forma fechada da enzima para a forma aberta, com o sítio ativo acessível ao substrato. Esse fenômeno é chamado de ativação interfacial (JAEGER; DIJKSTRA; REETZ, 1999; SCHRAG *et al.*, 1997).

O destaque das lipases como biocatalisadores deve-se principalmente à sua especificidade e seletividade frente a diferentes substratos. Em termos de classificação, lipases podem ser regiosseletivas, quando hidrolisam ligações éster de acordo com a posição na molécula, podendo ser *sn*-1,3 ou *sn*-1,2 específicas, ou aleatoriamente em qualquer ligação, e, neste caso, são inespecíficas; tipossseletivas, quando atuam sobre ácidos graxos com comprimentos de cadeia e graus de insaturação específicos; e enantiosseletivas, quando discriminam enantiômeros em misturas racêmicas. As lipases podem também apresentar a combinação de mais de um tipo de especificidade (JENSEN; GALLUZZO; BUSH, 1990).

Lipases enantiosseletivas são de grande destaque em biocatálise devido à sua utilização na resolução de misturas racêmicas para a produção de compostos opticamente puros e de alto valor agregado. A aplicação de lipases nessas reações baseia-se na capacidade da enzima atuar, com diferentes velocidades, sobre dois enantiômeros, catalisando a reação com formação

preferencial de um enantiômero em relação ao outro. O enantiômero que permitir a melhor interação dos grupos substituintes com o sítio ativo da enzima será, portanto, o enantiômero preferido (FABER, 2000).

Para lipases, a enantiosseletividade frente a álcoois secundários é explicada pela chamada "Regra de Kazlauskas". O reconhecimento quiral ocorre em duas regiões de diferentes tamanhos, uma grande e outra pequena, localizadas no sítio ativo da enzima (KAZLAUSKAS *et al.*, 1991). Quando o álcool secundário é submetido à resolução por uma lipase enantiosseletiva, um dos enantiômeros liga-se ao sítio ativo da enzima com maior eficiência do que o outro, numa geometria denominada de "modo produtivo" (FIGURA 1.3A). Quando o outro enantiômero interage com a enzima, é então forçado a acomodar seu maior substituinte na menor região do sítio ativo, denominado de "modo não produtivo" (FIGURA 1.3B). Esse encaixe é energicamente desfavorável e provoca um impedimento estérico, diminuindo assim, a velocidade de reação para este enantiômero (GHANEM, 2007).

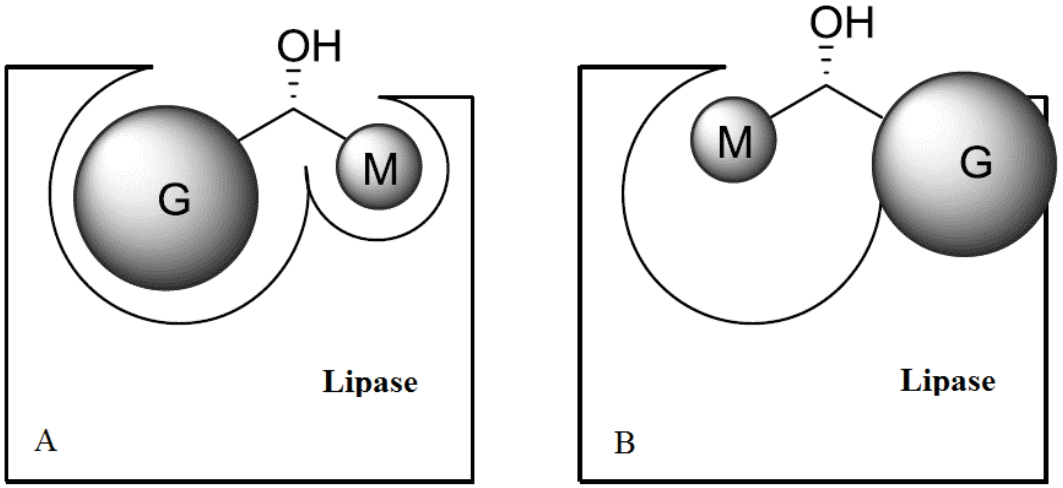

FIGURA 1.3 – MODELO ESTRUTURAL DO SÍTIO ATIVO DE LIPASES SEGUNDO A REGRA DE KAZLAUSKAS

FONTE: Adaptado de GHANEM (2007)
LEGENDA: (A) Enantiômero de reação mais rápida, "modo produtivo"; (B) enantiômero de reação mais lenta, "modo não produtivo". Utilizando como referência o grupo hidroxila do álcool, o grupo grande (G) fica à esquerda, e o grupo médio (M) à direita, ao contrário do seu enantiômero.

As lipases, além de serem comumente usadas na indústria farmacêutica, para resolução de misturas racêmicas, são também amplamente empregadas na indústria oleoquímica para síntese de ésteres do biodiesel em reações de esterificação e transesterificação; na indústria alimentícia, para conferir ou modificar propriedades organolépticas dos alimentos; na área médica, para dosagem de triglicerídeos no sangue; na engenharia ambiental, para tratamento de

efluentes e na indústria de papel e na produção de cosméticos (HASAN; SHAH; HAMEED, 2006; JAEGER; REETZ, 1998; LIU *et al.*, 2012).

1.3.1.2 A lipase de *Pseudomonas cepacia* (LipPS)

A lipase extracelular de *Pseudomonas cepacia* (PS ou PCL, da Amano, aqui denominada de LipPS) utilizada no presente trabalho, tem cadeia polipeptídica composta por 320 resíduos de aminoácidos, peso molecular de 34,1 kDa e ponto isoelétrico (pI) de 5,1 (BORNSCHEUER *et al.*, 1994; SUGIHARA *et al.*, 1992). LipPS é atualmente uma das enzimas mais aplicadas em processos biotecnológicos devido à sua capacidade de atuação em uma grande variedade de substratos, à resistência a altas temperaturas (até 75 °C), à atuação em ampla faixa de pH (3,5 – 11) e à tolerância a diversos solventes orgânicos, incluindo álcoois de cadeia curta (BORNSCHEUER *et al.*, 1994; SASSO *et al.*, 2016). Além disso, LipPS exibe atividade de hidrólise sobre diferentes triacilgliceróis, independentemente do comprimento da cadeia dos ácidos graxos e não é regiosseletiva (KIM *et al.*, 1992). Entretanto, em reações de esterificação, na presença de solventes hidrofílicos, LipPS apresenta preferência pelas posições *sn*-1,3 (BI *et al.*, 2015) e marcante seletividade para ácidos graxos C8, C10 e C18:2 (CHANG; LEE; PARKIN, 1999; LEE; PARKIN, 2003).

LipPS já foi caracterizada e sua estrutura 3D determinada por difração de raio-X (BORNSCHEUER *et al.*, 1994; KIM *et al.*, 1992) . O sítio ativo de LipPS inclui Ser87, His286 e Asp264 (FIGURA 1.4), sendo que os principais resíduos hidrofóbicos da enzima constituem as paredes ao redor do sítio ativo, *lid* (BARBE *et al.*, 2009; LANG *et al.*, 1996; SCHRAG *et al.*, 1997). A estrutura de LipPS em sua conformação aberta e ativa está mostrada na FIGURA 1.4, na qual a tríade catalítica do sítio ativo está representada por bastões vermelhos. As alterações conformacionais de LipPS durante o fenômeno de ativação interfacial estão associadas ao deslocamento da α5-hélice (*lid*) (verde, FIGURA 1.4) acompanhada pela reorientação da α9-hélice (azul, FIGURA 1.4).

LipPS comercial utilizada neste trabalho é produzida pela empresa Amano Enzyme Inc. (Elgin, Illinois, U.S.A.) e comercializada pela Sigma-Aldrich (St. Louis, Missouri, U.S.A.) na forma livre, imobilizada no suporte comercial Immobead 150 (um polipropileno que contém o anel oxirano ligado covalentemente à enzima) ou encapsulada em Sol-gel-AK (uma matriz de sílica gel, feita pelo processo sol-gel). LipPS na forma livre é um pó branco, solúvel em água, que contém dextrina e outros estabilizantes não divulgados pela empresa na composição (BORNSCHEUER e KAZLAUSKAS, 2006).

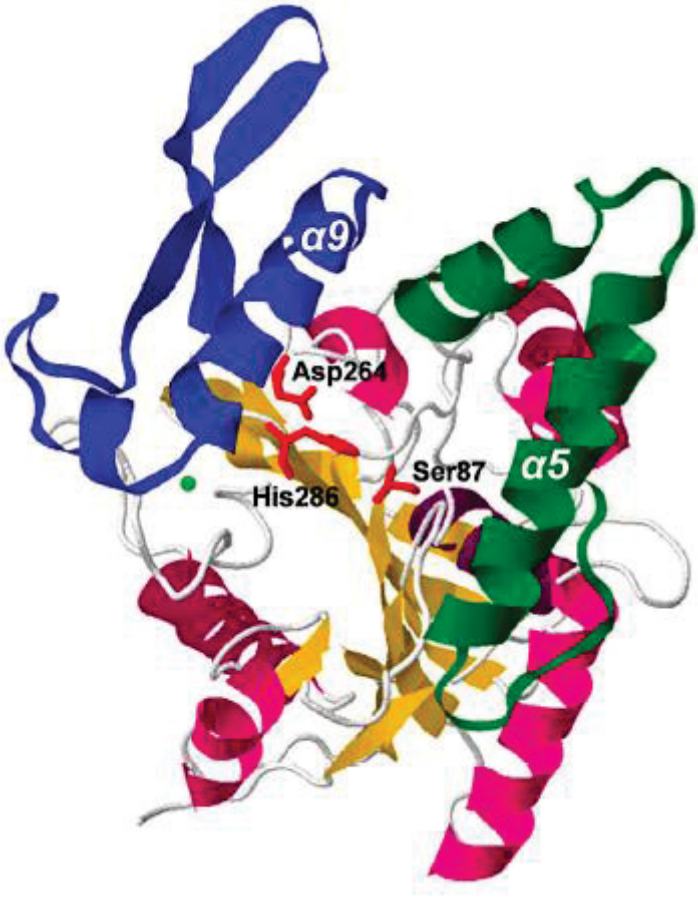

FIGURA 1.4 – ESTRUTURA DA LIPASE DE P*seudomonas cepacia* NA CONFORMAÇÃO ABERTA

FONTE: LIU; YANG; LI (2015)
LEGENDA: O sítio de ligação ao íon cálcio é destacado em verde claro, a tríade catalítica do sítio ativo está representada por bastões vermelhos.

Diferentes suportes já foram usados para imobilizar LipPS, como cerâmica, terra de diatomácea, compósitos de sílica-PVA e epóxi-sílica-PVA e Accurel EP-100 (polipropileno macroporoso) (HARA; HANEFELD; KANERVA, 2008; HASAN; SHAH; HAMEED, 2006; LIU *et al.*, 2012; MOREIRA *et al.*, 2007; SALIS *et al.*, 2008; SÁNCHEZ; TONETTO; FERREIRA, 2018; SUAN; SARMIDI, 2004; TONGBORIBOON; CHEIRSILP; H-KITTIKUN, 2010). A imobilização de LipPS em todos esses materiais conferiu maior atividade e melhor estabilidade para a enzima. A melhoria da estabilidade térmica e o aumento da resistência a solventes e álcoois após a imobilização também foram relatados por vários autores (CAO *et al.*, 2009; LIU, *et al.*, 2011; PENCREAC'H; BARATTI, 1997).

1.3.2 Imobilização de lipases

Imobilização é uma técnica de "aprisionar" ou "ligar" enzimas a suportes inertes por meios físicos ou químicos, tornando-as insolúveis em qualquer meio, para possibilitar a recuperação e reutilização do biocatalisador, mas permitindo com que a enzima entre em contato com o substrato, catalisando as reações (MATEO *et al.*, 2007). A imobilização de enzimas é fundamental em biocatálise visto que essa técnica, além de propiciar a reutilização do biocatalisador, pode também minimizar problemas decorrentes do uso de enzimas em sua forma livre, como as baixas atividade e estabilidade nas condições reacionais utilizadas em biocatálise, como faixas de temperatura relativamente elevadas, ampla faixa de pH e solventes orgânicos (VILLENEUVE *et al.*, 2000).

A imobilização pode tornar as lipases mais estáveis através de vários mecanismos dependendo do tipo de interação lipase-suporte. Por exemplo, a imobilização por adsorção em um suporte poroso pode estabilizar a lipase por impedir que ela interaja com ela própria ou outras moléculas do próprio extrato enzimático, prevenindo a agregação. Quando em meios orgânicos, uma vez imobilizadas, as lipases também não formarão agregados e tornam-se mais estáveis, ao contrário do que ocorre quando as enzimas livres são colocadas em contato direto com solventes (COLOMBIÉ; GAUNAND; LINDET, 2001).

Várias técnicas de imobilização têm sido estudadas e utilizadas para propiciar o desenvolvimento de processos industriais. Para imobilização de lipases, há vários tipos de métodos. A escolha do suporte e do método de imobilização deve levar em consideração as características da lipase e do substrato, o tipo de interação, o caráter hidrofóbico/hidrofílico do suporte, a capacidade de ligação com a enzima, a estabilidade química e mecânica do suporte (que pode ser sólido, poroso, membranoso, fibroso, entre outros) e o custo (SOARES *et al.*, 1999). Parâmetros como eficiência de imobilização, retenção de atividade, volume e tamanho de poros, limitações de transferência de massa e perda de atividade enzimática durante o processo de imobilização também são fatores que devem ser considerados (VILLENEUVE *et al.*, 2000).

1.3.2.1 Principais métodos de imobilização

Os métodos de imobilização de enzimas (FIGURA 1.5) são classificados de acordo com a natureza da interação enzima-suporte e dividem-se em dois tipos: a) métodos físicos, que incluem a adsorção da enzima no suporte por interações hidrofóbicas, ligações de hidrogênio

ou por interações iônicas, e o encapsulamento ou aprisionamento da enzima em géis de matrizes poliméricas ou em micelas inversas; b) métodos químicos, que se caracterizam pelo estabelecimento de ligações covalentes entre a enzima e o suporte, por ligações intercruzadas entre as próprias moléculas de enzima e por entrelaçamento entre polímeros geleiformes como, por exemplo, a poliacrilamida (MURTY; BHAT; MUNISWARAN, 2002).

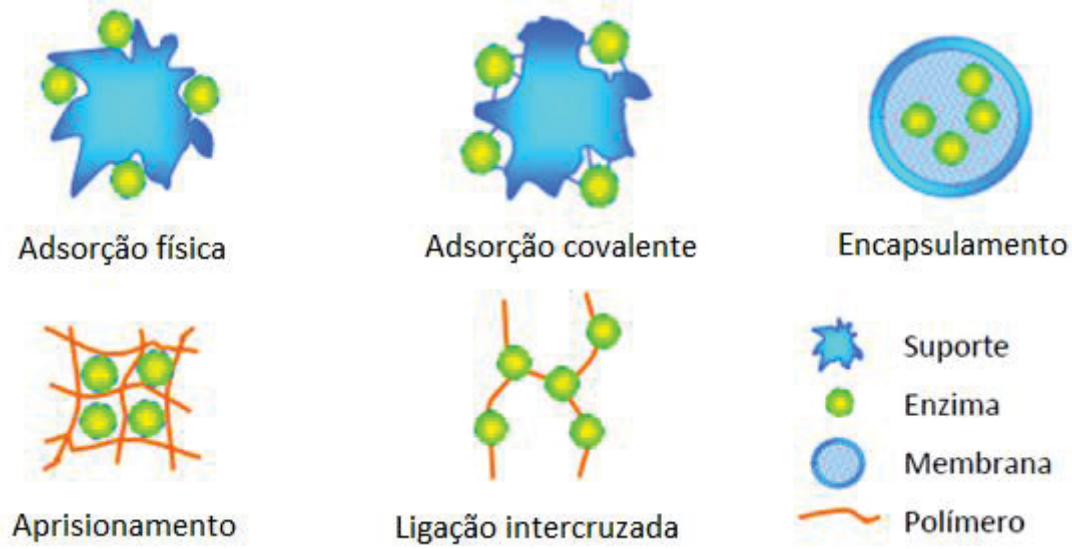

FIGURA 1.5 – MÉTODOS DE IMOBILIZAÇÃO DE ENZIMAS
FONTE: Adaptado de SALLEH; RAHMAN; BASRI (2006)

O método baseado na adsorção de lipases em suportes sólidos é o mais utilizado e, também por ter sido objeto de estudo no presente trabalho, será descrito com mais detalhes na próxima seção (1.3.3.2).

A imobilização por ligação covalente ocorre pela formação de ligações covalentes entre grupos reativos do suporte e alguns grupos funcionais dos resíduos de aminoácidos na superfície da enzima (MURTY; BHAT; MUNISWARAN, 2002). A principal vantagem desse tipo de imobilização é a formação de ligações muito resistentes entre a enzima e o suporte que, além de conferir maior estabilidade, evita a dessorção da enzima do suporte mesmo em meios aquosos. Como desvantagens, têm-se os custos mais elevados de imobilização e o efeito negativo da ligação covalente na conformação cataliticamente ativa da enzima (HANEFELD; GARDOSSI; MAGNER, 2009; MATEO *et al.*, 2007).

Já o método de imobilização por encapsulamento envolve a inclusão de enzimas em membranas semipermeáveis, impondo a elas uma barreira física, que, ao mesmo tempo, não restringe a mobilidade dos substratos e produtos no interior das membranas (BRADY; JORDAAN, 2009; FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). Uma

vantagem desse método é que diferentes enzimas podem ser imobilizadas simultaneamente via processo sol-gel. Além disso, a imobilização por encapsulamento praticamente não induz a alterações conformacionais na estrutura da enzima, o que previne a sua desnaturação. No entanto, apesar de o método do encapsulamento ser simples e robusto, enzimas encapsuladas podem ter o desempenho catalítico comprometido devido às limitações de transferência de massa nos meios reacionais (BRADY; JORDAAN, 2009; SASSOLAS; BLUM; LECA-BOUVIER, 2012).

No processo de imobilização por ligação intercruzada não se utiliza suporte para a imobilização, como cristais enzimáticos reticulados (*cross-linked enzyme crystals - CLECs*) e agregados enzimáticos reticulados (*cross-linked enzyme aggregates - CLEAs*) (SHELDON; PELT, 2013). Nesses métodos, grupos funcionais dos resíduos de aminoácidos das enzimas formam ligações covalentes cruzadas entre si através de grupos nucleofílicos de reagentes bifuncionais, tais como dialdeídos, diaminas ativadas com carbodiimida, diisocianatos, entre outros, sendo o glutaraldeído mais utilizado para esse fim (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013; HANEFELD; GARDOSSI; MAGNER, 2009). A principal desvantagem do método de ligação intercruzada é a possibilidade da perda de atividade enzimática devido às distorções sofridas pelas enzimas, que alteram a sua estrutura cataliticamente ativa (SASSOLAS; BLUM; LECA-BOUVIER, 2012).

Apesar de todas essas técnicas apresentadas já estarem consolidadas para a imobilização de enzimas, não há um método universal de imobilização, cuja metodologia se aplique eficazmente para qualquer enzima. Logo, não há uma regra que prediga a magnitude da atividade e estabilidade de uma enzima após o processo de imobilização (HANEFELD; GARDOSSI; MAGNER, 2009; VILLENEUVE *et al.*, 2000). A abordagem para o processo de imobilização envolve tipicamente procedimentos empíricos, até que um sistema satisfatório seja desenvolvido. Assim, para confirmar o sucesso na imobilização deve-se levar em conta, especialmente, a estabilidade e a atividade alcançada pelas enzimas após imobilizadas (HANEFELD; GARDOSSI; MAGNER, 2009; SHELDON; PELT, 2013).

1.3.2.2 Método de imobilização por adsorção de lipases em superfícies sólidas

A adsorção física de lipases em superfícies sólidas é o método mais comum para imobilização de lipases, pois envolve procedimentos simples e é menos dispendiosa quando comparada aos outros tipos de imobilização. Nesse método, as lipases são imobilizadas em um suporte sólido através interações com baixas energias de ligação, como forças Van der Waals,

ligações de hidrogênio, interações hidrofóbicas e interações eletrostáticas ou iônicas (MENDES *et al.*, 2011; VILLENEUVE *et al.*, 2000).

Vários parâmetros são determinantes para o sucesso e eficiência da adsorção física, como o tamanho da enzima a ser adsorvida, a área específica do material suporte, suas propriedades morfológicas, sua química de superfície e o pH do meio de imobilização, particularmente no caso de imobilização por interação eletrostática (VILLENEUVE *et al.*, 2000). Para este método, frequentemente são utilizados suportes porosos uma vez que a enzima pode ser adsorvida tanto na superfície quanto no interior dos poros.

Além de simples e de custo mais baixo, a imobilização de lipases por adsorção física não provoca alterações significativas na estrutura tridimensional das enzimas, o que evita a desnaturação. Adicionalmente, a *lid* pode exercer um papel fundamental na imobilização por adsorção quando utilizados suportes hidrofóbicos, uma vez que ela, juntamente com os resíduos hidrofóbicos em torno do sítio ativo da lipase, vai interagir fortemente com a superfície do suporte. Nesses casos, lipases sofrem ativação interfacial na presença da interface hidrofóbica do suporte, sendo adsorvidas em sua superfície, mantendo a *lid* em sua conformação aberta (FERNANDEZ-LAFUENTE *et al.*, 1998; PALOMO *et al.*, 2002), facilitando a entrada do substrato. As desvantagens desse método são resultantes das interações fracas estabelecidas entre as lipases e o suporte que podem levar à baixa estabilidade das enzimas imobilizadas, além da possibilidade de dessorção das lipases do suporte, quando utilizadas em ambientes aquosos (HANEFELD; GARDOSSI; MAGNER, 2009). Entretanto, quando interações iônicas são estabelecidas, essa desvantagem é menos pronunciada devido à maior energia da interação iônica.

O método de imobilização de lipases por adsorção iônica é baseado nas interações eletrostáticas atrativas entre cargas residuais de aminoácidos da enzima e cargas opostas presentes na superfície do suporte (RABE; VERDES; SEEGER, 2011; TALBERT; GODDARD, 2012). Disto resulta que as interações eletrostáticas favoráveis à imobilização serão fortemente dependentes do pH do meio reacional, do ponto isoelétrico (pI) da enzima, bem como da distribuição de cargas na superfície do suporte. As lipases possuem resíduos de aminoácidos com cadeias laterais com grupos positivamente carregados nos resíduos de lisina e arginina, e com grupos negativamente carregados em resíduos de ácido aspártico ou ácido glutâmico (TALBERT E GODDARD, 2012) e, desse modo, o pH do meio determinará o estado eletrostático das moléculas de lipase e influenciará favorável ou desfavoravelmente a adsorção por adsorção iônica.

Quando o pH do meio de imobilização é igual ao ponto isoelétrico (pI) da enzima, a somatória de cargas positivas e negativas da proteína será muito próxima ou igual a zero, o que indica uma carga líquida neutra. Em condições de pH abaixo do pI, as enzimas estarão positivamente carregadas, enquanto em pH acima do ponto isoelétrico as moléculas estarão negativamente carregadas. Assim, as interações eletrostáticas serão favoráveis quando as repulsões enzima-enzima e enzima-suporte na interface forem mínimas, sendo que geralmente a capacidade máxima de adsorção é observada em valores de pH próximos ao pI da enzima (KNEZEVIC; SILER-MARINKOVIC; MOJOVIC, 2004; RABE; VERDES; SEEGER, 2011). A FIGURA 1.6 mostra um esquema de interação eletrostática entre uma molécula de lipase e um suporte com diferentes distribuições de cargas na superfície.

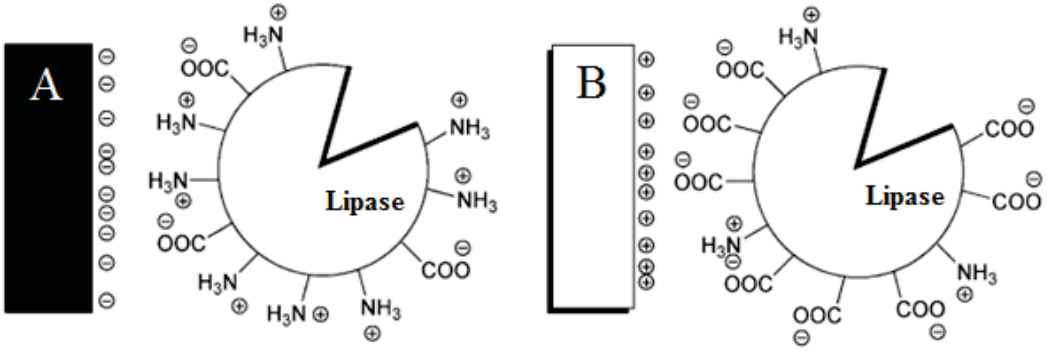

FIGURA 1.6 – REPRESENTAÇÃO ESQUEMÁTICA DE INTERAÇÕES ELETROSTÁTICAS ENTRE LIPASES E SUPORTES CARREGADOS

FONTE: Adaptado de HANEFELD; GARDOSSI; MAGNER (2009)
LEGENDA: (A) Suporte negativamente carregado e lipase positivamente carregada (pH<pI); (B) Suporte positivamente carregado e lipase negativamente carregada (pH>pI).

1.3.3 Suportes para imobilização de lipases

O sucesso na imobilização de enzimas depende, fundamentalmente, do tipo de suporte utilizado. A escolha do suporte ideal é um grande desafio na biocatálise, uma vez que a técnica de imobilizar enzimas sempre é executada visando obter biocatalisadores insolúveis, ativos, estáveis, recuperáveis e reutilizáveis. Assim, no momento da imobilização, um dos principais parâmetros a ser considerado é o conhecimento da natureza química e estrutura física do suporte, pois essas características irão influenciar na capacidade de adsorção e na manutenção da estrutura cataliticamente ativa da enzima, que por consequência afetam, respectivamente a estabilidade e a atividade enzimática (TALBERT; GODDARD, 2012).

Há uma enorme variedade de suportes naturais ou sintéticos, orgânicos ou inorgânicos, hidrofóbicos ou hidrofílicos e também híbridos orgânico-inorgânicos sintéticos que podem ser

empregados para a imobilização de lipases. Inicialmente, materiais inorgânicos como sílica, alumina e terra diatomácea eram os mais utilizados. Atualmente, outros materiais vêm sendo explorados para imobilização de lipases como biopolímeros (quitosana e celulose), resinas de troca iônica, argilas modificadas, silicatos mesoporosos, materiais híbridos nanoestruturados, filmes poliméricos, polímeros hidrofóbicos, nanopartículas magnéticas, entre outros (BELLEZZA; CIPICIANI; QUOTADAMO, 2005; KAMEL *et al.*, 2011; KANG *et al.*, 2007; LIU *et al.*, 2011; MAHMOOD *et al.*, 2008; SALIS *et al.*, 2009; SHYLESH; SCHÜNEMANN; THIEL, 2010; VILLENEUVE *et al.*, 2000).

No presente trabalho, foi estudada a imobilização de lipases por adsorção iônica utilizando compostos lamelares como suportes. Compostos lamelares são assim denominados por serem formados por lamelas, que são unidades bidimensionais cujos átomos estão ligados entre si por ligações covalentes. Lamelas empilham-se umas sobre outras para formação do composto lamelar e mantêm-se unidas por meio de interações do tipo Van der Waals, caso sejam neutras. No caso de lamelas carregadas, a união é mantida por interações eletrostáticas (KRYSZEWSKI, 2000; LERF, 2004). Assim, com base na carga elétrica das lamelas, os compostos lamelares são classificados em três grupos: a) compostos lamelares com cargas neutras, como o grafite e o talco; b) compostos lamelares com cargas negativas, como argilominerais trocadores catiônicos e c) compostos lamelares com cargas positivas, como os hidróxidos duplos lamelares (HDLs) e hidróxidos sais lamelares (HSLs). Esses dois últimos materiais foram usados no presente trabalho e serão descritos com mais detalhes a seguir.

1.3.3.1 Hidróxidos duplos lamelares (HDLs)

No grupo dos compostos lamelares com cargas positivas, HDLs são os mais explorados e há diversos trabalhos que descrevem os seus métodos de síntese, suas propriedades físico-químicas e sua vasta possibilidade de aplicações como agentes de reforço em polímeros, suportes para biomoléculas, agentes para remoção de poluentes em águas, liberadores controlados de medicamentos, pesticidas, catalisadores, entre outros (ALLOU *et al.*, 2017; CONSTANTINO; PINNAVAIA, 1995; CREPALDI; VALIM, 1991; RIVES; ULIBARRI, 1999; THEISS; AYOKO; FROST, 2016). HDLs também têm se mostrado apropriados para a imobilização de enzimas via interações eletrostáticas, pois são considerados materiais de baixo custo, apresentam grande capacidade de troca iônica além de serem sintetizados a partir de diferentes composições químicas que propicia variação de estrutura e textura (FORANO; VIAL; MOUSTY, 2006; TAVIOT-GUÉHO *et al.*, 2018).

A estrutura dos HDLs é derivada do hidróxido de magnésio, conhecido como brucita ($Mg(OH)_2$), um mineral de ocorrência natural, que pertence ao grupo dos compostos lamelares de cargas neutras. A brucita pode sofrer mudanças em sua composição, com alterações sutis na sua estrutura, quando um cátion metálico trivalente qualquer (M^{3+}) substitui isomorficamente alguns dos cátions Mg^{2+}, o que gera um excesso de carga nas lamelas. Essas cargas extras são então compensadas pela presença de ânions, dando origem aos HDLs (FIGURA 1.7). A composição química dos HDLs pode ser representada genericamente por $[M^{+2}_{1-x} M^{3+}_x(OH)_2]^{x+}(A^{m-})_{x/m}.yH_2O$, onde M^{2+} são os cátions metálicos bivalentes, M^{3+} são cátions metálicos trivalentes, e A^{m-} são diferentes tipos de ânions, como ânion inorgânico, ânion orgânico ou pequenas moléculas biológicas. Esses ânions têm a função de manter a eletroneutralidade e a consequente estabilidade da estrutura. Os ânions são acomodados no espaço entre as lamelas (interlamelar), geralmente estão hidratados e possuem uma relação estequiométrica direta com o número de M^{3+} na lamela (ALLOU *et al.*, 2017; NEWMAN; JONES, 1998).

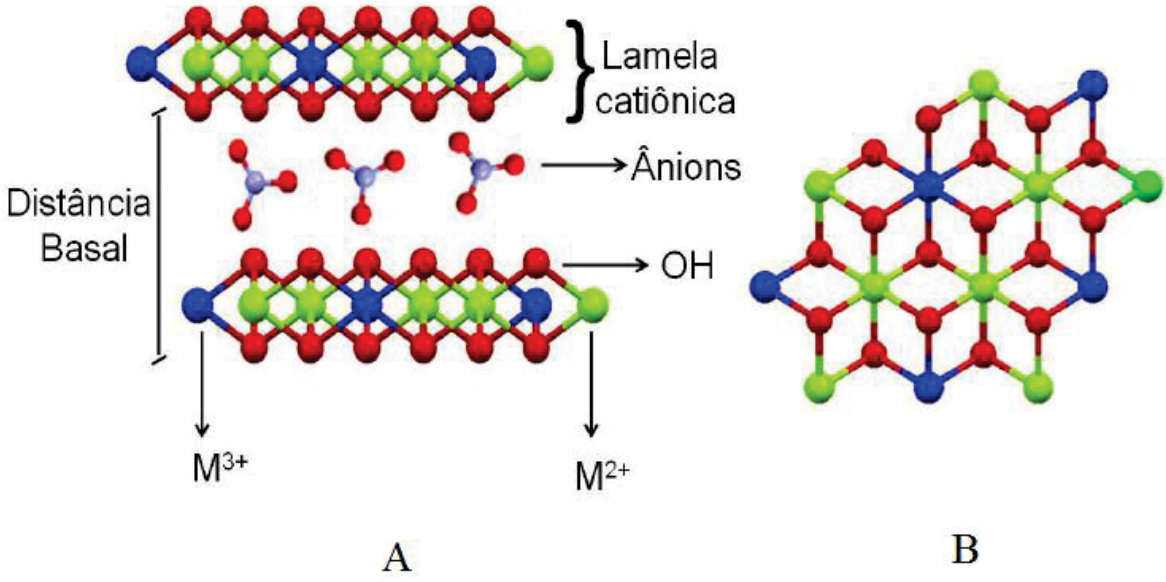

FIGURA 1.7 – REPRESENTAÇÃO ESQUEMÁTICA DA ESTRUTURA DE UM HIDRÓXIDO DUPLO LAMELAR GENÉRICO

FONTE: MANTOVANI (2015)
LEGENDA: (A) Visão lateral das lamelas; (B) Visão superior de uma lamela.

Nos HDLs, a razão molar entre os cátions metálicos bi e trivalentes (M^{2+}:M^{3+}) pode variar numa faixa de 1:1 a 8:1. Entretanto, a faixa ideal para se obter HDL puro e cristalino está entre 2:1 a 4:1 (RIVES, 2001). Essa variação é um fator importante, pois determina a densidade de carga na lamela dos HDLs, uma vez que a substituição isormórfica parcial dos metais

proporciona o excesso de cargas positivas. Portanto, a redução na razão molar implica no aumento da densidade de cargas e, de maneira inversa, o aumento da razão molar resulta na redução da densidade de cargas (CAVANI; TRIFIRO; VACCARI, 1991; CREPALDI; VALIM, 1991).

As células unitárias dos HDLs são normalmente de simetria hexagonal, com exceção da razão $M^{2+}/M^{3+} = 1$ (célula unitária é ortorrômbica). Ao ser analisado pela técnica de difração de raios X de pó, é observado para HDLs um padrão típico nos planos de difração para esta família de sólidos. Os planos basais (*00l*) estão relacionados ao empilhamento das lamelas, as reflexões (*hk0*) estão relacionadas a organização da estrutura no interior das lamelas e as reflexões (*0kl*) são relacionadas com a ordenação de uma lamela em relação à outra (CONTEROSITO *et al.*, 2018; CREPALDI; VALIM, 1991).

Os ânions responsáveis por conferir eletroneutralidade aos HDLs podem ser de natureza orgânica ou inorgânica, sendo mais comuns os ânions divalentes (carbonato ou sulfato) ou monovalentes (hidróxido, cloreto ou nitrato). A carga e o tamanho do ânion têm influência na estabilidade dos HDLs nos processos de troca iônica. Por exemplo, um HDL intercalado com íons NO_3^- será mais suscetível à troca iônica que um HDL intercalado com íons CO_3^{2-} (CREPALDI; VALIM, 1991).

HDLs são facilmente sintetizados em laboratório, o que permite um controle preciso na sua composição e estrutura, que são características importantes para muitas aplicações. Existem numerosos métodos para a preparação de HDLs, que incluem coprecipitação, sol-gel, síntese hidrotérmica, moagem mecânica, entre outros (THEISS; AYOKO; FROST, 2016). A síntese por coprecipitação ocorre a partir da precipitação simultânea de soluções contendo $M(OH)_2$ e $M(OH)_3$, sobre a solução do ânion a ser intercalado. Geralmente, esse tipo de síntese é feita a pH constante, que é mantido através da adição de uma solução de hidróxido de sódio ou potássio ao meio reacional (NEWMAN; JONES, 1998). As vantagens desse método são a maior homogeneidade, pureza de fase e cristalinidade do HDL obtido. A coprecipitação é o método mais comum para síntese de HDLs e é frequentemente descrito como um método "*one pot*" (THEISS; AYOKO; FROST, 2016) e, por ser simples e barato, foi o método utilizado para a preparação dos HDLs utilizados no presente trabalho.

1.3.3.1.1 HDLs para imobilização de lipases

O uso de HDLs como suporte na imobilização de enzimas ou células inteiras é descrito por diversos autores, sendo que os métodos mais utilizados são a adsorção das biomoléculas

nos cristais do HDL ou o aprisionamento entre as lamelas após os cristais do HDL passarem por processo de esfoliação. HDLs já foram utilizados como suporte para lipases em cinco trabalhos (TABELA 1.1) e, em todos eles, os autores focaram os estudos na síntese e caracterização dos HDLs e posterior comparação entre as lipases imobilizadas e livres em relação à parâmetros como estabilidade térmica, estabilidade em solventes orgânicos, atividade e estabilidade em ampla faixa de pH e estabilidade ao armazenamento (AGHAEI *et al*., 2020; DONG *et al.*, 2014; RAHMAN *et al.*, 2008; RAHMAN *et al.*, 2004a; RAHMAN *et al.*, 2004b).

Rahman *et al*. (2004a e 2004b) utilizaram a reação de esterificação do ácido oleico com 1-butanol, utilizando *n*-hexano como solvente para avaliar parâmetros como estabilidade térmica, estudo de lixiviação, estabilidade em solventes orgânicos e estabilidade de armazenamento das lipases imobilizadas em comparação com a sua forma livre. Nestes estudos, não foi feita a otimização da reação, que serviu apenas como método para avaliação da atividade enzimática. Em trabalho posterior, Rahman *et al.* (2008) otimizaram a síntese do metil adipato a partir de ácido adípico e metanol em *n*-hexano e observaram conversões acima de 80% em 2,5 h de reação.

TABELA 1.1 – ESTUDOS SOBRE O USO DE HIDRÓXIDOS DUPLOS LAMELARES (HDL) PARA A IMOBILIZAÇÃO DE LIPASES

Fonte da lipase	**HDL (razão molar)**	**Ânion interlamelar**	**Maior eficiência de imobilização (EI) relatada (%)**	**Aplicação**	**Referência**
Candida rugosa (Sigma Chemical)	Zn/Al Mg/Al (2:1)	Nitrato, carbonato e dodecil sulfato	[1]ni	Hidrólise do azeite de oliva	(AGHAEI *et al*., 2020)
Candida lipolytica (Shanghai Fu Xiang Biotechnology Co. Ltd)	Mg/Al (2; 2,5; 3; 3,5 e 4:1)	Dodecil sulfato	56,4	Hidrólise do azeite de oliva	(DONG *et al.*, 2014)
Candida rugosa (Sigma Chemical)	Zn/Al Mg/Al Ni/Al (4:1)	Nitrato	71	Esterificação (síntese do metil adipato)	(RAHMAN *et al.*, 2008)
Candida rugosa (Sigma Chemical)	Mg/Al (4:1)	Dodecil sulfato e nitrato	70,8	Esterificação (síntese do oleato de butila)	(RAHMAN, *et al.*, 2004a)
Candida rugosa (Sigma Chemical)	Zn/Al (4:1)	Dioctil sulfocinato e nitrato	78,5	Esterificação (síntese do oleato de butila)	(RAHMAN, *et al.*, 2004b)

[1]ni: não informado pelos autores ou não foi possível calcular com os dados fornecidos.
FONTE: O autor (2019)

Levando-se em consideração que os estudos relatados na literatura mostram apenas a prova de conceito (*proof of concept*) da imobilização de lipases em HDL e que as publicações que mostram a aplicação desses derivados imobilizados em meios orgânicos são escassas, é

necessário que se realizem em novos estudos que demonstrem não só a viabilidade da imobilização de lipases em HDL, mas também as vantagens da aplicação desses derivados imobilizados em processos biocatalisados.

1.3.3.2 Hidróxidos sais lamelares (HSLs)

HSLs, embora menos estudados que HDLs, também são alvos de diversos trabalhos relacionados à síntese, caracterização, resolução estrutural por difração de raios X, estabilidade térmica, dentre outros (MARKOV; IONCHEVA, 1990; MEYN; BENEKE; LAGALY, 1993; NEWMAN; JONES, 1999; PETROV; PETKOV; RACHEV, 1985; STÄHLIN; OSWALD, 1971). Em estrutura, HSLs, assim como HDLs, também são considerados derivados da brucita, embora com uma complexidade estrutural maior. Nos HSLs, a capacidade de troca iônica provém de outra natureza, pois não ocorre substituição isomórfica parcial dos cátions divalentes por trivalentes. Para formação dos HSLs, uma fração dos sítios correspondentes às hidroxilas é ocupada por outros ânions ou por moléculas de água, o que exige a presença de contraíons para estabilizar as cargas das lamelas que alocam os cátions (ARIZAGA; SATYANARAYANA; WYPYCH, 2007). HSLs são representados pela fórmula genérica $M^{2+}(OH)_{2-x}(A^{n-})_{x/n}.yH_2O$, onde M^{2+} corresponde ao cátion metálico e A^{n-} o ânion de carga n, denominado de contraíon, sendo os mais comuns os íons cloreto, nitrato, sulfato ou acetato (MARANGONI *et al.*, 2001; STÄHLIN; OSWALD, 1971).

HSLs podem se organizar para dispor os cátions, hidroxilas, contraíons e moléculas de água, em conformações distintas (LOUËR; LOUËR; GRANDJEAN, 1973). Uma dentre essas conformações é a do hidroxinitrato de zinco (HNZ), utilizado pioneiramente no presente trabalho para imobilização de lipases. As lamelas de HNZ são compostas por octaedros de zinco que possuem um quarto dos sítios vazios, sendo que ao lado superior e posterior dos octaedros vazios estão alocados átomos de zinco tetraedricamente coordenados. Nestes tetraedros, três dos vértices são ocupadas por íons hidroxila, pertencentes à folha de octaedros e o quarto por uma molécula de água. Em HNZ, os íons nitrato estão localizados entre as lamelas, e não coordenados ao metal, o que gera um sólido com distância basal de aproximadamente 9,9 Å (Figura 1.8 – A). (ARIZAGA, SATYANARAYANA e WYPYCH, 2007; MACHADO *et al.*, 2010)

Outro tipo de HSL utilizado neste trabalho como suporte para imobilização de lipases foi o hidroxicloreto de zinco (HCZ) cujas as lamelas são formadas por átomos de zinco que estão coordenados de forma octaédrica à grupamentos hidroxila (Figura 1.8 – B), sendo que um

quarto dos sítios octaédricos está vazio, assim como observado para a estrutura lamelar do HNZ, criando uma vacância de átomos na lamela. Nas partes superior e inferior destas vacâncias estão alocados átomos de zinco em sítios tetraédricos, e isto origina a carga positiva da lamela. No átomo de zinco tetraédrico, três vértices estão ocupados por grupos hidroxila e a quarta posição do metal está ligada diretamente com o átomo de cloro. Na estrutura do HCZ ainda estão presentes moléculas de água interlamelares, o que gera um sólido com distância basal de aproximadamente 7,9 Å (ARIZAGA, SATYANARAYANA e WYPYCH, 2007; MACHADO *et al.*, 2010; MACHADO, WYPYCH e NAKAGAKi, 2012).

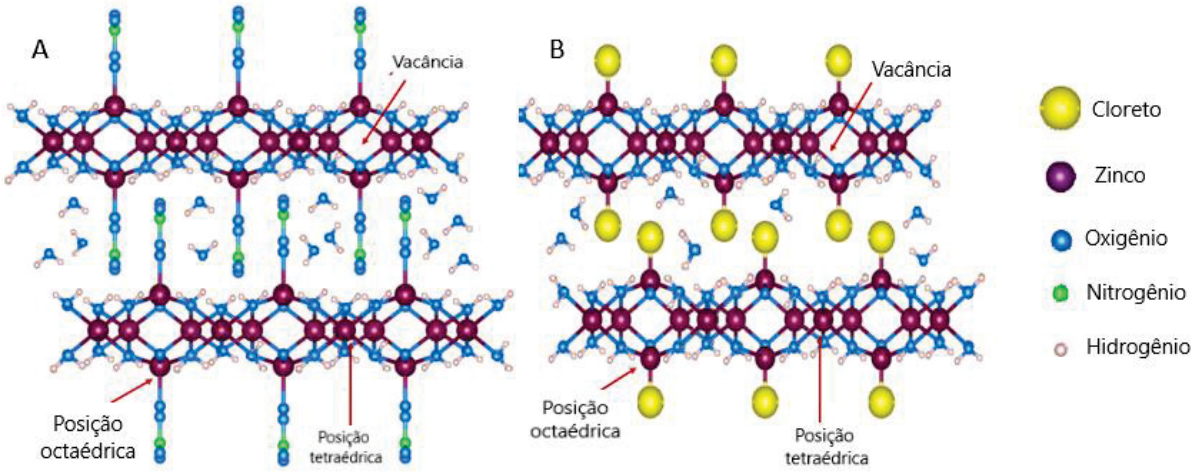

FIGURA 1.8 – REPRESENTAÇÃO ESQUEMÁTICA DA ESTRUTURA DO HIDROXINITRATO E HIDROXICLORETO DE ZINCO

FONTE: O Autor (2020)
LEGENDA: (A) Visão lateral das lamelas de hidroxinitrato de zinco; (B) Visão lateral das lamelas de hidroxicloreto de zinco.

Não foram encontrados estudos de imobilização de enzimas em HSLs. Portanto, utilizar pioneiramente HSLs para imobilização por adsorção iônica de lipases se apresenta como uma alternativa viável e interessante além de abrir novas perspectivas de uso para esses materiais.

1.4 CONSIDERAÇÕES FINAIS

A busca por novas metodologias e materiais para imobilização tem sido explorada para viabilizar ainda mais a aplicação de lipases e, nesse contexto, compostos lamelares podem ser explorados. Dentre os materiais lamelares, HDLs e HSLs merecem destaque, principalmente pelo baixo custo de produção destes materiais e pela possível capacidade de imobilizar lipases por adsorção física, via interações eletrostáticas. Portanto, imobilizar lipases em compostos

lamelares pode ser promissor, e servir de base para estudos adicionais não só de imobilização, mas para aplicação desses derivados imobilizados em reações de interesse industrial.

CAPÍTULO II – IMMOBILIZATION OF *Pseudomonas cepacia* LIPASE ON LAYERED DOUBLE HYDROXIDE OF Zn/Al-Cl FOR KINETIC RESOLUTION OF *rac*-1-PHENYLETHANOL

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ABSTRACT

Layered double hydroxides (LDHs) are cheap materials suitable for immobilization of enzymes. In this study, we prepared Zn/Al-Cl LDHs with different Zn:Al molar ratios for immobilization of the lipase from *Pseudomonas cepacia*. The best values for activity retention (188%), immobilization efficiency (96%) and hydrolytic activity in organic medium (279 U g^{-1}) were obtained with a molar ratio of Zn:Al of 4:1, a protein loading of 162 mg g^{-1} and Tris-HCl buffer (10 mmol L^{-1}, pH 7.5) as the solvent for preparing the lipase solution. The immobilized lipase keeps its activity when stored at 4 °C during 30 days. The immobilized lipase gave a conversion of 50% in 1 h for the kinetic resolution of the alcohol *rac*-1-phenylethanol, with both ee_s and ee_p higher than 99% and E higher than 200. In the reutilization study, 30 successive 1-h kinetic resolutions were done with the same batch of immobilized enzyme. For all 30 resolutions, 50% conversion was maintained, with ee_s and ee_p higher than 99% and E higher than 200. These are promising results that lay the basis for further studies of immobilization of lipases onto LDHs for applications in organic media.

Keywords: lipases, immobilization, layered double hydroxides, enzymatic kinetic resolution.

2.1 Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) are widely used in biocatalysis due to their ability to catalyze a wide range of reactions, including esterification, transesterification and interesterification [1,2]. Lipases also find application in the kinetic resolution of racemic compounds [2–4]. Kinetic resolution (also referred to as kinetically controlled resolution, not to be confused with dynamic kinetic resolution or thermodynamically controlled resolution) requires the specificity constant (i.e. k_{cat}/K_M) of the lipase for the reaction with one of the enantiomers to be much higher than it is for the same reaction with the other enantiomer. For example, if the ratio of the specificity constant for the *R*-enantiomer (i.e. k_{catR}/K_{MR}) to the specificity constant for the S-enantiomer (i.e. k_{catS}/K_{MS}) is very high, then, by the time that the *R*-enantiomer has been fully converted to the product, essentially none of the *S*-enantiomer has reacted. This ratio of specificity constants is referred to as the enantiomeric ratio and given the symbol *E*.

Industrial processes require the use of lipases immobilized on inert solid supports to allow their recovery from the reaction media and subsequent reuse, thereby reducing process costs. Different methods have been used to immobilize lipases, such as adsorption, covalent bonding, entrapment, cross-linked enzyme aggregates (CLEAs), and cross-linked enzyme crystals (CLECs) [5]. The most commonly used method is adsorption, given that it is simple and tends to have little deleterious effect on enzyme activity, since the lipase is bound to the support through relatively weak forces (in comparison to covalent bonding) such as hydrophobic and ionic interactions [6]. In fact, the immobilization of lipases on hydrophobic supports commonly leads to an increase of activity, which is attributed to the opening of the hydrophobic lid, in a process analogous to the interfacial activation that occurs with emulsions of hydrophobic substrates [7,8]. Immobilization may also increase the stability of

the lipase, reduce inhibition by substrates or products and render it more selective or change its enantiopreference, however, these effects vary, depending on the support, the immobilization technique and on the lipase itself [6,7,9].

Layered double hydroxides (LDHs) are cheap materials suitable for immobilization of enzymes. They have been studied for the immobilization of lipases, ureases, kinases, transketolases, penicillin G acylase, fructose-6-phosphate aldolase, lactate dehydrogenase, triosephosphate isomerase and acetylcholinesterase [10,11]. LDHs are crystalline structures that are produced from the stacking of positively charged two-dimensional layers, with these layers being separated by hydrated anions. Their chemical composition can be represented by the general formula $[M^{2+}_{1-x} M^{3+}_{x} (OH)_2]^{x+}(A^{m-})_{x/m}.yH_2O$, where M^{2+} and $M3^{+}$ are divalent and trivalent metal cations, respectively, and A^{m-} can be various interlayer anions, such as inorganic anions, organic anions, and small biological molecules [12,13]. LDHs act as anion exchangers due to the positive charges exposed on their surfaces and anion exchange is the main mechanism by which enzymes are immobilized. LDHs have the advantageous features of various supports that are widely used for immobilization, but at a lower cost. These features include: i) LDHs can be synthesized with a wide variation of chemical compositions, both inter- and intralayer; ii) The structural and textural properties of LDHs can be adjusted through the production of different particle sizes (from nanometers to micrometers); iii) LDHs have low toxicity; iv) The LDH particles grow with a platelet-like morphology, producing a high superficial area, which is adequate for immobilizing enzymes [10,11,14,15]; v) LDHs are highly hydrophilic materials, but the layers can be intercalated with organic compounds, such as the anionic detergents dodecyl sulfate or dioctyl sulfosuccinate, giving a slight amphipathic nature to the support [10,11]. This might affect the immobilization of lipases, which might be activated by the presence of the hydrophobic regions of these detergents [7,8].

One of the most used lipases for racemic resolution of organic compounds is the lipase from *Pseudomonas cepacia* (lipase PS or PCL, from Amano), which has good heat resistance and good stability in organic solvents, especially short chain alcohols [3,5]. With these properties, it has found applications in transesterification reactions of prochiral or racemic alcohols with acetates [3], in the synthesis of biodiesel and pharmaceutical drugs, in the synthesis and modification of structured lipids and in the synthesis and degradation of polymers [5].

Lipase PS has been immobilized through different methods, including adsorption and covalent bonding, onto various supports. These supports include Immobead 150 (a polypropylene containing an oxirane ring that links covalently to the enzyme), modified ceramic, diatomaceous earth, silica-PVA and epoxy-silica-PVA composites and also Accurel EP-100 (a macroporous polypropylene) [2,4,5,16–20]. However, some of these supports have high costs, are difficult to prepare, can be toxic or can pollute the environment after use.

There are only four studies of the immobilization of lipases on LDHs [15,21–23]. These studies focus on the synthesis and characterization of the LDHs and a subsequent comparison between immobilized and free lipases, with respect to parameters like thermal stability, stability in organic solvents, activity and stability as a function of pH and stability during storage. However, they do not address the application of these immobilized preparations in biocatalytic processes in organic media.

In the present work, we investigated the immobilization of lipase PS onto LDHs of Zn/Al synthesized using different molar ratios, with interlayer chloride ions. The immobilized

preparation with the best immobilization efficiency and retention of activity was used in the kinetically controlled resolution of racemic rac-1-phenylethanol, which is used as a chiral building block and synthetic intermediate in the fine chemical and pharmaceutical industries.

2.2 Material and methods

2.2.1 Material

Pseudomonas cepacia lipase (Lipase PS Amano) was acquired from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). The enzyme is provided in white lyophilized powder form, with hydrolytic activity in aqueous medium against olive oil higher than 23,000 U g^{-1}, according to the manufacturer (Sigma-Aldrich, St. Louis, Missouri, U.S.A.). The hydrolytic activities obtained experimentally for this powder were 24,000 U g^{-1} against olive oil in aqueous medium (Section 2.6) and 125 U g^{-1} against triolein in organic medium (Section 2.7). The lipase PS powder contained 13% (w/w) of protein, as determined by the bicinchoninic acid method (BCA) (Section 2.2.8). Novozym 435, which is lipase B from *Candida antarctica* (now *Pseudozyma antarctica*) immobilized in a microporous resin, was kindly donated by Novozymes Latin America (Araucária, Paraná, Brazil). $ZnCl_2$, $AlCl_3.6H_2O$ and NaOH, which were used in the synthesis of LDHs, were of analytical grade and supplied by Vetec (Duque de Caxias, Rio de Janeiro, Brazil).

The substrates used to determine the hydrolytic activities in aqueous and organic media were, respectively, olive oil with maximum acidity ≤ 0.50% (Cargill Agrícola S.A., São Paulo, Brazil) and triolein (65%) (Sigma-Aldrich, St. Louis, Missouri, U.S.A.).

The alcohol *rac*-1-phenylethanol, which was used in the enzymatic kinetic resolution studies, was prepared through the reduction of acetophenone with sodium borohydride in methanol

with 97% yield. Vinyl acetate (>99%) was obtained from Neon (São Paulo, Brazil) and n-hexane (99.5%) was obtained from Vetec (Duque de Caxias, Rio De Janeiro, Brazil). All other chemicals used were of analytical grade.

2.2.2 Synthesis of Zn/Al-Cl LDHs at different molar ratios

All LDHs used in this study were synthesized by the co-precipitation method at constant pH [24]. For this purpose, NaOH (1 mol L^{-1}), $AlCl_3.6H_2O$ and $ZnCl_2$ solutions were prepared in decarbonated water. Metal-ion-containing solutions were prepared in distinct concentrations, in order to achieve LDHs with the desired molar ratios. These solutions were dripped concomitantly and slowly into a reactor under N_2 flow (to minimize contamination by atmospheric CO_2), with constant stirring and at room temperature. The synthesis occurred at pH 8.0, for a period of 2 h. After this time, the pH was adjusted to 8.5 and the product remained under magnetic stirring for a further 24 h, after which it was centrifuged at 3622 × g. The recovered white precipitate was repeatedly washed with distilled water and dried in a vacuum oven at 60 °C for 48 h. After drying, the material was triturated and then sieved to obtain particles smaller than 0.85 mm. Three types of Zn and Al LDHs with interlayer Cl- ions were prepared: with molar ratios of Zn to Al of 2:1, 3:1 and 4:1. These are referred to as Zn_2Al-Cl, Zn_3Al-Cl and Zn_4Al-Cl, respectively.

2.2.3 Lipase immobilization

For the immobilization experiments, lipase PS powder (hereafter referred to as LipPS) was used as provided by the manufacturer, without purification. Immobilizations were made in 25 mL Erlenmeyer flasks, with the addition of 0.1 g of LDH to 5 mL of an enzyme solution that was prepared by dissolving the stated amount of enzyme in ultrapure water or a buffer. This mixture was incubated in an orbital shaker at 110 rpm and 25 °C for 24 h. The solid was then

recovered by filtration with qualitative filter paper (Whatman no. 1), placed in a desiccator under vacuum (produced using a primary vacuum pump) for 16 h to dry and then stored at 4 °C or room temperature.

In the initial studies aimed at selecting the best support, the masses used for immobilization were 0.1 g of LDH and 0.1 g of LipPS dispersed in 5 mL of ultra-pure water at pH 6.7. After choosing the best support, the effect of the solvent used for preparing the LipPS solution during immobilization was studied. The solvents tested were ultra-pure water pH 6.7, sodium phosphate buffer 10 mmol L^{-1} pH 7.5 and Tris-HCl buffer 10 mmol L^{-1} pH 7.5. To evaluate the best protein loading for immobilization, LDH (0.1 g) was added to a LipPS solution (5 mL), with loadings ranging from 32.5 to 195 mg g^{-1} (mass of protein per mass of LDH).

In all the assays of immobilization, the kinetics of immobilization were evaluated during 24 h. The hydrolytic activity of the aqueous lipase solution (hereafter referred to as the supernatant) was monitored using the titrimetric method (Section 2.2.6). The immobilization efficiency (IE, %) was calculated as:

$$\mathrm{IE}(\%) = \frac{(A_i - A_f)}{A_i} \times 100 \qquad (1)$$

where A_i is the hydrolytic activity (U) of the supernatant before immobilization and A_f is the hydrolytic activity (U) remaining in the supernatant at the end of the immobilization procedure, both measured in aqueous medium.

The retention of activity (R, %) was calculated as:

$$R(\%) = \frac{A_o}{A_t} \times 100 \quad (2)$$

where A_o is the hydrolytic activity measured in organic medium (Section 2.7) of the immobilized preparation and A_t is the theoretical activity of the immobilized enzyme (both in U g^{-1} of LDH). The theoretical activity was calculated as:

$$A_t = (M_{ip} - M_{fp}) \times SA_o \quad (3)$$

where M_{ip} is the mass of protein per mass of LDH (mg g^{-1}) originally in the supernatant before immobilization (also referred to as the "protein loading"), M_{fp} is the mass of protein per mass of LDH (mg g^{-1}) remaining in the supernatant at the end of the immobilization procedure and SA_o is the specific hydrolytic activity of the free enzyme (U mg^{-1} of protein), measured in organic medium. It should be noted that values of R above 100% are possible if the lipase is activated upon immobilization.

Controls were performed for all experiments, in which the enzyme solution was incubated for 24 h under the immobilization conditions, but in the absence of LDH. There was no loss of enzymatic activity from the supernatant in these controls.

For evaluation of storage stability, immobilized LipPS, hereafter referred to as "ImLipPS", was kept for 30 days at 4 °C or room temperature. The residual hydrolytic activity in organic medium (Section 2.2.7) was determined as a percentage of residual activity compared to the initial activity at day one.

2.2.4 Enzymatic Kinetic Resolution

The performances of ImLipPS, LipPS and Novozym 435 were compared in transesterification of *rac*-1-phenylethanol with vinyl acetate as the acyl moiety donor. The (*R*)-enantiopreference of LipPS and Novozym 435 for secondary alcohols is already described in the literature [25,26].

The reactions were carried out in 4-mL screw-capped bottles. Sufficient enzyme preparation to give 12 U of hydrolytic activity in organic medium (43 mg of ImLipPS, 96 mg of LipPS or 24 mg of Novozym 435) was added to a solution of *rac*-1-phenylethanol (0.1 mmol) and vinyl acetate (0.4 mmol) in n-hexane (2 mL). The reaction was carried out at 35 °C and 180 rpm in an orbital shaker. At fixed intervals, 0.01-mL samples of the mixture were removed and analyzed by chiral GC analysis (Section 2.9). Experiments with pure LDH samples were done and produced no reaction. The enantiomeric excesses of substrate (ees, %) and product (eep, %) were determined as:

$$ee(\%) = \left[\frac{R - S}{R + S}\right] \times 100 \qquad (4)$$

where R is the concentration of the (*R*)-enantiomer (substrate or product) and S is the concentration of the (*S*)-enantiomer (substrate or product).

The conversion (c, %) and enantiomeric ratio (E) were calculated from the values of ee_s and ee_p according to Chen et al. (Chen *et al.*, 1982):

$$c(\%) = \frac{ee_s}{ee_s + ee_p} \times 100 \qquad (5)$$

$$E = \frac{ln\,[(1\text{-}c)(1\text{-}ee_s)]}{ln\,[(1\text{-}c)(1\text{+}ee_s)]} \quad (6)$$

For the study of the reutilization of ImLipPS, the reaction was repeated for thirty cycles, each of 1 h. The experimental procedure, the amount of ImLipPS and the reaction mixtures used were as described above. After each cycle, the reaction mixture was removed and then replaced with a new mixture.

2.2.5 Characterization of the LDHs

LDH particles were characterized by X-ray diffractometry (XRD) and Fourier-transform infrared spectroscopy (FTIR). The analysis of XRD and FTIR confirmed that the synthesized materials are LDHs with different Zn:Al molar ratios (Supplementary material).

The measurements of surface area and porosity were analyzed by nitrogen adsorption/desorption isotherms that were obtained using a Quantachrome gas sorption analyzer, model NOVA 2000e. The samples were degassed at 110 °C under vacuum for 2 h and the analyses were carried out in liquid nitrogen (−196 °C). The adsorption/desorption isotherms for N2 were obtained over the relative pressure range of 0.05–0.99. The specific surface areas of the samples were calculated using the multi-point Brunauer–Emmet–Teller (BET) method for relative pressures ranging from 0.05 to 0.30. The specific pore volume was calculated from the desorption isotherm using the Barrett–Joyner–Halenda method (BJH).

2.2.6 Determination of the hydrolytic activity in aqueous medium

In the immobilization studies, the olive-oil hydrolyzing activity of the supernatant was determined by titration using an automatic titrator pHStat (Metrohm 718 Stat Titrino) in aqueous medium, from which the immobilization efficiencies for each protein loading were calculated. In these assays, the substrate emulsions consisted of 67 mmol L^{-1} olive oil, 3% (w/v) gum arabic, 2 mmol L^{-1} $CaCl_2$, 2.5 mmol L^{-1} Tris–HCl pH 8.0 and 150 mmol L^{-1} NaCl, dispersed in distilled water [28]. The free enzyme was added to 20 mL of the emulsion under magnetic stirring (300 rpm) at 30 °C and the reaction was followed for 5 min. One unit of hydrolytic activity in aqueous medium (U) corresponds to the release of 1 μmol of fatty acid per minute, under the assay conditions. There was no reaction in controls performed with pure LDH samples.

2.2.7 Determination of the hydrolysis activity in organic medium

This methodology was used to measure the activities of ImLipPS, LipPS and Novozym 435 and to calculate the activity retention of the preparations of LipPS immobilized on Zn_4Al-Cl LDH (i.e. ImLipPS). The hydrolytic activities were measured in organic medium to prevent enzyme leaching. The activity of LipPS was determined by adding the powder to the reaction medium, using the same conditions as for ImLipPS.

The hydrolytic activity in organic medium was determined by adding 5 mL of a reaction medium containing 4.9 mL of n-hexane, 70 mmol L^{-1} triolein and 2% (v/v) distilled water to a 25-mL Erlenmeyer flask. The mixture was incubated in an orbital shaker at 200 rpm and 40 °C, with the reaction being started by the addition of the enzyme preparation [29]. Every 5 min until 30 min, 100-μL samples of the mixture were collected and analyzed for free fatty acids by the Lowry and Tinsley method [30]. Initial rates were calculated. One unit of

hydrolytic activity in organic medium (U) corresponds to the release of 1 μmol of fatty acid per minute, under the assay conditions. The hydrolytic activity was expressed in units of activity per gram of enzymatic preparation (U g^{-1}). There was no reaction in controls performed with pure LDH samples.

2.2.8 Protein determination

Protein content was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich, St. Louis, EUA) with bovine serum albumin as the standard [31].

2.2.9 GC analysis

The samples from the enzymatic kinetic resolution studies were analyzed by gas chromatography in a GC-17A chromatographer (Shimadzu Co., Kyoto, Japan), equipped with a hydrogen flame ionization detector, and a chiral column CP Chirasil-DEX CB (25m × 0.25mm diameter, 0.25-μm film thickness). A sample of 0.5 μL was injected using N_2 as the carrier gas (1 mL min^{-1}). The injector and detector were set at 220 °C. The oven program was as follows: initial value of 110 °C, with heating at 1 °C min^{-1} to 120 °C.

2.3 Results

2.3.1 Effects of LDHs charge density on LipPS immobilization

The initial step was to select the best LDH for LipPS immobilization. In these assays, the LipPS to LDH ratio was 1:1 (w/w) with ultra-pure water at pH 6.7 being used to prepare the lipase solution. The kinetics of immobilization were followed by determining the residual hydrolytic activity in the supernatant (Fig. 2.1).

The immobilization efficiency after 24 h increases as the molar ratio between Zn and Al atoms in the LDH increases (Fig. 2.1): 0% for Zn_2Al-Cl, 88% for Zn_3Al-Cl, and 97% for Zn_4Al-Cl. This increase in the immobilization efficiency can be explained by ionic interactions between the lipases and the LDHs: The pH of the ultra-pure water (6.7) is above the isoelectric point (pI) of LipPS, which is 5.1 [32], and therefore LipPS will assume a negative overall charge. For Zn_4Al-Cl LDH, which has the lowest positive charge density, the lipase molecules are probably immobilized in a well-spaced manner [33]. On the other hand, the Zn_2Al-Cl LDH, which has the highest positive charge density, attracts more lipase molecules to its surface; the immobilization does not occur probably due to the formation of strong lipase-lipase repulsive interactions [33]. The phenomenon of repulsion between proteins immobilized on high charge density surfaces has been observed previously. For example, Rabe et al. [34], who studied the immobilization of β-lactoglobulin on a hydrophobic glass surface, and Tie et al. [35], who studied the immobilization of fibronectin on $Si(Ti)O_2$, showed that the interaction between proteins leads to a loss of binding energy and, subsequently, a decrease in the immobilization rate.

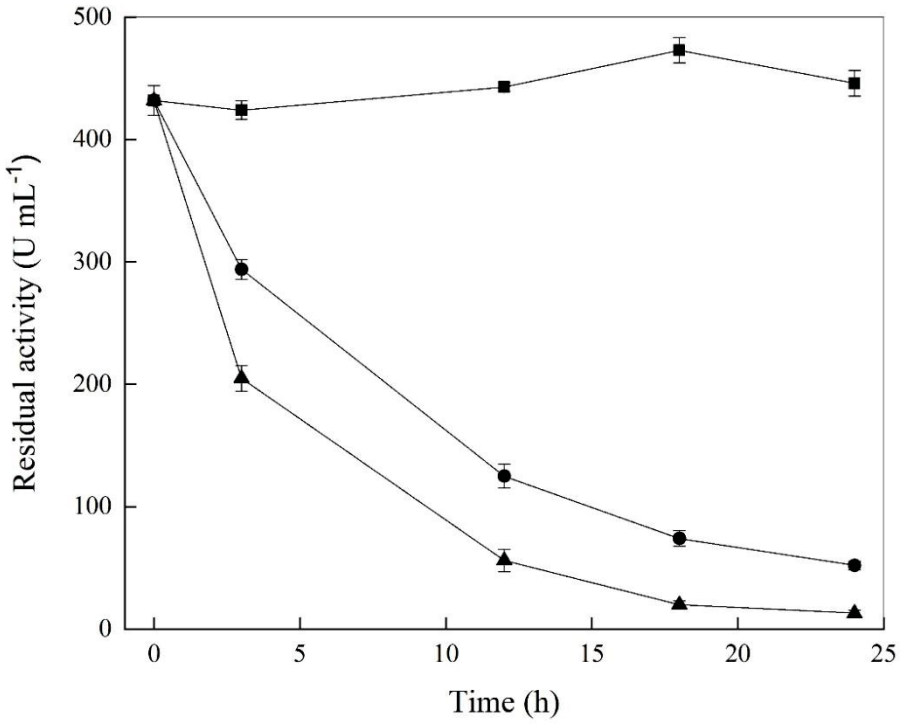

Fig. 2.1 – Kinetics of immobilization of Pseudomonas cepacia lipase (LipPS) on ZnAl-Cl LDHs with different Zn:Al molar ratios. Key: (■) Zn2Al-Cl; (●) Zn3Al-Cl and (▲) Zn4Al-Cl. The residual olive-oil hydrolyzing activity in the supernatant was determined by the titrimetric method using a pHStat, at 40 °C, pH 7.0 for 5 min. The values are plotted as the means of triplicate analyses±the standard errors of the means. When the error bar is not visible, it is smaller than the symbol. There was no loss of activity from the supernatant in a control experiment in which the enzyme solution was incubated in the absence of LDH for 24 h under the immobilization conditions.

There is another factor that might explain the increase of lipase adsorption with increase in the Zn:Al molar ratio of the LDHs. Both the specific surface area and the specific pore volume of the LDHs increase as the Zn:Al molar ratio increases (Table 2.1). The specific surface area of Zn4Al-Cl LDH (33.7m^2 g^{-1}) is similar to that of Accurel EP100, a commercial hydrophobic support that is successfully used for immobilization of lipases by adsorption and which has a

specific surface area varying between 20 and 43m^2 g^{-1} [36,37]. The LDH denominated Zn_4Al-Cl was used in subsequent experiments.

Table 2.1 – BET specific surface area and specific pore volume of Zn/Al-Cl LDHs with different Zn:Al molar ratios

HDL	BET specific surface area (m^2 g^{-1})	BET correlation coefficient	Specific pore volume (cm^3 g^{-1})
Zn_2Al-Cl	8.6	0.9992	2.7
Zn_3Al-Cl	9.9	0.9963	3.4
Zn_4Al-Cl	33.7	0.9996	10.6

2.3.2 Effect of the solvent used for preparing the LipPS solution during immobilization

Most of the studies in which LDHs are used for enzyme immobilization use ultra-pure or distilled water to prepare enzyme solutions for immobilization, in order to avoid interference by the anions contained in the buffers [21,22,38,39]. However, one needs to be careful about using purified or distilled water since, if it is not used immediately, its pH will change due to the dissolution of CO_2 from the atmosphere. We therefore used the best LDH identified in the previous section to determine whether immobilization would be successful in buffers. We compared ultra-pure water pH 6.7, sodium phosphate buffer (10 mmol L^{-1} pH 7.5) and Tris HCl buffer (10 mmol L^{-1} pH 7.5). All pH values are above the pI of LipPS and therefore ensure that the lipase has a net negative charge, which should favor immobilization by anion exchange with the positively charged surface of the LDH.

For both ultra-pure water and Tris-HCl buffer, the residual hydrolytic activities in the supernatants decreased from the initial value of 446 U mL^{-1} to about 10 U mL^{-1} at 24 h (Fig. 2.2), which corresponds to an immobilization efficiency of 97%. In contrast, for

immobilization in sodium phosphate buffer, the immobilization efficiency was only 20% at 24 h. This relativity low immobilization efficiency can be explained by the preference of the positively charged groups of the LDH for the negative ions of the phosphate buffer ($H_2PO_4^-$ and HPO_4^{2-}, pKa 7.2 at 25 °C) compared to the negatively charged lipase. This is consistent with the results of Badreddine et al. [40], where phosphate ions completely replaced chloride ions in Zn/Al LDH, molar ratio 2:1, suggesting a strong selectivity of Zn/Al LDH for phosphate ions with different sizes and charges (PO_3^-, $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}, $P_2O_7^{4-}$ and $P_3O_{10}^{5-}$). In the case of Tris-HCl, although Cl− is present as the counter-ion of the LDH preparations used in our work, the Cl^- anion of the buffer does not hinder the immobilization of LipPS. This is consistent with the relatively low preference of LDHs for Cl^- [41].

In the current work, as there was no difference between the immobilization results obtained with ultra pure water and those obtained with Tris-HCl buffer, the remaining studies were done with Tris-HCl buffer, in order to assure the reproducibility of the experiments, given that it is possible to keep the pH at 7.5.

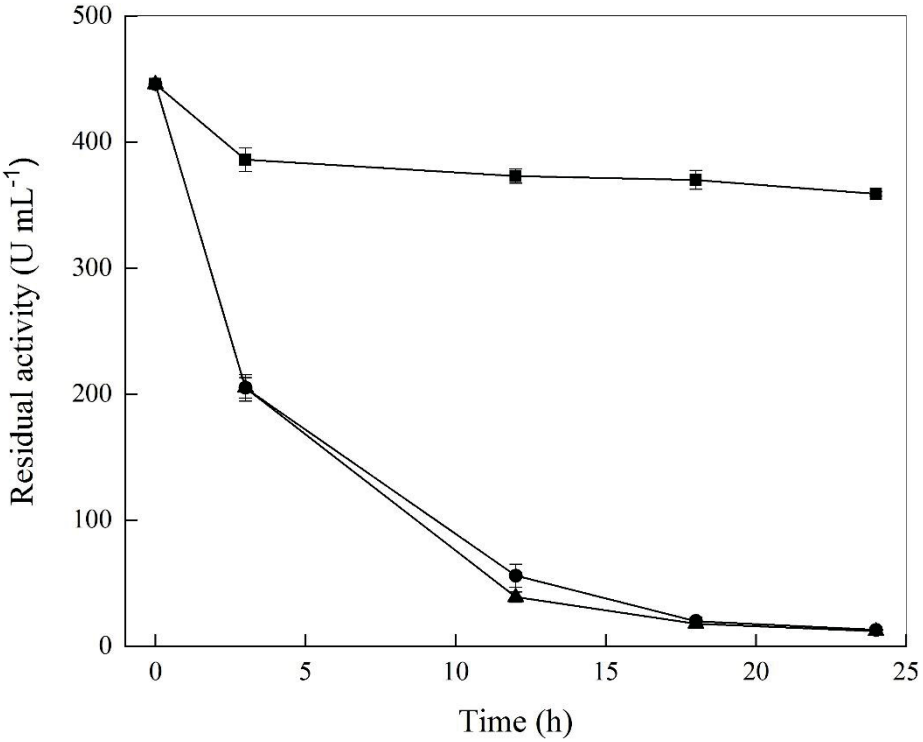

Fig. 2.2 – Kinetics of immobilization of *Pseudomonas cepacia* lipase (LipPS) on Zn_4Al-Cl LDH in ultra-pure water and buffers. Key: (■) Sodium phosphate buffer (10 mmol L-1 pH 7.5); (●) Tris-HCl buffer (10 mmol L-1 pH 7.5) and (▲) Ultra-pure water pH 6.7. The residual olive-oil-hydrolyzing activity in the supernatant was determined by the titrimetric method using a pHStat, at 40 °C, pH 7.0 for 5 min. The values are plotted as the means of triplicate analyses± the standard errors of the means. When the error bar is not visible, it is smaller than the symbol. There was no loss of activity from the supernatant in a control experiment in which the enzyme solution was incubated in the absence of LDH for 24 h under the immobilization conditions.

2.3.3 Effect of protein loading on LipPS immobilization

As described above, Zn_4Al-Cl LDH was chosen as the support and Tris-HCl (10 mmol L^{-1} pH 7.5) as the buffer for preparing the lipase solution. In order to evaluate the effect of the protein loading, five different ratios of LipPS to LDH were tested: 250, 500, 1000, 1250 and 1500 mg of the LipPS powder per g of LDH (mg g^{-1}), which correspond, respectively, to protein

loadings of 32.5, 65, 130, 162.5 and 195 mg g^{-1}. Activities were measured for the hydrolysis of triolein, with the assays being done in organic medium in order to prevent the possibility of enzyme leaching from the support (See Section 2.2.7).

For the protein loadings of 32.5 and 65 mg g^{-1}, the triolein-hydrolyzing activities in the supernatant at 12 h were very low, giving immobilization efficiencies (IE, Table 2) of 96% and 99%, respectively (Fig. 2.3). For the protein loadings of 130 and 162.5 mg g^{-1}, the IE values were similar, reaching 97% and 96%, respectively, but after a longer time (24 h). For the highest protein loading (195 mg g^{-1}), the IE value at 24 h was 93%.

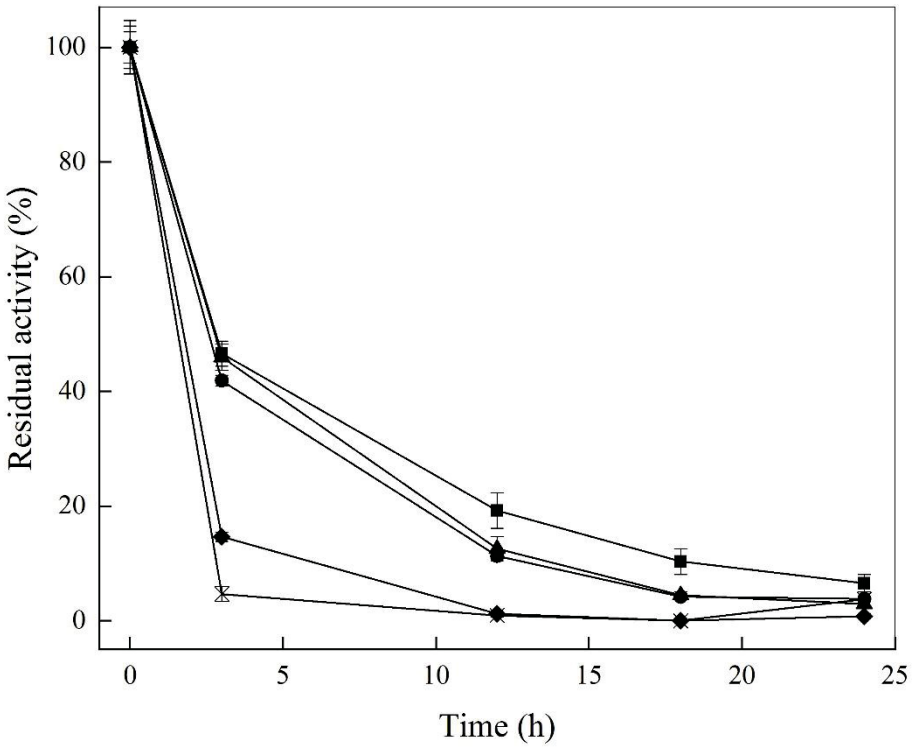

Fig. 2.3 –Effect of protein loading on the kinetics of immobilization of *Pseudomonas cepacia* lipase (LipPS). Key: (×) 32.5 mg g^{-1}, (♦) 65 mg g^{-1}, (▲) 130 mg g^{-1}, (●) 162.5 mg g^{-1} and (■) 195 mg g^{-1}. The residual olive-oil-hydrolyzing activity in the supernatant was determined by the titrimetric method using a pHStat, at 40 °C, pH 7.0 for 5 min. The values are plotted as the means of triplicate analyses±the standard errors of the means. When the error bar is not

visible, it is smaller than the symbol. There was no loss of activity from the supernatant in a control experiment in which the enzyme solution was incubated in the absence of LDH for 24 h under the immobilization conditions.

The activity retention values (Table 2.2) suggest that multilayer formation occurred at the highest protein loading: The activity retention was stable at around 180% for the protein loadings of 32.5–162.5 mg g^{-1}, but then fell sharply to only 123% at the protein loading of 195 mg g^{-1}. Immobilization isotherm studies show that LipPS forms multiple layers at high protein loadings during immobilization on the hydrophobic support Accurel [42]. Lipases can also form multiple layers on charged surfaces: lipase D of *Rhizopus oryzae* formed multiple layers when immobilized on a porous anion-exchange hollow-fiber membrane [43].

Values higher than 100% of activity retention (R) were found for all protein loadings, indicating enzyme activation upon immobilization (Table 2.2). The R value was obtained by comparing the hydrolytic activity in organic medium of immobilized LipPS with that of free LipPS (i.e. in lyophilized powder form, as supplied by Sigma) (see Section 2.2.3). The activation can be attributed to the fact that free lipases tend to aggregate in organic medium, leading to mass transfer limitations [44,45]. This phenomenon does not occur when lipases are dispersed across the surface of the immobilization support [44,46].

Desorption assays were performed to give an insight into the nature of the interaction between LipPS and the surface of the LDH (Supplementary material, section S3). In these assays, ImLipPS was incubated for 3 h in different aqueous media: ultra-pure water, 3% (v/v) Triton X-100, 1 mol L^{-1} NaCl and 2 mol L^{-1} NaCl. Activities were compared to that of ImLipPS without incubation. ImLipPS did not lose activity after incubation in ultra-pure water (Fig.

S4). The activity of ImLipPS decreased only 9% after incubation in Triton X-100, suggesting that a small portion of the lipases may be immobilized in a multilayer fashion. On the other hand, the activity of ImLipPS decreased 56% after incubation in 1 mol L^{-1} NaCl, suggesting that most of the LipPS immobilizes onto LDH by ion exchange. Surprisingly, there was no further leaching of LipPS from the LDH even when incubated in 2 mol L^{-1} NaCl, showing that the interaction between LipPS and the support is very strong.

Table 2.2 – Effect of the protein to support ratio (protein loading) on the immobilization of *Pseudomonas cepacia* lipase on Zn_4Al-Cl LDH

Protein loading (mg g^{-1})	Lipase powder (mg g^{-1})	IE (%)[a]	ImLipPS hydrolytic activity in organic media (U g^{-1})[b]	R (%)[c]
32.5	250	96	54 ± 10	180
65	500	99	112 ± 9	181
130	1000	97	216 ± 18	178
162.5	1250	96	279 ± 20	186
195	1500	93	215 ± 2	123

a) Calculated using Eq. (1); b) The values are given as the means of triplicate analyses ± the standard errors of the means; c) Calculated using Eq. (2).

The protein loading of 162.5 mg g^{-1}, which gave high values for activity retention and immobilization efficiency and the highest hydrolytic activity in organic medium (279 U g^{-1}), was chosen to produce the batch of ImLipPS that was used in the remaining assays. Samples of this batch were evaluated by techniques of XRD and FTIR in comparison with pure LipPS and pure LDH (Supplementary material). The XRD pattern of ImLipPS shows a decrease of crystallinity compared to the XRD pattern of LDH, suggesting that LipPS is adsorbed on the LDH surface (Supplementary material, Fig. S1). In the FTIR analyses, the vibration bands in the spectrum of ImLipPS were at the same range of wavelengths as in the spectra of LipPS

and Zn_4Al-Cl LDH, confirming the presence of LipPS in the immobilized preparation (Supplementary material, Fig. S2).

2.3.4 Stability of ImLipPS during storage

The stability of LipPS immobilized on Zn4Al-Cl LDH was evaluated during thirty days at two temperatures (Fig. 2.4). ImLipPS kept its activity when stored at 4 °C ($p > 0.05$), but at room temperature (average of 22 °C) the activity decreased by 20%.

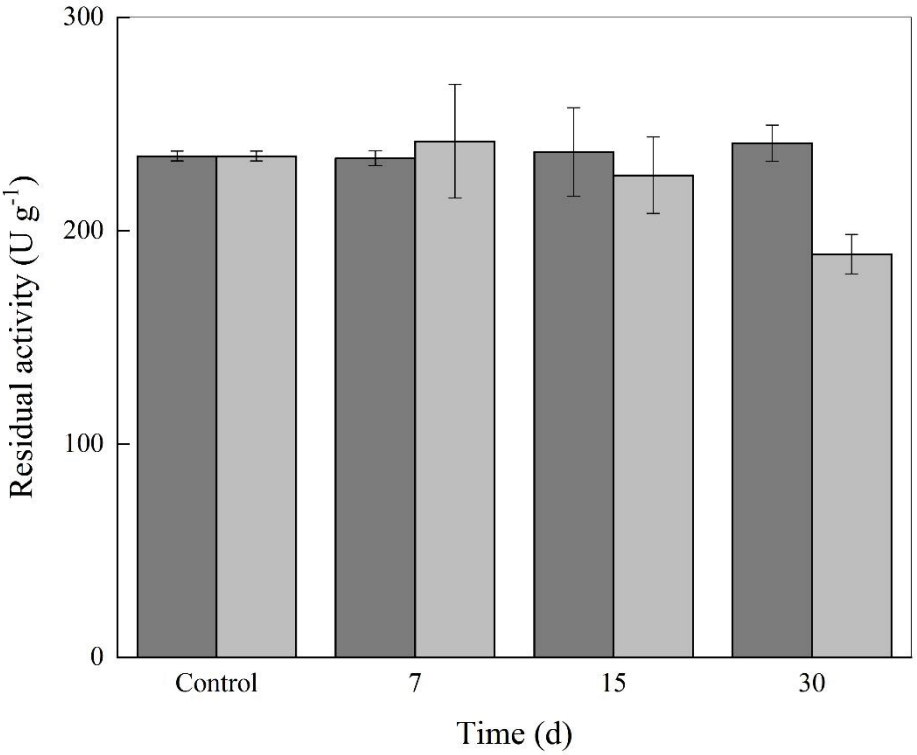

Fig. 2.4 – Stability during storage of *Pseudomonas cepacia* lipase (LipPS) immobilized on Zn_4Al-Cl LDH. Key: (black bars) Storage at 4 °C and (~ 22 ºC, grey bars) Room temperature. Residual activity was measured by the hydrolysis of triolein in organic media. The values are plotted as the means of triplicate analyses ± the standard errors of the means.

Although ImLipPS can be stored at 4 °C for at least 30 days with no loss of activity, it was less stable during storage at room temperature than the free enzyme powder. This powder (i.e.

the lyophilized commercial preparation sold by Sigma, which most probably contains stabilizers, although this is not specified on the product specification sheet) maintained its activity after one year in our laboratory (data not shown). The stability during storage of ImLipPS at 4 °C is better than that of Candida rugosa lipase immobilized on two Zn/Al-LDHs prepared at a Zn to Al ratio of 4:1 (w/w), one with nitrate and the other with dioctyl sulfosuccinate as the intercalated anion. The residual activities of these immobilized preparations fell to around 75% when they were stored at 4 °C during thirty days [22].

2.3.5 Enzymatic kinetic resolution

To confirm the stability of Zn_4Al-Cl LDH against the solvents used in the kinetic resolution reactions, we incubated it for 30 h at 35 °C in a solution of *rac*-1-phenylethanol (0.1 mmol) and vinyl acetate (0.4 mmol) in n-hexane. We also incubated it in methanol (99.8% purity) and ethanol (99.5% purity). X-ray analysis confirmed that these treatments did not affect the LDH structure (Supplementary material, section S4).

ImLipPS and LipPS were then used in the resolution of the racemic alcohol *rac*-1-phenylethanol, which is a substrate commonly used to determine the enantioselectivity of lipases [47]. The reaction was followed during 60 min and samples were analyzed by chiral GC analysis (Supplementary material, Fig. S3).

Table 2.3 presents the values obtained in the reactions for the conversion (c), the enantiomeric excesses of the substrate (ee_s) and of the product (ee_p) and the enantiomeric ratio (E). LipPS has enantiopreference for the (*R*)-isomer for the resolution of *rac*-1-phenylethanol [26] and this did not change upon immobilization on LDH. Novozym 435 gave a conversion of 50% in 15 min, with both ees and eep higher than 99% and E higher than 200. At the same time,

ImLipPS had converted 33% of the substrate, with an ee_s of 51, an ee_p higher than 99% and a value of E higher than 200 (Table 2.3). There was no conversion at 15 min in the reaction with free LipPS. No conversion was detected when the support alone (Zn_4Al-Cl LDH) was added to the reaction medium.

Table 2.3 – Resolution of *rac*-1-phenylethanol using the lipase from *Pseudomonas cepacia* in free and immobilized form and *Candida antarctica* lipase B (Novozym 435).

Lipase/Support	Time (min)	*c* (%)	ee_s (%)	ee_p (%)	*E*
Novozym 435	12	44	78	> 99	>200
	15	50	> 99	> 99	>200
	30	50	> 99	> 99	>200
	60	50	> 99	> 99	>200
Pseudomonas cepacia (Zn_4Al-Cl LDH)	15	33	51	> 99	>200
	30	44	77	> 99	>200
	60	50	> 99	> 99	>200
Pseudomonas cepacia (free)	15	0	-	-	-
	30	7	7	> 99	>200
	60	15	17	> 99	>200
Zn_4Al-Cl LDH (control)	15	0	-	-	-
	30	0	-	-	-
	60	0	-	-	-

(*c*) Ester conversion calculated using Eq. (5); (ee_s) excess enantiomeric of substrate and (ee_p) excess enantiomeric of product calculated using Eq. (4); (*E*) enantiomeric ratio calculated using Eq. (6). Reaction conditions: 0.1 mmol of *rac*-1-phenylethanol and 0.4 mmol of vinyl acetate in 2 mL of *n*-hexane. The enzymatic resolution was carried out at 35 °C.

After 60 min, the conversion with ImLipPS reached 50%, with both ees and eep higher than 99% and E higher than 200, whereas for LipPS the conversion was 15%, with an ee_s of 17% (Table 2.3). The results for ImLipPS are comparable to those obtained for Novozym 435, demonstrating the viability of using ImLipPS for the kinetic resolution of racemic

mixtures in organic media.

In the reutilization study, 30 successive 1-h kinetic resolutions were done with the same batch of ImLipPS. The conversion of 50% and the ees higher than 99% that were obtained in the first cycle remained the same over the 30 cycles (Fig. 2.5).

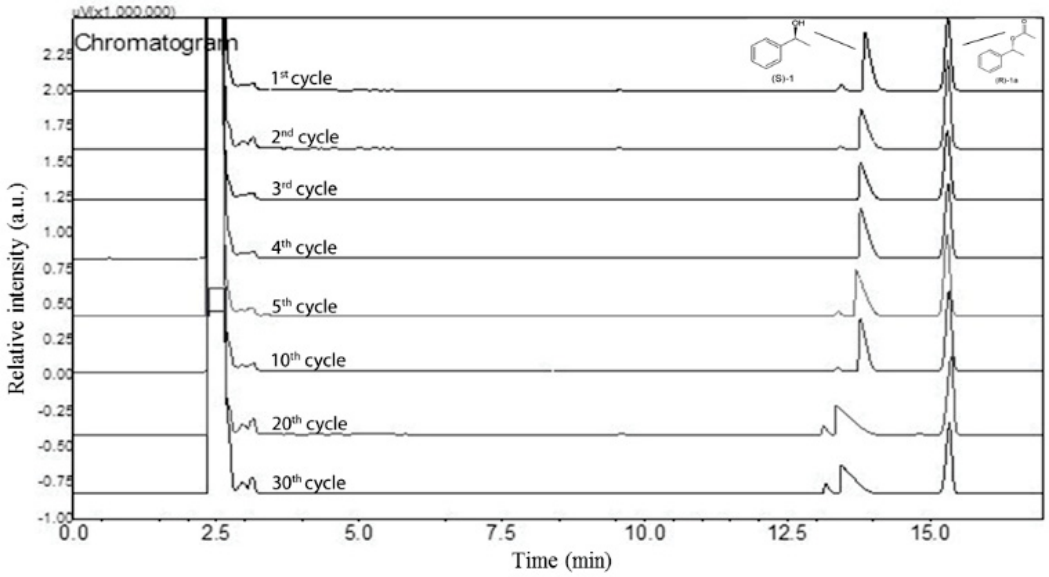

Fig. 2.5 – Chromatograms of different reaction cycles in the enzymatic kinetic resolution of *rac*-1-phenylethanol by *Pseudomonas cepacia* lipase immobilized on Zn_4Al-Cl LDH. Analyzes were performed on a gas chromatograph equipped with chiral capillary column Chirasil-Dex CB. The reaction was repeated for thirty cycles, each of 1 h. After each cycle, the reaction mixture was collected and then replaced with a new mixture. Reaction conditions: 0.1 mmol of rac-1-phenylethanol and 0.4 mmol of vinyl acetate in 2 mL of n-hexane. The enzymatic resolution was carried out at 35 °C.

2.4 Discussion

This study makes two contributions in the area of immobilization of lipases. First, it not only represents the highest conversion obtained for *rac*-1-phenylethanol using *Pseudomonas cepacia* lipase, but it also demonstrates that the immobilized preparation can maintain its performance for 30 cycles of kinetic resolution. Second, it demonstrates the possibility of using LDHs for immobilization of lipases for application in organic media. These points are discussed separately below.

2.4.1 Enzymatic kinetic resolution of rac-1-phenylethanol by LipPS

The optically pure isomers of 1-phenylethanol are used as chiral building blocks and synthetic intermediates in the fine chemical and pharmaceutical industries. Specifically, the isomer (*R*)-1-phenylethanol is widely used as a fragrance in cosmetics due to its soft floral scent. (*R*)- 1-phenylethanol is also used as a solvatochromic dye, ophthalmic preservative and inhibitor of intestinal absorption of cholesterol [16].

LipPS has been used, both as free enzyme and immobilized on a support, for the kinetic resolution of *rac*-1-phenylethanol. In these assays, it has shown enantiopreference for the (*R*)-isomer, with values of E higher than 200 and high ee_s and ee_p values, in reaction times as long as 24 h (Table 2.4). The best result for kinetic resolution of *rac*-1-phenylethanol catalyzed by commercial LipPS (Amano PS-C, immobilized on ceramic) is a conversion of 50% in 2 h, with cyclohexane as solvent and vinyl acetate as acyl donor, at 60 °C [48]. In the present work, the same conversion was obtained in half the time and at a lower temperature (35 °C).

The reuse of immobilized lipases in repeated reaction cycles is indispensable for industrial processes, since it significantly reduces the cost of the process. The reutilization assays with ImLipPS gave results that were amongst the best reported to date (Table 2.4), since it was reused for 30 reaction cycles of 1 h each, with no losses in the conversion, nor in the purity of the product. The reusability of ImLipPS makes LDHs promising for lipase immobilization.

Table 2.4 – Recent studies on kinetic resolution of *rac*-1-phenylethanol with *Pseudomonas cepacia* lipase (LipPS)

Support	Acyl donor / Solvent	Temp.	Conversion / Time	ee_s	ee_p	E	Reutilization / number of cycles	Reference
Zn_4Al-Cl LDH	Vinyl acetate / *n*-hexane	35 °C	50 % / 1 h	>99%	>99%	>200	Yes / 30	This work
Free	Vinyl acetate / *n*-hexane	35 °C	15 % / 1 h	17%	>99%	>200	No	This work
Mesoporous Silica	Vinyl acetate / Hexane	30 °C	50 % / 6 h	[1]ni	99%	[1]ni	Yes / 6	(Liu *et al.*, 2012)
Sol-gel	Vinyl acetate / Toluene	[2]Room	50 % / 24 h	>99%	>99%	>200	Yes / 8	(Hara, Hanefeld e Kanerva, 2008)
Ceramic	Vinyl acetate / Cyclohexane	60 °C	50 % / 2 h	97%	99%	>200	Yes / 10	(Souza, De *et al.*, 2009)

Support	Acyl donor / Solvent	Temp.	Conversion / Time	ee_s	ee_p	E	Reutilization / number of cycles	Reference
[3]DPP	Vinyl acetate / Ionic liquid	35 °C	47 % / 24 h	[1]ni	99%	[1]ni	Yes / 10	(Li *et al.*, 2013)
Free	Decanoate / Diethyl ether	40 °C	50 % / 24 h	>99%	>99%	>200	No	(Melais, Aribi-Zouioueche Riant, 2016)

[1]Not informed by the authors; [2]The authors did not mention the mean room temperature; [3]DPP: 2,5-di(2-thienyl)-1H-pyrrole-1-(*p*-phenylacetic acid)

2.4.2 LDHs as supports for lipase immobilization

In the present work, values of immobilization efficiencies (IE) above 90% were obtained for all protein loadings studied (Table 2.2). These values are higher than the results reported in the literature for lipases immobilized on LDHs (Table 2.5). However, this parameter by itself is not sufficient to determine the success of the enzymatic immobilization, since the removal of active enzyme from the solution does not ensure that the enzyme is immobilized in its active form [49]. The retention of activity parameter (*R*) is also important. In our case, *R* was more than 100% (Table 2). Dong et al. [15] used Mg/Al LDH, at a molar ratio of Mg to Al of 3:1, to immobilize Candida lipolytic lipase (Table 2.5) and they obtained an R of only 69%. However, they measured the activity of the immobilized preparation by the titrimetric method, using an emulsion of olive oil in water. This methodology for evaluating lipase activity may allow desorption of the lipases from the LDH, leading to errors, since the desorbed lipases might have an activity that is different from that of the immobilized lipase.

Besides, there might be mass transfer problems during the hydrolysis of olive oil when the lipases are immobilized in a porous support, since olive oil can form small droplets that are inaccessible to the immobilized lipases [50]. In our work, the retention of activity was evaluated in organic medium, which, in addition to avoiding desorption, mimics the conditions of the final application in which the immobilized enzyme will be used.

Our work shows, for the first time, the resolution of racemic compounds using LDH as a support for lipases. However, some other applications of lipases immobilized on LDHs have been studied previously (Table 2.5). Rahman et al. [21,22] used the esterification of oleic acid with 1-butanol to evaluate parameters such as thermal stability, leaching, stability in organic solvent and stability during storage of the lipase from Candida rugosa immobilized on LDHs. In a subsequent work, Rahman et al. [23] optimized the synthesis of methyl adipate from adipic acid and methanol in *n*-hexane using lipases from *Candida rugosa* immobilized on LDHs, with conversions above 80% in 2.5 h of reaction.

LDHs have the potential to be used as low-cost environmentally friendly supports for the immobilization of lipases; they have good chemical and thermal stability, they do not swell in water and their chemical and structural properties can be easily manipulated [11,51]. Stability of the support is a key issue if immobilized preparations are to be reused over many cycles. Our LDH was stable not only in the reaction medium used for the racemic resolution of rac-1-phenylethanol, but also in ethanol and methanol, alcohols that are commonly present in lipase-catalyzed reactions in organic media. This gives our immobilized preparation a potential advantage over Novozym 435, in which lipase B from *Candida antarctica* is immobilized on a microporous acrylic polymer resin (Lewatit VP OC 1600). Components of this acrylic polymer can solubilize in reaction media containing alcohols, especially methanol

and ethanol, leading to the desorption of the lipase and to the contamination of the reaction products with components extracted from the support [52–54]. Thus, although Novozym 435 gave a faster reaction time than ImLipPS over one kinetic resolution cycle (Table 2.3), the stability of the LDH means that ImLipPS is better suited to processes involving many cycles in organic reaction media.

Table 2.5 – Studies about the use of layered double hydroxides (LDHs) for the immobilization of lipases

Source of lipases	LDHs (molar ratio)	Anion interlayer	Greatest immobilization efficiency (IE) reported (%)	Application	Reference
Candida lipolytica (Shanghai Fu Xiang Biotechnology Co. Ltd)	Mg/Al (2, 2.5, 3, 3.5 and 4:1)	Dodecyl sulfate	56.4	Hydrolysis of olive oil	(Dong *et al.*, 2014)
Candida rugosa (Sigma Chemical)	Mg/Al (4:1)	Dodecyl sulfate and nitrate	70.8	Esterification (butyl oleate synthesis)	(Rahman, Mohd Basyaruddin Abdul *et al.*, 2004)
Candida rugosa (Sigma Chemical)	Zn/Al (4:1)	Diocytyl sulfosuccinate and nitrate	78.5	Esterification (butyl oleate synthesis)	(Rahman, M.B.A. *et al.*, 2004)
Candida rugosa (Sigma Chemical)	Zn/Al Mg/Al Ni/Al (4:1)	Nitrate	71	Esterification (methyl adipate synthesis)	(Rahman *et al.*, 2008)

2.5 Conclusions

In the present work, we studied the immobilization of *Pseudomonas cepacia* lipase onto Zn/Al LDHs with interlayer Cl^- ions. The LDH synthesized at the highest molar ratio studied (4:1, Zn:Al) was the most effective for lipase immobilization. After optimization, the immobilization efficiency was 96% and the retention of activity was 186% for a protein loading of 162.5 mg g^{-1}. In the transesterification of *rac*-1- phenylethanol with vinyl acetate in *n*-hexane, the immobilized preparation showed high conversion (50%) in 1 h of reaction, with enantiomeric excesses of substrates and products higher than 99% and an enantioselectivity coefficient higher than 200. This performance was maintained over 30 kinetic resolution cycles.

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References

[1] B. Joseph, P.W. Ramteke, G. Thomas, Cold active microbial lipases: Some hot issues and recent developments, Biotechnol. Adv. 26 (2008) 457–470. doi:10.1016/j.biotechadv.2008.05.003.

[2] F. Hasan, A.A. Shah, A. Hameed, Industrial applications of microbial lipases, Enzyme Microb. Technol. 39 (2006) 235–251. doi:10.1016/j.enzmictec.2005.10.016.

[3] G.M. Ziarani, P. Gholamzadeha, P. Asadiatoueia, N. Lashgari, The role of *Pseudomonas cepacia* lipase in the asymmetric synthesis of heterocyclic based compounds, J. Mol. Catal. B Enzym. 122 (2015) 93–116. doi:10.1016/j.molcatb.2015.08.022.

[4] J. Liu, S. Bai, Q. Jin, H. Zhong, C. Li, Q. Yang, Improved catalytic performance of lipase accommodated in the mesoporous silicas with polymer-modified microenvironment, Langmuir. 28 (2012) 9788–9796. doi:10.1021/la301330s.

[5] D.A. Sánchez, G.M. Tonetto, M.L. Ferreira, *Burkholderia cepacia* lipase: A versatile catalyst in synthesis reactions, Biotechnol. Bioeng. 115 (2018) 6–24. doi:10.1002/bit.26458.

[6] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. 40 (2007) 1451–1463. doi:10.1016/j.enzmictec.2007.01.018.

[7] R. Fernandez-Lafuente, P. Armisén, P. Sabuquillo, G. Fernández-Lorente, J.M. Guisán, Immobilization of lipases by selective adsorption on hydrophobic supports, Chem. Phys. Lipids. 93 (1998) 185–197. doi:10.1016/S0009-3084(98)00042-5.

[8] R.C. Rodrigues, J.J. Virgen-ortíz, C.S. José, Á. Berenguer-murcia, A.R. Alcantara, O. Barbosa, C. Ortiz, R. Fernandez-lafuente, Immobilization of lipases on hydrophobic supports : immobilization mechanism , advantages , problems , and solutions, Biotechnol. Adv. (2019) 0–1. doi:10.1016/j.biotechadv.2019.04.003.

[9] E. Pereira Cipolatti, E. Andrade Manoel, R. Fernandez-lafuente, D. Maria Guimarães Freire, Support engineering : relation between development of new supports for

immobilization of lipases and their applications, Biotechnol. Res. Innov. 1 (2017) 26–34. doi:10.1016/j.biori.2017.01.004.

[10] C. Taviot-Guého, V. Prévot, C. Forano, G. Renaudin, C. Mousty, F. Leroux, Tailoring hybrid layered double hydroxides for the development of innovative applications, Adv. Funct. Mater. 28 (2018) 1–33. doi:10.1002/adfm.201703868.

[11] C. Forano, S. Vial, C. Mousty, Nanohybrid enzymes - layered double hydroxides: potential applications, Curr. Nanosci. 2 (2006) 283–294. doi:10.2174/1573413710602030283.

[12] V. Rives, M.A. Ulibarri, Layered double hydroxides (LDH) intercalated with metal coordination compounds and oxometalates, Coord. Chem. Rev. 181 (1999) 61–120. doi:10.1016/S0010-8545(98)00216-1.

[13] V.R.L. Constantino, T.J. Pinnavaia, Basic Properties of Mg2+1−xAl3+x Layered Double Hydroxides Intercalated by Carbonate, Hydroxide, Chloride, and Sulfate Anions, Inorg. Chem. 34 (1995) 883–892. doi:10.1021/ic00108a020.

[14] N.B. Allou, P. Saikia, A. Borah, R.L. Goswamee, Hybrid nanocomposites of layered double hydroxides: an update of their biological applications and future prospects, Colloid Polym. Sci. 295 (2017) 725–747. doi:10.1007/s00396-017-4047-3.

[15] L. Dong, C. Ge, P. Qin, Y. Chen, Q. Xu, Immobilization and catalytic properties of *Candida lipolytic* lipase on surface of organic intercalated and modified MgAl-LDHs, Solid State Sci. 31 (2014) 8–15. doi:10.1016/j.solidstatesciences.2014.02.006.

[16] C. Suan, M.R. Sarmidi, Immobilised lipase-catalysed resolution of (*R,S*)-1-phenylethanol in recirculated packed bed reactor, J. Mol. Catal. B Enzym. 28 (2004) 111–119. doi:10.1016/j.molcatb.2004.02.004.

[17] K. Tongboriboon, B. Cheirsilp, A. H-Kittikun, Mixed lipases for efficient enzymatic synthesis of biodiesel from used palm oil and ethanol in a solvent-free system, J. Mol.

Catal. B Enzym. 67 (2010) 52–59. doi:10.1016/j.molcatb.2010.07.005.

[18] P. Hara, U. Hanefeld, L.T. Kanerva, Sol-gels and cross-linked aggregates of lipase PS from *Burkholderia cepacia* and their application in dry organic solvents, J. Mol. Catal. B Enzym. 50 (2008) 80–86. doi:10.1016/j.molcatb.2007.09.004.

[19] A.B.R. Moreira, V.H. Perez, G.M. Zanin, H.F. de Castro, Biodiesel synthesis by enzymatic transesterification of palm oil with ethanol using lipases from several sources immobilized on silica-PVA composite, Energy and Fuels. 21 (2007) 3689–3694. doi:10.1021/ef700399b.

[20] A. Salis, M. Pinna, M. Monduzzi, V. Solinas, Comparison among immobilised lipases on macroporous polypropylene toward biodiesel synthesis, J. Mol. Catal. B Enzym. 54 (2008) 19–26. doi:10.1016/j.molcatb.2007.12.006.

[21] M.B.A. Rahman, M. Basri, M.Z. Hussein, M.N.H. Idris, R.N.Z.R.A. Rahman, A.B. Salleh, Immobilisation of lipase from *Candida rugosa* on layered double hydroxides of Mg/Al and its nanocomposite as biocatalyst for the synthesis of ester, Catal. Today. 93–95 (2004) 405–410. doi:10.1016/j.cattod.2004.06.048.

[22] M.B.A. Rahman, M. Basri, M.Z. Hussein, R.N.Z.A. Rahman, D.H. Zainol, A.B. Salleh, Immobilization of lipase from Candida rugosa on layered double hydroxides for esterification reaction, Appl. Biochem. Biotechnol. 118 (2004) 313–320. doi:10.1385/ABAB:118:1-3:313.

[23] M.B.A. Rahman, U.H. Zaidan, M. Basri, M.Z. Hussein, R.N.Z.R.A. Rahman, A.B. Salleh, Enzymatic synthesis of methyl adipate ester using lipase from *Candida rugosa* immobilised on Mg, Zn and Ni of layered double hydroxides (LDHs), J. Mol. Catal. B Enzym. 50 (2008) 33–39. doi:10.1016/j.molcatb.2007.09.020.

[24] F.L. Theiss, G.A. Ayoko, R.L. Frost, Synthesis of layered double hydroxides containing Mg2+, Zn2+, Ca2+and Al3+layer cations by co-precipitation methods - A

review, Appl. Surf. Sci. 383 (2016) 200–213. doi:10.1016/j.apsusc.2016.04.150.

[25] H. Frykman, N. Öhrner, T. Norin, K. Hult, S-ethyl thiooctanoate as acyl donor in lipase catalysed resolution of secondary alcohols, Tetrahedron Lett. 34 (1993) 1367–1370. doi:10.1016/S0040-4039(00)91797-0.

[26] R.J. Kazlauskas, A.N.E. Weissfloch, A.T. Rappaport, L.A. Cuccia, A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*, J. Org. Chem. 56 (1991) 2656–2665. doi:10.1021/jo00008a016.

[27] C.S. Chen, Y. Fujimoto, G. Girdaukas, C.J. Sih, Quantitative analysis biochemical kinetic resolution of enantiomer, J. Am. Chem. Soc. 104 (1982) 7294–7299. doi:10.1021/ja00389a064.

[28] A. Tiss, F. Carrière, R. Verger, Effects of gum arabic on lipase interfacial binding and activity, Anal Biochem. 294 (2001) 36–43. doi:10.1006/abio.2001.5095.

[29] R.C. Alnoch, V.P. Martini, A. Glogauer, A.C.D.S. Costa, L. Piovan, M. Muller-Santos, E.M. De Souza, F.D.O. Pedrosa, D.A. Mitchell, N. Krieger, Immobilization and characterization of a new regioselective and enantioselective lipase obtained from a metagenomic library, PLoS One. 10 (2015) 1–18. doi:10.1371/journal.pone.0114945.

[30] R.R. Lowry, I.J. Tinsley, Rapid colorimetric determination of free fatty acids., J. Am. Oil Chem. Soc. 53 (1976) 470–472. doi:10.1007/BF02636814.

[31] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Frovenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85. doi:10.1016/0003-2697(85)90442-7.

[32] A. Sugihara, M. Ueshima, Y. Shimada, S. Tsunasawa, Y. Tominaga, Purification and characterization of a novel thermostable Lipase from *Pseudomonas cepacia*, J.

Biochem. 603 (1992) 598–603. doi:10.1093/oxfordjournals.jbchem.a123946.

[33] M. Rabe, D. Verdes, S. Seeger, Understanding protein adsorption phenomena at solid surfaces, Adv. Colloid Interface Sci. 162 (2011) 87–106. doi:10.1016/j.cis.2010.12.007.

[34] M. Rabe, D. Verdes, M. Rankl, G.R.J. Artus, S. Seeger, A comprehensive study of concepts and phenomena of the nonspecific adsorption of β-lactoglobulin, ChemPhysChem. 8 (2007) 862–872. doi:10.1002/cphc.200600710.

[35] Y. Tie, C. Calonder, P.R. Van Tassel, Protein adsorption: Kinetics and history dependence, J. Colloid Interface Sci. 268 (2003) 1–11. doi:10.1016/S0021-9797(03)00516-2.

[36] K. Singh, R. Bharose, V.K. Singh, S.K. Verma, Sugar decolorization through selective adsorption onto functionalized accurel hydrophobic polymeric support, Ind. Eng. Chem. Res. 50 (2011) 10074–10082. doi:10.1021/ie200501p.

[37] B. Al-Duri, R. Goddard, J. Bosley, Characterisation of a novel support for biocatalysis in supercritical carbon dioxide, J. Mol. Catal. B Enzym. 11 (2001) 825–834. doi:10.1016/S1381-1177(00)00195-8.

[38] Y. Tokudome, M. Fukui, N. Tarutani, S. Nishimura, V. Prevot, C. Forano, G. Poologasundarampillai, P.D. Lee, M. Takahashi, High-Density protein loading on hierarchically porous layered double hydroxide composites with a rational mesostructure, Langmuir. 32 (2016) 8826–8833. doi:10.1021/acs.langmuir.6b01925.

[39] C. Mousty, O. Kaftan, V. Prevot, C. Forano, Alkaline phosphatase biosensors based on layered double hydroxides matrices: Role of LDH composition, Sensors Actuators, B Chem. 133 (2008) 442–448. doi:10.1016/j.snb.2008.03.001.

[40] M. Badreddine, A. Legrouri, A. Barroug, A. De Roy, J.P. Besse, Ion exchange of different phosphate ions into the zinc-aluminum-chloride layered double hydroxide,

Mater. Lett. 38 (1999) 391–395. doi:10.1016/S0167-577X(98)00195-5.

[41] Z. Liu, R. Ma, M. Osada, N. Iyi, Y. Ebina, K. Takada, T. Sasaki, Synthesis , Anion Exchange , and Delamination of Co - Al Layered Double Hydroxide : Assembly of the Exfoliated Nanosheet / Polyanion Composite Films and Magneto-Optical Studies, (2006) 4872–4880. doi:10.1021/ja0584471.

[42] B. Al-Duri, Y.P. Yong, Characterisation of the equilibrium behaviour of lipase PS (from *Pseudomonas*) and lipolase 100L (from *Humicola*) onto Accurel EP100, J. Mol. Catal. - B Enzym. 3 (1997) 177–188. doi:10.1016/S1381-1177(96)00052-5.

[43] M. Goto, H. Kawakita, K. Uezu, S. Tsuneda, K. Saito, M. Goto, M. Tamada, T. Sugo, Esterification of Lauric Acid Using Lipase Immobilized in the Micropores of a Hollow-Fiber Membrane, J. Am. Oil Chem. Soc. 83 (2006) 209–213. doi:10.1007/s11746-006-1195-x.

[44] M. Persson, I. Mladenoska, E. Wehtje, P. Adlercreutz, Preparation of lipases for use in organic solvents, Enzyme Microb. Technol. 31 (2002) 833–841. doi:10.1016/S0141-0229(02)00184-9.

[45] A.M. Klibanov, Why are enzymes less active in organic solvents than in water ?, Trends Biotechnol. 15 (1997) 97–101. doi:10.1016/S0167-7799(97)01013-5.

[46] R.C. Rodrigues, C. Ortiz, R. Ferna, C. Ortiz, Modifying enzyme activity and selectivity by immobilization, Chem. Soc. Rev. 42 (2013) 6290–6307. doi:10.1039/c2cs35231a.

[47] K.P. Dhake, K.M. Deshmukh, Y.S. Wagh, R.S. Singhal, B.M. Bhanage, Investigation of steapsin lipase for kinetic resolution of secondary alcohols and synthesis of valuable acetates in non-aqueous reaction medium, J. Mol. Catal. B Enzym. 77 (2012) 15–23. doi:10.1016/j.molcatb.2012.01.009.

[48] R.O.M.A. De Souza, O.A.C. Antunes, W. Kroutil, C.O. Kappe, Kinetic resolution of *rac*-1-phenylethanol with immobilized lipases: A critical comparison of microwave and

conventional heating protocols, J. Org. Chem. 74 (2009) 6157–6162. doi:10.1021/jo9010443.

[49] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: Why, what and how, Chem. Soc. Rev. 42 (2013) 6223–6235. doi:10.1039/c3cs60075k.

[50] J.A. Bosley, A.D. Peilow, Immobilization of lipases on porous polypropylene: Reduction in esterification efficiency at low loading, J. Am. Oil Chem. Soc. 74 (1997) 107–111. doi:10.1007/s11746-997-0153-6.

[51] N. Chubar, R. Gilmour, V. Gerda, M. Mičušík, M. Omastova, K. Heister, P. Man, J. Fraissard, V. Zaitsev, Layered double hydroxides as the next generation inorganic anion exchangers: Synthetic methods versus applicability, Adv. Colloid Interface Sci. 245 (2017) 62–80. doi:10.1016/j.cis.2017.04.013.

[52] C. Ortiz, M.L. Ferreira, O. Barbosa, J.C.S. dos Santos, R.C. Rodrigues, Á. Berenguer-Murcia, L.E. Briand, R. Fernandez-Lafuente, Novozym 435: the "perfect" lipase immobilized biocatalyst?, Catal. Sci. Technol. 9 (2019) 2380–2420. doi:10.1039/c9cy00415g.

[53] C. José, R.D. Bonetto, L.A. Gambaro, M.D.P. Guauque Torres, M.L. Foresti, M.L. Ferreira, L.E. Briand, Investigation of the causes of deactivation-degradation of the commercial biocatalyst Novozym® 435 in ethanol and ethanol-aqueous media, J. Mol. Catal. B Enzym. 71 (2011) 95–107. doi:10.1016/j.molcatb.2011.04.004.

[54] C. José, L.E. Briand, Deactivation of Novozym® 435 during the esterification of ibuprofen with ethanol: Evidences of the detrimental effect of the alcohol, React. Kinet. Mech. Catal. 99 (2010) 17–22. doi:10.1007/s11144-009-0103-4.

[55] M. Li, H. Shan, L. Zhou, Y. Yin, Z. Li, Novel bioreactor for resolution of (*R,S*)-1-phenylethanol using the functional conducting polymer and ionic liquid with excellent catalytic activity and stability, J. Chem. Technol. Biotechnol. 88 (2013) 2091–2097.

doi:10.1002/jctb.4077.

[56] N. Melais, L. Aribi-Zouioueche, O. Riant, The effect of the migrating group structure on enantioselectivity in lipase-catalyzed kinetic resolution of 1-phenylethanol, Comptes Rendus Chim. 19 (2016) 971–977. doi:10.1016/j.crci.2016.05.002.

Supplementary material

"Immobilization of *Pseudomonas cepacia* lipase on layered double hydroxide of Zn/Al-Cl for kinetic resolution of *rac*-1-phenylethanol"

by

Glauco Silva Dias, Pamela Taisline Bandeira, Silvia Jaerger, Leandro Piovan, David Alexander Mitchell, Fernando Wypych, Nadia Krieger

S1. Characterization of layered double hydroxides before and after immobilization

The layered structures of the synthesized materials were confirmed by X-ray diffraction (XRD) measurements. For this, the powdered samples were deposited on glass sample holders. The measurements were obtained in the reflection mode using a Shimadzu XRD-6000 diffractometer operating at 40 kV and 20 mA, using CuKα radiation (λ = 1.5418 Å) and a dwell time of 2° min^{-1}; the data were collected between 3 and 70° (in 2θ).

The XRD measurements (Fig. S1) display the characteristic diffraction patterns of the layered double hydroxides (LDHs), exhibiting, in addition to the 00*l* series of diffraction peaks, the *hk*0 reflections, which characterize the ordering inside the layer [1]. The XRD peaks were indexed assuming a hexagonal lattice with a *R*-3*m* symmetry, which is the most common polytype for synthetic LDHs [1,2]. The cell parameters *d,* obtained from the higher order possible 00*l* reflections (Fig. S1, parts a, b and c), were determined according to Bragg as 7.76, 7.86 and 7.93 Å for the LDHs Zn2Al-Cl, Zn3Al-Cl and Zn4Al-Cl, respectively. These basal distances are typical for the intercalation of hydrated chloride ions [3,4]. The value of *d* increases slightly with the increase of zinc content in the layered structure due to a higher

presence of water molecules between the layers [5]. In addition, there is a displacement of the diffraction line (110) in the XRD patterns of the three LDHs (Fig. S1, parts a, b and c). This diffraction line is related to the intralayer structure organization, for which the cell parameter a gives the distances between the metal ions inside the layers, these distances being 3.07, 3.08 and 3.10 Å for LDHs Zn_2Al-Cl, Zn_3Al-Cl and Zn_4Al-Cl, respectively. The increase of the distance between the metals is due to a higher molar ratio Zn^{2+} to Al^{3+}, as the ionic radius of Zn^{2+} is 0.74 Å while that of Al^{3+} is 0.535 Å [6].

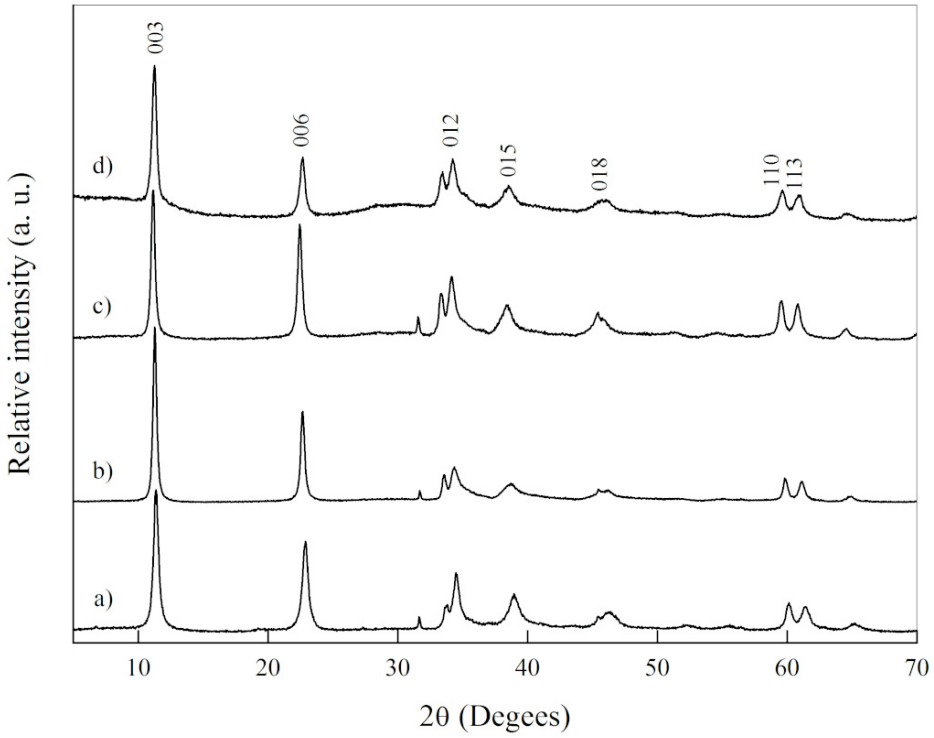

Fig. S2.1. X-ray diffraction (XRD) patterns of ZnAl-Cl LDHs with different molar ratios of Zn to Al and of Zn_4Al-Cl LDH on which *Pseudomonas cepacia* lipase (LipPS) has been immobilized (i.e. ImLipPS). Key: (a) Zn_2Al-Cl LDH, (b) Zn_3Al-Cl LDH, (c) Zn_4Al-Cl LDH and (d) ImLipPS.

When compared to the XRD patterns of pure Zn4Al-Cl LDH, XRD patterns of ImLipPS (Fig. S1, part d) display identical characteristic diffraction reflections, except for the reduction of

the intensity of diffraction peaks due to the decrease of crystallinity, suggesting that LipPS is adsorbed onto the surface of LDH. The preservation of the (012) and (110) diffraction lines for ImLipPS proves the maintenance of the layer structure and indicates that there was no delamination/intercalation process during the immobilization.

The layered materials also were analyzed by Fourier-transform infrared spectroscopy (FTIR). For this, FTIR spectra were recorded with a Bio-Rad FTS 3500GX spectrophotometer in the 400-4000 cm^{-1} range, using KBr pellets. KBr was crushed with a small amount of the appropriate solid sample. Spectra were recorded with a resolution of 4 cm^{-1} and an accumulation of 32 scans.

The FTIR spectra of the LDH preparations (Fig. S2) also presented typical characteristics of LDHs with interlayer Cl^- ions [7]. For all the molar ratios studied (Fig. S2, parts a, b and c), the broad band near 3500 cm^{-1} is due to vibrations of hydroxyl groups of LDHs and physisorbed/intercalated water molecules. Absorption bands that are due to the angular deformation of water molecules are present around 1620 cm^{-1} and the adsorption bands near 600 cm^{-1} and 430 cm^{-1} are related to M-O, M-OH bending and chain connection vibrations of O-M-O. In all the spectra, there is a band at 1365 cm^{-1}, which is due to a small contamination of the materials with carbonate ions. Carbonate is probably physisorbed between the LDH particles as well co-intercalated with the chloride ions (Fig. S2 a, b and c).

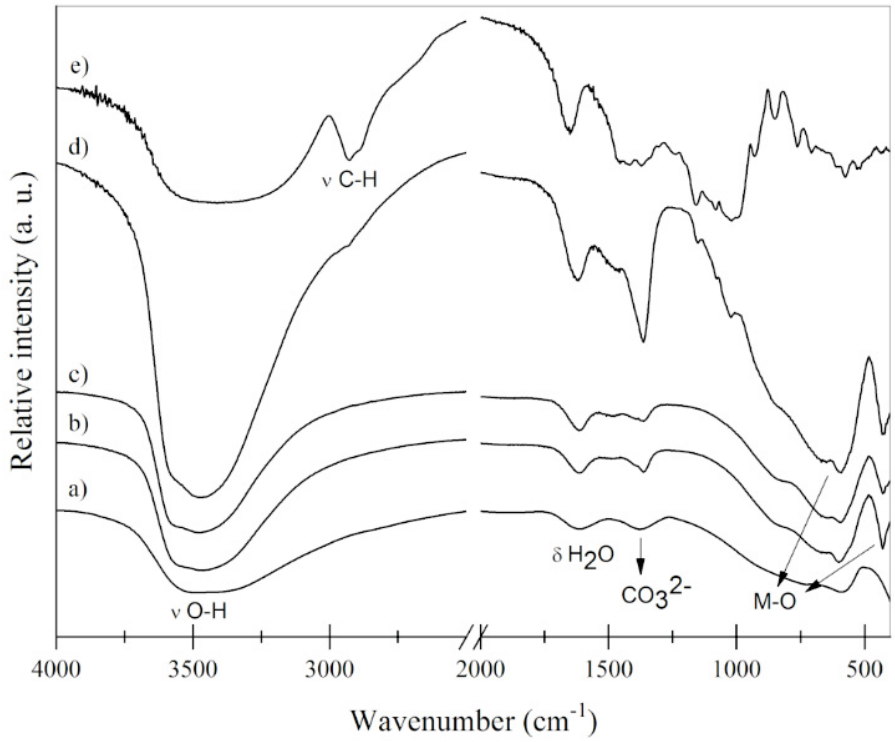

Fig. S2.2. Fourier-transform infrared spectroscopy (FTIR) spectra of ZnAl-Cl LDHs with different molar ratios and *Pseudomonas cepacia* lipase (LipPS) in free form and immobilized on Zn_4Al-Cl LDH. Key: (a) Zn_2Al-Cl LDH, (b) Zn_3Al-Cl LDH, (c) Zn_4Al-Cl LDH, (d) ImLipPS and (e) LipPS.

ImLipPS and LipPS were also analyzed by FTIR (Fig. S2, parts d and e). Similar transmittance profiles were observed for Zn_4Al-Cl LDH and ImLipPS, however, for ImLipPS, the bands associated with LipPS overlap the characteristic bands of pure Zn_4Al-Cl LDH, with the exception of the band at 2920 cm^{-1} (Fig. S2, part e) which corresponds to the vibrations of C-H of LipPS [8]. In the spectrum of ImLipPS, this band appears weakly, probably due to low protein concentration on the support. In the LipPS spectrum (Fig. S2, part e), the bands at 1656 and 1451 cm^{-1} correspond to the stretching and deformation of C-O (amide I) and N-H (amide II), respectively. The bands between 1175 and 990 cm^{-1} are due to C-N and N-H vibrations (amide III) [8]. The fact that the vibration bands found for ImLipPS are at the same

wavelengths as those obtained in the spectra for LipPS and Zn_4Al-Cl LDH confirms the presence of LipPS in the immobilized preparation.

S2. Enzymatic kinetic resolution

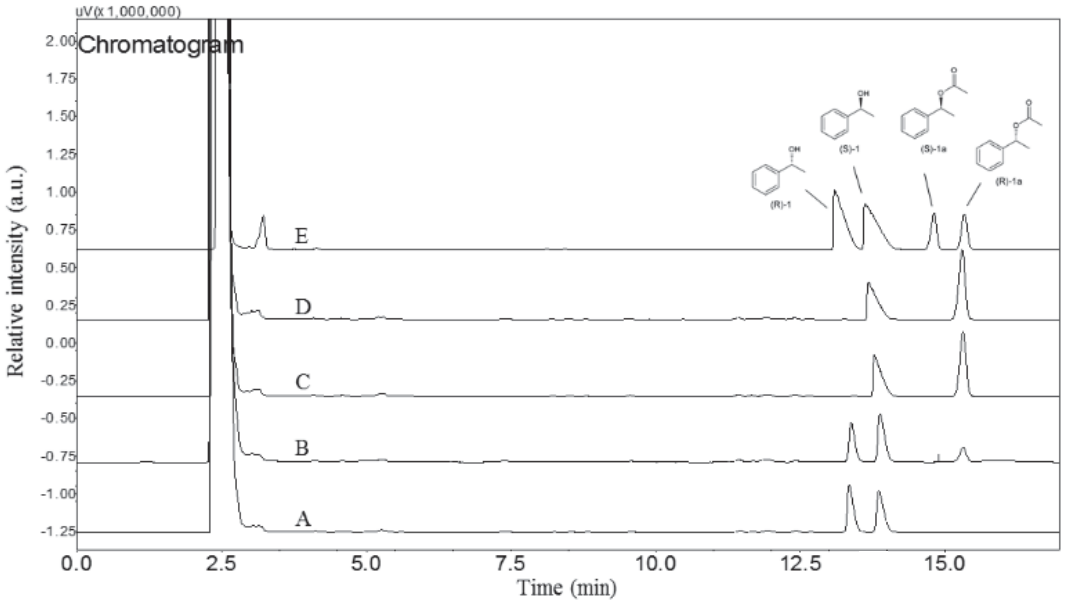

Fig. S2.3. Chromatograms of separation, confirmation of standards and enzymatic kinetic resolution of *rac*-1-phenylethanol by *Pseudomonas cepacia* lipase PS in free and immobilized form and *Candida antarctica* lipase B (Novozym 435). Key to chromatograms: (A) reaction catalyzed by Zn_4Al-Cl LDH (control); (B) reaction catalyzed by LipPS; (C) reaction catalyzed by ImLipPS; (D) reaction catalyzed by Novozym 435; (E) separation and confirmation of standards *rac*-1-phenylethanol. The peaks correspond, respectively, to (*R*)-1-phenylethanol, (*S*)-1-phenylethanol, (*S*)-1-phenylethyl acetate and (*R*)-1-phenylethyl acetate, according to the molecular structures presented. Analyses were performed on a gas chromatograph equipped with chiral capillary column (Chirasil-Dex CB). Reaction conditions: 0.1 mmol of *rac*-1-phenylethanol and 0.4 mmol of vinyl acetate in 2 mL of n-hexane. The enzymatic

resolution was carried out at 35 °C.

S3. Desorption assays

Desorption assays were done in 25-mL Erlenmeyer flasks, with the addition of 0.1 g of ImLipPS to 5 mL of solutions containing (i) NaCl (1 or 2 mol L^{-1}), (ii) 3% (v/v) Triton X-100 and (iii) ultra-pure water. The mixtures were incubated in an orbital shaker at 110 rpm and 25 °C for 3 h. The solid was then recovered by filtration with qualitative filter paper (Whatman no. 1) and dried for 16 h in a desiccator under vacuum (produced using a primary vacuum pump). The residual hydrolytic activity in organic medium was determined as a percentage of residual activity compared to the ImLipPS without incubation (control).

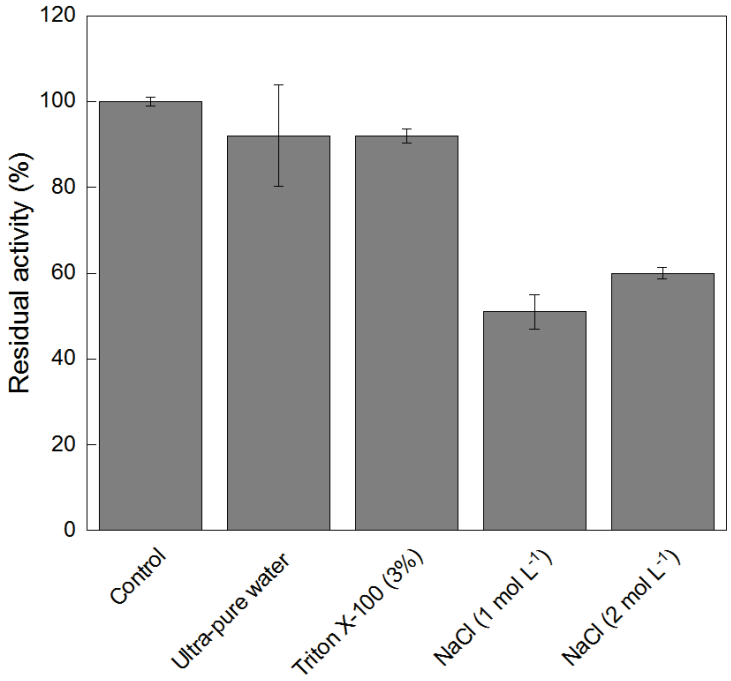

Fig. S2.4. Residual hydrolytic activity of *Pseudomonas cepacia* lipase immobilized on Zn_4Al-Cl LDH (i.e. ImLipPS) after incubation in different solutions. The residual activity was determined in organic medium and expressed as a percentage the activity of ImLipPS without

incubation (i.e. the control). The values are plotted as the mean of duplicate analyses ± the standard errors of the means.

S4. Stability of Zn4Al-Cl-LDH in organic media

The effect of organic solvents on the stability of Zn_4Al-Cl LDH was studied. These assays were performed in closed vials, with the addition of 0.2 g of Zn_4Al-Cl LDH to 2 mL of ethanol (99.5% purity), 2 mL of methanol (99.8% purity), or a solution of *rac*-1-phenylethanol (0.1 mmol) and vinyl acetate (0.4 mmol) in n-hexane (2 mL) (to mimic the conditions of the kinetic resolution reactions). The mixtures were incubated in an orbital shaker at 180 rpm and 35 °C for 30 h (the same conditions as those of the kinetic resolution reactions). The solid was then recovered by filtration with qualitative filter paper (Whatman no. 1) and dried for 16 h in a desiccator under vacuum (produced using a primary vacuum pump). LDH particles were characterized by XRD (Section S1).

The XRD measurements of the Zn_4Al-Cl LDHs incubated in all media tested were identical to those of the control (i.e. without incubation) (Fig. S5). The preservation of all the diffraction lines after the incubation shows that the layer structure was maintained and that there was no delamination/intercalation/deterioration.

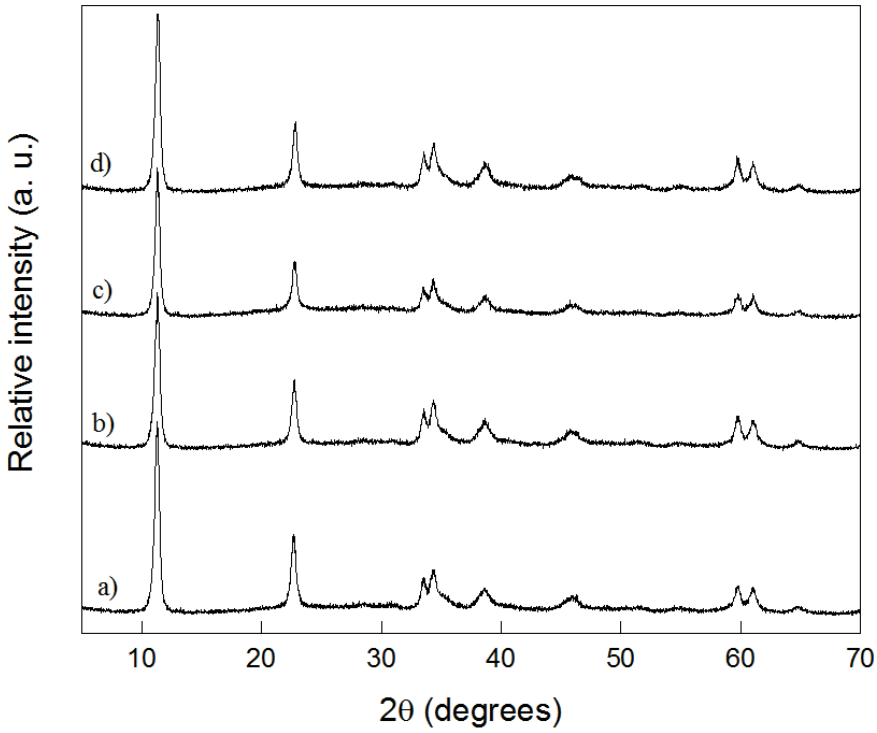

Fig. S2.5. X-ray diffraction (XRD) patterns of Zn_4Al-Cl LDH after 30 h of incubation in different organic solvents. Key: (a) Zn_4Al-Cl LDH without incubation (control), (b) Zn_4Al-Cl LDH incubated in n-hexane containing *rac*-1-phenylethanol (0.1 mmol) and vinyl acetate (0.4 mmol); (c) Zn_4Al-Cl LDH incubated in ethanol and (d) Zn_4Al-Cl LDH incubated in methanol. The incubations were performed in an orbital shaker at 180 rpm and 35 °C for 30 h.

References

[1] E. Conterosito, V. Gianotti, L. Palin, E. Boccaleri, D. Viterbo, M. Milanesio, Facile preparation methods of hydrotalcite layered materials and their structural characterization by combined techniques, Inorganica Chim. Acta. 470 (2018) 36–50. doi:10.1016/j.ica.2017.08.007.

[2] F.L. Theiss, G.A. Ayoko, R.L. Frost, Synthesis of layered double hydroxides containing Mg2+, Zn2+, Ca2+and Al3+layer cations by co-precipitation methods - A review, Appl. Surf. Sci. 383 (2016) 200–213. doi:10.1016/j.apsusc.2016.04.150.

[3] S. Vial, C. Forano, D. Shan, C. Mousty, H. Barhoumi, C. Martelet, N. Jaffrezic, Nanohybrid-layered double hydroxides/urease materials: Synthesis and application to urea biosensors, Mater. Sci. Eng. C. 26 (2006) 387–393. doi:10.1016/j.msec.2005.10.069.

[4] C. Mousty, O. Kaftan, V. Prevot, C. Forano, Alkaline phosphatase biosensors based on layered double hydroxides matrices: Role of LDH composition, Sensors Actuators, B Chem. 133 (2008) 442–448. doi:10.1016/j.snb.2008.03.001.

[5] K.G. Wypych, F. Satyanarayana, In Clay surfaces – Fundamentals and applications, Elsevier, 2004.

[6] I.G. Richardson, Zn- and Co-based layered double hydroxides: Prediction of the a parameter from the fraction of trivalent cations and vice versa, Acta Crystallogr. Sect. B Struct. Sci. Cryst. Eng. Mater. 69 (2013) 414–417. doi:10.1107/S2052519213017545.

[7] E.M. Seftel, E. Popovici, M. Mertens, K. De Witte, G. Van Tendeloo, P. Cool, E.F. Vansant, Zn-Al layered double hydroxides: Synthesis, characterization and photocatalytic application, Microporous Mesoporous Mater. 113 (2008) 296–304. doi:10.1016/j.micromeso.2007.11.029.

[8] J. Liu, W. Wang, H. Liu, Y. Zhou, H. Zhang, X. Zhou, Penicillium expansum lipase-coated magnetic Fe3O 4-polymer hybrid hollow nanoparticles: A highly recoverable and magnetically separable catalyst for the synthesis of 1,3-dibutylurea, RSC Adv. 4 (2014) 25983–25992. doi:10.1039/c4ra04156a

CAPÍTULO III – LAYERED HYDROXIDE SALTS AS NEW SUPPORTS FOR ENZYME IMMOBILIZATION: APPLICATION IN THE IMMOBILIZATION OF *Pseudomonas cepacia* LIPASE ON ZINC HYDROXIDE NITRATE AND ZINC HYDROXIDE CHLORIDE

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Keywords: lipase, layered hydroxide salts, immobilization, anion exchange, enzymatic kinetic resolution.

ABSTRACT

Layered double hydroxides (LDHs) are positive-charge lamellar compounds that derive from the mineral brucite ($Mg(OH)_2$) and they have been used successfully for the immobilization of several enzymes. Layered hydroxide salts (LHSs), materials of the same class as LDHs, have not yet been studied as support for enzymes. In this work, we prepared two types of LHSs, the zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC) to investigate its use as a support in the immobilization of *Pseudomonas cepacia* lipase (LipPS), a versatile enzyme widely used in biocatalysis. Different ratios of LipPS to LHSs were tested and for both ZHN and ZHC and the best protein loading was 162.5 mg g^{-1}; this value represented a compromise between high values of triolein-hydrolyzing activity in organic medium (103 and 105 U g^{-1}, respectively), with high immobilization efficiency (above 90%) and activity retention (above 170%). These immobilized preparations were evaluated for their stability in different organic solvents and storage at different temperatures (4 ºC and 21 ºC). Insights on the nature and strength of the interactions between the enzyme and the supports were evaluated by desorption experiments in solutions of detergents and salts of different ionic forces. The immobilized LipPS on ZHN and ZHC were evaluated for the kinetic resolution of the alcohol (*R,S*)-1-phenylethanol and gave conversions of 50 and 40% in 2 h and ee_s of 99% and 73%, respectively. For both immobilized preparations, the ee_p was higher than 99% and *E* higher than 200. In the reutilization study, 5 successive 2-h kinetic resolutions were done with the same batches of immobilized lipase. The conversions and ee_s values were maintained for both immobilized preparations. The results obtained in this work provide the basis for the development of applications in biocatalysis using ZHN and ZHC as support for lipases.

Keywords: *Pseudomonas cepacia* lipase, immobilization, layered hydroxide salts, enzymatic kinetic resolution.

3.1 Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) are one of the most important enzymes used in biocatalysis, mainly due to their activity and stability in organic media and their ability of catalyzing different types of reactions of interest in organic synthesis, such as esterification, interesterification and transesterification reactions [1,2]. Among lipase applications, one of the most important is the kinetic resolution of chiral compounds to produce enantiomerically pure isomers, which are used in the pharmaceutical industry (for example, ibuprofen, ketoprofen, fenprofen, benoxaprophen, oxazepam and benzodiazepine) [3].

The application of lipases in the industry generally requires its immobilization on a solid support. The enzymatic immobilization technique consists in the attachment or entrapping of enzymes onto inert supports by physical adsorption through ionic or hydrophobic interactions, or by covalent bonding, making them insoluble to reaction media but allowing their active sites to be accessible to substrates [4]. The advantages of using immobilized enzymes are mainly: i) recovery and possible reuse or the possibility of continuous use; ii) simplified downstream processing; iii) generally decreases the conversion time of substrates into products; iv) obtaining high purity products; v) higher volumetric productivity; vi) precise control on reaction time; vii) possibility of greater stability, especially toward organic solvents and higher temperatures and viii) reduction in the cost of the biocatalyzed processes [4–6]. For all these advantages, the use of immobilized enzymes is required in many industrial processes and, therefore, the development of novel supports and immobilization techniques are very important research topics [5–7].

Positive-charge lamellar compounds that derive from the mineral brucite ($Mg(OH)_2$), such as layered double hydroxides (LDHs), have been studied as support for immobilization of

several enzymes, such as lipases, ureases, kinases, transketolases, penicillin G-acylase, fructose-6-phosphate aldolase, lactate dehydrogenase, triosephosphate isomerase and acetylcholinesterase [8,9], producing bioinorganic hybrid nanomaterials for application in biocatalysis or as biosensors. LDHs are formed from changes in composition and structure in brucite where a trivalent metallic cation (M^{3+}) isomorphically replaces part of Mg^{2+}, and forms excess charge in the layers that are neutralized by interlayer anions. As these materials are positively charged on their surface, the immobilization occurs by ionic interactions between the enzyme and the surface of the material. The advantage of the so called "bio-nanocomposites" is that they have well defined morphologies, can be synthesized in different ways and in relatively mild conditions that are compatible with enzymes and are non-toxic and biodegradable [9,10].

Another type of nanomaterial based on brucite-like structures are the layered hydroxide salts (LHSs), which are formed by isomorphic replace of Mg^{2+} of the brucite metallic center by a divalent metal cation, such as Ni^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, surrounded by hydroxyl groups, and occasionally water [10–14]. The deficiency of hydroxyl anions coordinating with the metal cations creates a positive charge at the layers, which needs to be compensated by the presence of interlayer anions, resulting in materials with different potential applications [10,15]. LHSs have been used for a variety of purposes such as intercalation reactions [16,17], pickering emulsifiers [18], oxide precursors [19,20], additives in polymers [10], drug carriers [21–23], and anti-corrosion agents [24]. Although LHSs were also used as support for inorganic catalysts (i. e. porphyrins) [10–12], so far, they were never used for enzyme immobilization.

There are two zinc LHSs that has been successfully explored as supports for inorganic catalysts, the zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC) (Fig. 3.1)

[11,12]. ZHN is represented by the formula $Zn_5(OH)_8(NO_3)_2.2H_2O$ and the solid layer is formed by zinc octahedra where one in every four sites are vacant, while zinc atoms coordinated in tetrahedral geometry are located on the superior and inferior sides of the octahedra. Three vertices of the zinc tetrahedra are occupied by hydroxyl ions from the octahedral sheet, whereas the fourth apical vertex contains a water molecule. This configuration, with five Zn^{2+} ions and eight hydroxyl ions, gives the layer its positive residual charge. The nitrate ions are changeable anions located in the interlayer space that stabilize ZHN structure, generating a solid with basal distance of approximately 9.8 Å [10,11,13]. Zinc hydroxide chloride (ZHC), represented by the formula $Zn_5(OH)_8Cl_2 \cdot H_2O$ is another type of LHS that has a structure similar to ZHN, but instead of water, tetrahedral zinc is directly bonded to the chloride ion, besides being coordinated with tree hydroxyl ions from the octahedral sheet. In the ZHC structure, water molecules are also present between the layers, generating a solid with basal distance of approximately 7.8 Å [10,11,13].

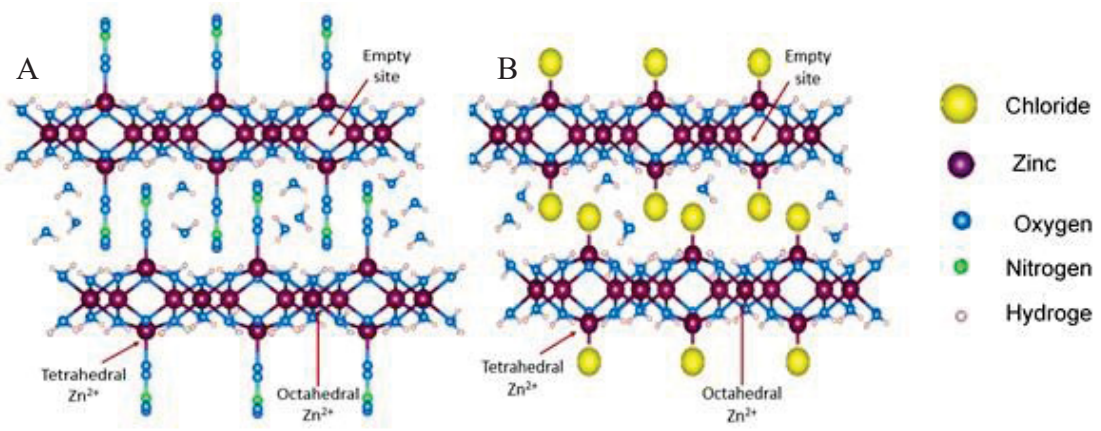

Fig. 3.1 – Schematic representation of the zinc hydroxide nitrate (A) and zinc hydroxide chloride (B) structures.

Recently, we have demonstrated that LDHs could be used as support for *Pseudomonas cepacia* lipase (LipPS) immobilization and that the immobilized preparations could be successfully used for the kinetic resolution of a racemic secondary alcohol, (*R,S*)-1-

phenylethanol. We obtained the maximum desirable conversion of 50% in only one hour, with enantiomeric excesses of substrate (ee_s) and product (ee_p) above 99%, which gave a high enantiomeric ratio (E>200). Moreover, the immobilized preparations were stable after being reused for 30 cycles of reaction, keeping high conversions and selectivity [25].

Considering the success, we had with LDHs for the immobilization of LipPS, and the need of expansion of the range of new materials for lipase immobilization, in this work we investigate the viability of the use of layered hydroxide salts ZHN and ZHC as supports for the immobilization of enzymes using the LipPS as a pattern. ZHN and ZHC were synthesized by coprecipitation and the lipase was immobilized by physical adsorption. Parameters of the immobilization process such as immobilization efficiencies, retention of activity, and the stability of the immobilized preparations in organic solvents with different log P values were studied. As a model reaction to investigate the performance of these nanohybrid materials in synthesis optically pure compounds, we used the transesterification of (*R,S*)-1-phenylethanol with vinyl acetate as the acyl moiety donor.

3.2 Material and methods

3.2.1 Material

Pseudomonas cepacia lipase (Lipase PS Amano) was acquired from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). The enzyme is provided in white lyophilized powder form, with hydrolysis activity in aqueous medium against olive oil higher than 23,000 U g^{-1}, according to the manufacturer (Sigma-Aldrich, St. Louis, Missouri, U.S.A.). The hydrolytic activity obtained experimentally for this powder was 125 U g^{-1} against triolein in organic medium (Section 3.2.7).

$ZnCl_2$, $Zn(NO_3)_2.6H_2O$ and NaOH, which were used in the synthesis of LHSs, were of analytical grade and supplied by Vetec (Duque de Caxias, Rio de Janeiro, Brazil). The alcohol (*R,S*)-1-phenylethanol, which was used in the enzymatic kinetic resolution studies, was from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). Vinyl acetate (> 99%) was obtained from Neon (São Paulo, Brazil) and *n*-hexane (99.5%) was obtained from Êxodo Científica (Hortolândia, São Paulo, Brazil). All other chemicals used were of analytical grade.

3.2.2 Synthesis of the layered hydroxide salts

Both hydroxide salts used in this study, ZHN and ZHC, were synthesized by the co-precipitation method at constant pH [10] and, for this purpose, NaOH (1 mol L^{-1}), $ZnCl_2$ (2 mol L^{-1}) and $AlCl_3.6H_2O$ (2 mol L^{-1}) solutions were prepared in distilled water. Using the ZHC synthesis as example, NaOH and metal-ion-containing solutions were dripped concomitantly and slowly into a reactor, with constant stirring and at room temperature (approximately 21 °C). The synthesis occurred at pH around 6.2, for a period of 1 h. After this time, the pH was adjusted to 7.0 and the product remained under magnetic stirring for a further 24 h, after which it was centrifuged at 3,622 × g. The recovered white precipitate was repeatedly washed with distilled water, dried in a vacuum oven at 60 °C for 48 h and then was triturated with the aid of an agate mortar and pestle.

3.2.3 Lipase immobilization

For the immobilization experiments, lipase PS powder (hereafter referred to as LipPS) was used as provided by the manufacturer, without purification. Immobilizations were made in 25-mL Erlenmeyer flasks, with the addition of 0.1 g of LHSs (ZHN or ZHC) to 5 mL of an enzyme solution that was prepared by dissolving the stated amount of enzyme in Tris-HCl

buffer (10 mmol L^{-1} pH 7.5). This mixture was incubated in an orbital shaker at 120 rpm and 25 °C for 24 h. The solid was then recovered by filtration with qualitative filter paper (Whatman no. 1), placed in desiccators under a vacuum (produced using a primary vacuum pump) for 16 h to dry and then stored at 4 °C or room temperature.

In all the assays of immobilization, the kinetics of immobilization were evaluated during 24 h. The hydrolytic activity of the aqueous lipase solution (hereafter referred to as the supernatant) was monitored using the titrimetric method (Section 3.2.6). The immobilization efficiency (IE, %) was calculated as:

$$IE(\%) = \frac{(A_i - A_f)}{A_i} \times 100\% \quad (1)$$

where A_i is the hydrolytic activity (U) of the supernatant before immobilization and A_f is the hydrolytic activity (U) remaining in the supernatant at the end of the immobilization procedure, both measured in aqueous medium.

The retention of activity (R, %) was calculated as:

$$R(\%) = \frac{A_o}{A_t} \times 100 \quad (2)$$

where A_o is the measured hydrolytic activity in organic medium (Section 3.2.7) of the immobilized preparation and A_T is the theoretical activity of the immobilized enzyme (both in U g^{-1} of LDH). The theoretical activity was calculated as:

$$A_t = (M_{ip} - M_{fp}) \times SA_o \qquad (3)$$

where M_{ip} is the mass of protein per mass of LDH (mg g^{-1}) originally in the enzyme solution (supernatant) before immobilization, M_{fp} is the mass of protein per mass of LDH (mg g^{-1}) remaining in the supernatant at the end of the immobilization procedure and SA_o is the specific hydrolytic activity of the free enzyme (U mg^{-1} of protein), measured in organic medium. It should be noted that values of R above 100% are possible if the lipase is activated upon immobilization.

Controls were performed for all experiments, in which the enzyme solution was incubated for 24 h under the immobilization conditions, but in the absence of LHSs. There was no loss of enzymatic activity from the supernatant in these controls.

For evaluation of storage stability, immobilized LipPS, hereafter referred to as "LipPSZHN" (to LipPS immobilized onto ZHN) or "LipPSZHC" (to LipPS immobilized onto ZHC), was kept for 30 days at 4 °C or room temperature (approximately 21 °C). The residual hydrolytic activity in organic medium (Section 3.2.7) was determined as a percentage of residual activity compared to the initial activity at day one.

LipPS desorption assays were done in 25-mL Erlenmeyer flasks, with the addition of 0.1 g of LipPSZHN or LipPSZHC to 5 mL of solutions containing (i) NaCl (1 or 2 mol L^{-1}), (ii) 3% (v/v) Triton X-100 and (iii) ultra-pure water. The mixtures were incubated in an orbital shaker at 120 rpm and 25 °C for 2 h. The solid was then recovered by filtration with qualitative filter paper (Whatman nº. 1) and dried for 16 h in a desiccator under vacuum (produced using a primary vacuum pump). The residual hydrolytic activity in organic medium (Section 3.2.7)

was determined as a percentage of residual activity compared to the LipPSZHN or LipPSZHC without incubation (control).

The effect of organic solvents on the stability of LipPSZHN, LipPSZHC and free LipPS was determined using the following solvents (with their log *P* values): *n*-heptane (4.0), *n*-hexane (3.5), toluene (2.5) methanol (-0.76) and ethanol (-0.31). In 25-mL Erlenmeyer flasks, 0.1 g of LipPSZHN, LipPSZHC or free LipPS was incubated in 5 mL of each solvent in an orbital shaker at 180 rpm and 35 °C for 2 h. The solvent was then removed by filtration with qualitative filter paper (Whatman nº. 1) and LipPSZHN, LipPSZHC or LipPS was then dried for 16 h, 4 ºC in a desiccator under vacuum (produced using a primary vacuum pump) and its residual triolein-hydrolyzing activity in organic medium (Section3.2.7) was determined as a percentage of residual activity compared to the LipPSZHN, LipPSZHC or LipPS without incubation (control).

3.2.4 Enzymatic Kinetic Resolution

The performances of LipPSZHN, LipPSZCN and free LipPS were compared in transesterification of (*R,S*)-1-phenylethanol with vinyl acetate as the acyl moiety donor. The (*R*)-enantiopreference of LipPS for secondary alcohols is already described in the literature (KAZLAUSKAS et al., 1991).

The reactions were carried out in 4-mL screw-capped bottles. Sufficient enzyme preparation to give 12 U of hydrolytic activity in organic medium of each enzyme preparation was added to a solution of (*R,S)*-1-phenylethanol (0.1 mmol) and vinyl acetate (0.4 mmol) in *n*-hexane (2 mL). The reaction was carried out at 35 °C and 180 rpm in an orbital shaker. At fixed

intervals, 0.01-mL samples of the mixture were removed and analyzed by chiral GC analysis (Section 3.2.9). Experiments with pure LHSs samples were done and produced no reaction.

The enantiomeric excesses of substrate (ee_s, %) and product (ee_p, %) were determined as:

$$ee(\%) = \left[\frac{R - S}{R + S}\right] \times 100 \quad (4)$$

where R is the concentration of the (*R*)-enantiomer (substrate or product) and S is the concentration of the (*S*)-enantiomer (substrate or product).

The conversion (c, %) and enantiomeric ratio (E) were calculated from the values of ee_s and ee_p according to CHEN et al. (1982)[26]:

$$c(\%) = \frac{ee_s}{ee_s + ee_p} \times 100\% \quad (5)$$

$$E = \frac{ln\,[(1 - c)(1 - ee_s)]}{ln\,[(1 - c)(1 + ee_s)]} \quad (6)$$

3.2.5 Characterization of the LHSs

The layered structures of the materials were confirmed by X-ray diffraction (XRD) measurements. For this, the powdered samples were deposited on glass sample holders. The measurements were obtained in the reflection mode using a Shimadzu XRD-6000

diffractometer operating at 40 kV and 20 mA, using CuKα radiation (λ = 1.5418 Å) and a dwell time of 2° min^{-1}; the data were collected between 3 and 70° (in 2θ).

Fourier-transform infrared spectroscopy (FTIR) spectra were recorded with a Bio-Rad FTS 3500GX spectrophotometer in the 400-4000 cm^{-1} range, using KBr pellets. KBr was crushed with a small amount of the solid samples, and the spectra were recorded with a resolution of 4 cm^{-1} and an accumulation of 32 scans.

The measurements of surface area and porosity were analyzed by nitrogen adsorption/desorption isotherms that were obtained using a Quantachrome gas sorption analyzer, model NOVA 2000e. The samples were degassed at 110 °C under vacuum for 2 h and the analyses were carried out in liquid nitrogen (−196 °C). The adsorption/desorption isotherms for N_2 were obtained over the relative pressure range of 0.05–0.99. The specific surface areas of the samples were calculated using the multi point Brunauer–Emmet–Teller (BET) method for relative pressures ranging from 0.05 to 0.30. The specific pore volume was calculated from the desorption isotherm using the Barrett–Joyner–Halenda method (BJH).

The zeta potential values for ZHN and ZHC in Tris-HCl buffer (10 mmol L^{-1} pH 7.5) were measured indirectly by streaming potential analysis performed with a Particle Metrix Stabino Zeta-Check device (Colloid Metrix, Meerbusch,Germany), according to eq (5):

$$SP = k\Delta v \times ZP \tag{7}$$

Were, *SP* is the streaming potential, *k* is an instrument constant, Δv is fluid velocity and *ZP* is the zeta potential. The zeta values were acquired in real time for 15 min and

the median of the zeta of samples.

3.2.6 Determination of the triolein-hydrolysing activity in aqueous medium

In the immobilization studies, the triolein-hydrolyzing activity of the supernatant of the immobilization process was determined by titration using an automatic titrator pHStat (Metrohm 718 Stat Titrino) in aqueous medium, from which the immobilization efficiencies for each enzyme loading were calculated. In these assays, the substrate emulsions consisted of 67 mmol L^{-1} triolein (65% purity, Sigma-Aldrich, St. Louis, Missouri, U.S.A.), 3% (w/v) gum arabic, 2 mmol L^{-1} $CaCl_2$, 2.5 mmol L^{-1} Tris–HCl pH 8.0 and 150 mmol L^{-1} NaCl, dispersed in distilled water (TISS; CARRIÈRE; VERGER, 2001). Samples of the supernatant were added to 20 mL of the emulsion under magnetic stirring (300 rpm) at 30 °C and the reaction was followed for 5 min. One unit of hydrolytic activity in aqueous medium (U) corresponds to the release of 1 μmol of fatty acid per minute, under the assay conditions.

3.2.7 Determination of the triolein-hydrolysing activity in organic medium

This methodology was used to measure the activities of immobilized and free LipPS and to calculate the activity retention of the preparations of LipPS immobilized onto LHSs (i.e. LipPSZHN or LipPSZHC). The activity of free LipPS was determined by adding the powder to the reaction medium, using the same conditions as for LipPSZHN and LipPSZHC.

The triolein-hydrolysing activity in organic medium was determined by adding 5 mL of a reaction medium containing 4.9 mL of *n*-hexane, 70 mmol L^{-1} triolein (65% purity, Sigma-Aldrich (St. Louis, Missouri, U.S.A.) and 2% (v/v) distilled water to a 25-mL Erlenmeyer flask. The mixture was incubated in an orbital shaker at 200 rpm and 40 °C, with the reaction being started by the addition of the enzyme preparation [27]. Every 5 min until 30 min, 0.1

mL samples of the mixture were collected and analyzed for free fatty acids by the Lowry and Tinsley method [28]. Initial rates were calculated. One unit of hydrolytic activity in organic medium (U) corresponds to the release of 1 μmol of fatty acid per minute, under the assay conditions. The hydrolytic activity was expressed in units of activity per gram of enzymatic preparation (U g^{-1}) or per gram of free LipPs powder. There was no reaction in blanks performed with pure LHSs samples.

3.2.8 Protein determination

Protein content was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich, St. Louis, Missouri, EUA) with bovine serum albumin as the standard [29].

3.2.9 GC analysis

The samples from the enzymatic kinetic resolution studies were analyzed by gas chromatography in a GC-2010 chromatograph (Shimadzu, Kyoto, Japan) equipped with a hydrogen flame ionization detector and a chiral column β-DEX 120 (30m × 0.25mm diameter, 0.25-μm film thickness). An autoinjector (AOC-20i, Shimadzu Co, Kyoto, Japão) was used to inject 0.5 μL of the medium using N_2 as the carrier gas. The injector and detector were set at 250 °C. The oven program was as follows: initial value of 110 °C, heating at 1 °C min^{-1} up to 120 °C, 120 °C maintained for 10 min. Retention times are: (*S*)-1-phenylethyl acetate 13.5 min, (*R*)-1-phenylethyl acetate 14.3 min, (*R*)-1-phenylethanol 14.8 min and (*S*)-1-phenylethanol 15.6 min.

3.3 Results

3.3.1 Characterization of layered hydroxide salts before and after immobilization

The layered structures of the synthesized materials were confirmed by X-ray diffraction (XRD) measurements. All XRD measurements (Fig. 3.2) show the presence of a crystalline phase containing peaks related to the reflection planes in the basal stacking direction of layered crystal (*h00*), which indicates that the obtained products retained their layered structure. Also, the patterns for all LHSs samples show the presence of two characteristics non basal peaks in the region of 33 (º 2θ) which indicates (*002*) and (*021*) plans [10,30].

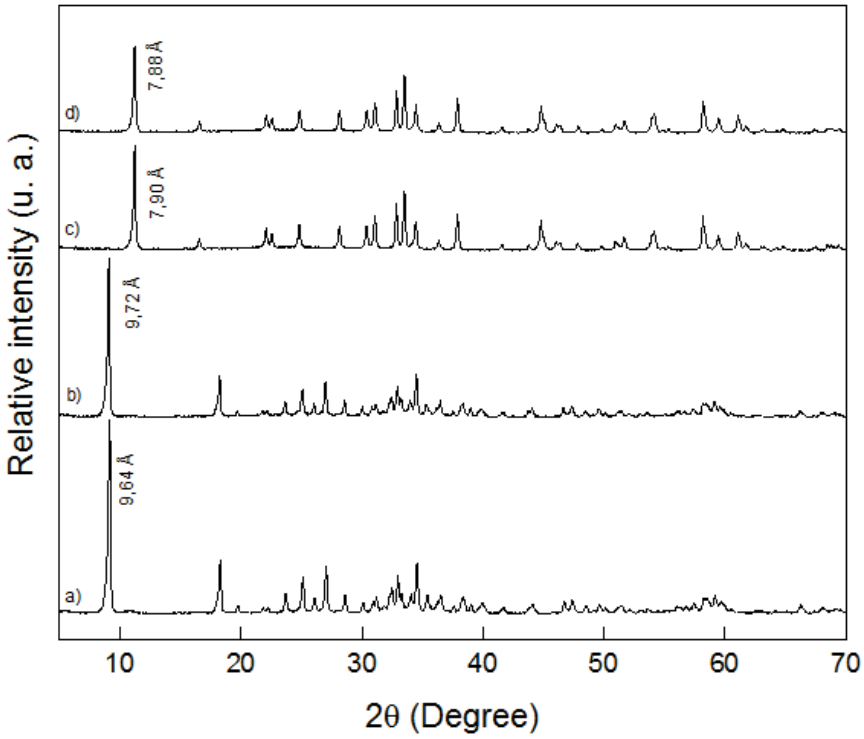

Fig. 3.2 – X-ray diffraction (XRD) patterns of layered hydroxide salts before and after immobilization. Key: (a) ZHN, (b) ZHNLipPS, (c) ZHC and (d) ZHCLipPS.

For XRD of ZHN and LipPSZHN, the basal distance obtained for the stacking (*200*) plan were around 9.70 Å (Fig. 3.2a and b), which is in accordance with the value reported in the literature for the intercalation of nitrate anion [31,32]. For the XRD of ZHC and LipPSZHC

(Fig. 3.2c and d), the (*200*) plan was around 7.90 Å and it is in accordance with the intercalation of chloride anion [12,33].

There was not significant difference between the calculated basal distance for pristine LHSs and their respective immobilized preparations (i. e. LipPSZHN and LipPSZHC), showing that crystallinity was not affected by the presence of LipPS on the materials.

To confirm the presence of LipPS in the immobilized preparation, the LHSs materials (before and after the immobilization) were also analyzed by Fourier-transform infrared spectroscopy (FTIR). For ZHN and LipPSZHN (Fig. 3.3a and b, respectively), it was observed a wide band in region of 3477 cm^{-1}, attributed to the O-H lattice vibration of water molecules. These spectra also show a narrow absorption band with very well-defined vibration energies in the region of 3574 cm^{-1}, related to hydroxyl groups of layered lattices. The intense band observed in the region of 1375 cm^{-1} is attributed to the v_3 mode, characteristic of the nitrate ion [10,31,34]. Bands near 700 and 470 cm^{-1} were related to the Metal-O bond, Metal-OH bending and chain connection vibration of O-Metal-O bond of the layer structure [25].

Spectra of FTIR of ZHC and LipPSZHC, (Fig. 3.3c and d, respectively), show bands characteristics of a layered hydroxide. Around 3448 cm^{-1}, it was observed a broad absorption band, attributed to the vibration of O-H bond of hydroxyl groups of the layered compound and water molecules, intercalated and adsorbed. The band in region of 1606 cm^{-1} is related to angular deformation of water molecules. Vibrational modes observed in 725 cm^{-1} and 472 cm^{-1} were related with O-Metal-O and Metal-O bonds, respectively [10,11].

For both pristine ZHN and ZHC and their corresponding immobilized preparations (LipPSZHC and LipPSZHN) no difference amongst the FTIR spectra was observed. This is probably due to the fact that bands associated with lipase (Fig. 3.3e) were overlapped by the typical bands of both layered compounds [25].

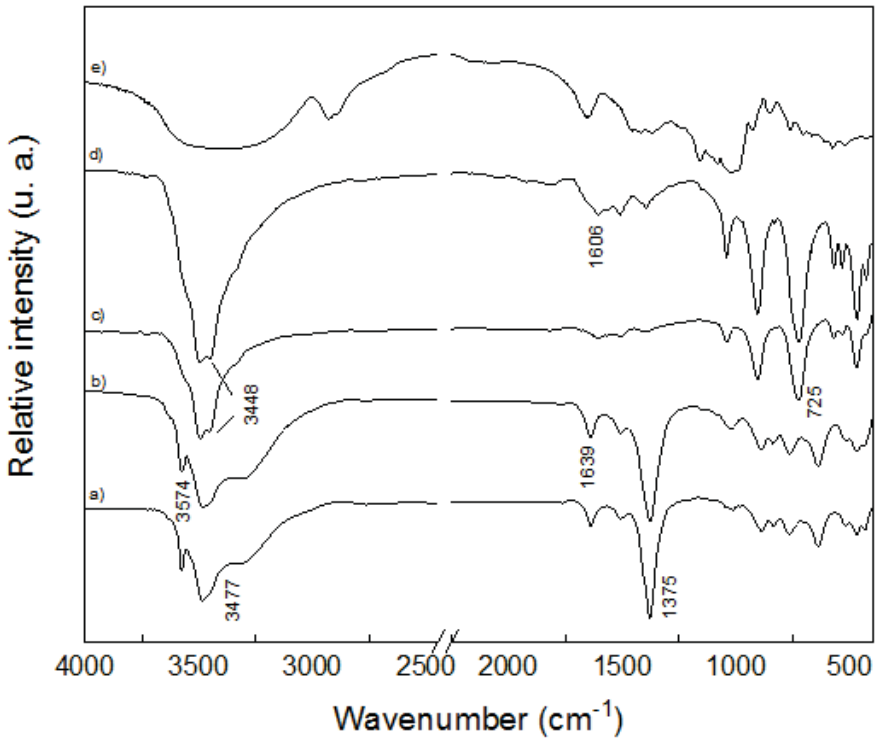

Fig. 3.3 – Fourier-transform infrared spectroscopy (FTIR) spectra of layered hydroxide salts before and after immobilization. Key: (a) ZHN, (b) ZHNLipPS, (c) ZHC, (d) ZHCLipPS and (e) free LipPS.

The surface area and porosity analysis of pristine LHSs showed that both the specific surface area and the specific pore volume of the ZHN is larger than that of ZHC (Table 3.1). In addition, the zeta potential (ζ) is more positive for ZHN (Fig. 3.4), due to the fact that, in ZHN, zinc is not directly bonded to the nitrate anion. This suggests that the immobilization of

LipPS will be more effective on ZHN, given that the enzyme molecule is negatively charged at pH 7.5, used to prepare the immobilization solution [25].

Table 3.1 – BET specific surface area and specific pore volume of zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC)

Sample	BET surface area (m^2/g)	BET average pore diameter (Å)	BET average pore volume (cm^3/g)
ZHC	5.5	45.1	1.8
ZHN	10.8	64.4	3.6

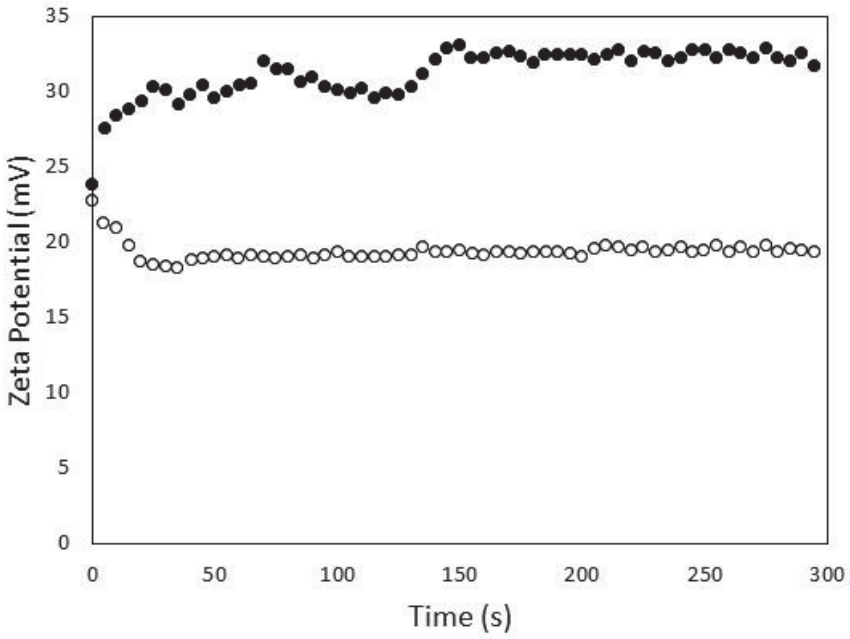

Fig. 3.4 – Zeta potential (ζ) of zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC). Key: (closed circles) ZHN, (opened circles) ZHC.

3.3.2 Immobilization of LipPS on layered hydroxide salts

In order to evaluate the effect of the enzyme loading, five different ratios of LipPS to LHSs were tested: 32.5, 65, 130, 162.5 and 195 mg of protein content in the powder of LipPS per g

of ZHN or ZHC (mg g^{-1}). These amounts of protein correspond to 250, 500, 1000, 1250 and 1500 mg of LipPS powder. The immobilized preparations were evaluated according to triolein-hydrolyzing activity in organic medium (See Section 3.2.7). Thus, in addition to avoiding protein leaching from the support, the performance of the biocatalyst in organic medium was tested.

For the protein loadings of 32.5 and 65 mg g^{-1}, in both supports, the lipase activities in the supernatant at 12 h were very low, giving immobilization efficiencies (Table 3.2) around 100% (Fig. 3.5). For the protein loadings of 130 and 163 mg g^{-1}, also for the both supports, the IE values were similar, reaching around 100%, but after 24 h. For the highest protein loading (195 mg g^{-1}), the IE at 24 h was 88 and 71% to ZHN and ZHC, respectively, indicating the support saturation.

Values higher than 100% of activity retention were found for all enzyme loadings, indicating enzyme activation upon immobilization. In lipases, this occurs due to the changes in enzymatic conformation. Most lipases have a lid domain that covers the active site: when free in aqueous solution, lipases are mainly in their closed conformation, which renders them less active. On the other hand, interactions with the support can provide immobilization of the enzyme with the lid open, producing activation of the enzyme [25,35].

The lower values of activity retention (132 and 114% to ZHN and ZHC, respectively) found for the highest protein loading (195 mg g^{-1}) is in accordance with the lowest immobilization efficiency value (Table 3.2). This is probably due to multilayer immobilization, preventing the access of the substrate to the inner layers of the enzyme, which does not happen at lower protein loadings [36].

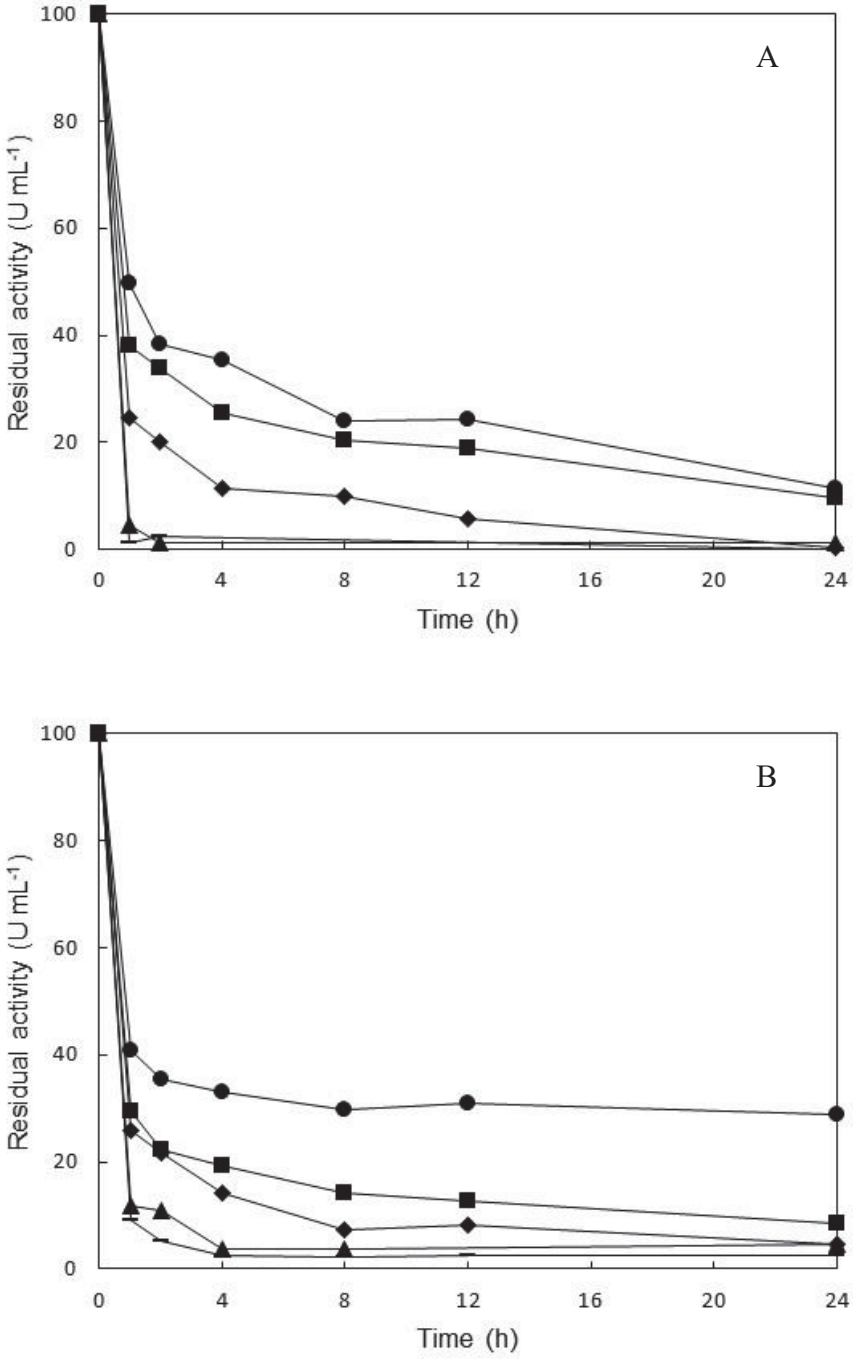

Fig. 3.5 – Effect of protein loading on the kinetics of immobilization of *Pseudomonas cepacia* lipase (LipPS). Key: (A) ZHN; (B) ZHC. (-) 32.5 mg g^{-1}, (▲) 65 mg g^{-1}, (♦) 130 mg g^{-1}, (■) 162.5 mg g^{-1} and (●) 195 mg g^{-1}. The residual triolein-hydrolyzing activity in the supernatant was determined by the titrimetric method using a pHStat, at 40 °C, pH 7.0 for 5 min. The values

are plotted as the means of triplicate analyses ± the standard errors of the means. When the error bar is not visible, it is smaller than the symbol.

For both ZHN and ZHC, the protein loading of 162.5 mg g^{-1} were chosen for further experiments. This protein loading represents a compromise between high values of triolein-hydrolyzing activity in organic medium (103 and 105 U g^{-1}, respectively), with high immobilization efficiency (above 90%) and activity retention (above 170%) (Table 3.2).

Table 3.2 – Principal parameters for immobilization of the LipPS on zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC)

Protein loading (mg g^{-1})	IE (%)[a]		R (%)[b]		Immobilized LipPS hydrolytic activity in organic medium (U g^{-1})[c]	
	ZHN	ZHC	ZHN	ZHC	ZHN	ZHC
32.5	100	97	180	176	25±1	28±0
65	99	97	178	175	45±6	46±1
130	96	95	181	175	91±3	94±3
162.5	90	92	171	172	103±3	105±2
195	88	71	132	114	135±3	114±7

[a] Calculated using Eq. (1).

[b] Calculated using Eq. (2).

[c] The values are given as the means of triplicate analyses ± the standard errors of the means.

3.3.3 Desorption assays

Desorption assays were performed to determine the interaction strength between LipPS and the LHSs and to have an insight of the type of interaction. In these assays, LipPSZHN or

LipPSZHC were incubated for 2 h in different media: (i) ultra-pure water and solutions containing (ii) 3% (v/v) Triton X-100, (iii) 1 or 2 mol L^{-1} NaCl (Fig. 3.6). No loss of LipPSZHN and LipPSZHC activity incubated in ultra-pure water was observed relative to the control (LipPSZHN or LipPSZHC without incubation).

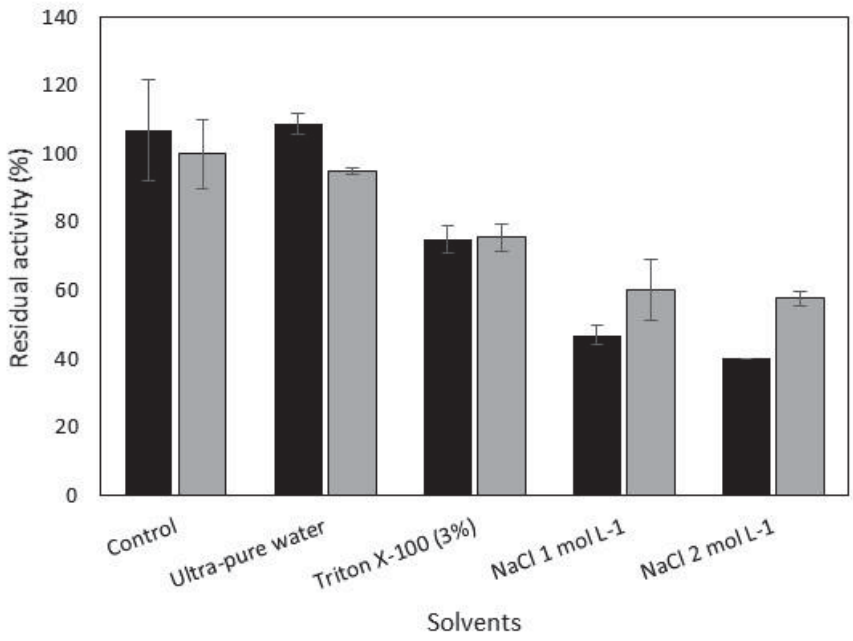

Fig. 3.6 – Residual triolein-hydrolyzing activity *Pseudomonas cepacia* lipase immobilized on zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC) after incubation in different solutions. Key: (black bars) ZHN and (grey bars) ZHC. Residual activity was measured in organic medium. The values are plotted as the mean of duplicate analyses ± the standard errors of the means.

For LipPSZHN and LipPSZHC incubated in Triton X-100 solution, 30 and 24%, respectively, of activity loss was observed, probably due to the leaching of the lipase molecules that were interacting with each other. Dias et al. [25], immobilized LipPS on a similar material, HDL of Zn/Al, and also observed a loss of activity of the immobilized derivative when incubated in

Triton X-110. They concluded that the effect is due to the multilayers formation during the immobilization and which is undone in the solution containing Triton X-100. On the other hand, when LipPSZHN and LipPSZHC were incubated in NaCl 1 mol L^{-1}, a loss of approximately 60% of enzymatic activity was observed for both supports, which confirms the immobilization of LipPS on both layered compounds surface by ionic interaction. When incubated in NaCl 2 mol L^{-1}, there was no further leaching of LipPS from the LHSs, which shows a strong interaction between the lipase and the support.

3.3.4 Stability in organic media

The effect of *n*-heptane, *n*-hexane, toluene and ethanol on the triolein-hydrolyzing activity of LipPSZHN and LipPSZHC was studied after incubation for 2 h at 35 °C. In all the tested solvents, LipPSZHN lost an average of 28% of its initial activity, with the highest loss in ethanol (42%, Fig. 3.7). LipPSZHC, on the other hand, remained stable when compared to the control ($p > 0.05$) for the solvents *n*-heptane, *n*-hexane and toluene, with a decrease of the residual activity observed only when incubated in ethanol (26%, Fig. 3.7). Hydrophilic organic solvents such as ethanol remove the water solvation layer around the enzyme, which causes a drastic distortion of its conformation, leading to the inactivation of the enzyme [37,38].

The XRD measurements of LipPSZHC and LipPSZHN after incubation in all organic solvents remained identical to the control (without incubation) (Fig. S 3.1). The preservation of the all diffraction lines after the incubation proves the maintenance of the layer structure and indicates that there was no delamination/intercalation/deterioration process when LipPSZHC and LipPSZHN were incubated in *n*-heptane, *n*-hexane, toluene and ethanol.

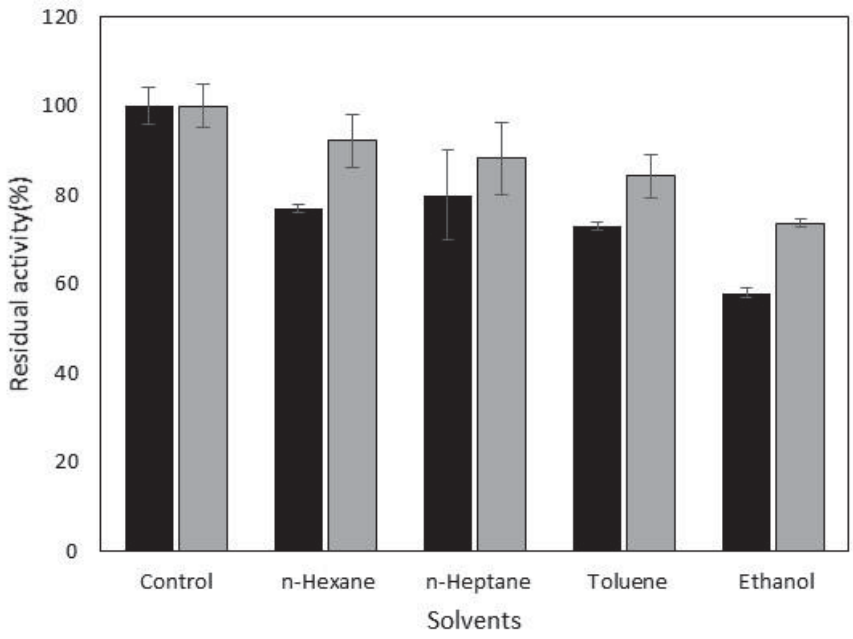

Fig. 3.7 – Residual triolein-hydrolyzing activity *Pseudomonas cepacia* lipase immobilized on zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC) after incubation for 2 h at 35 °C in different organic solvents. Residual activity was measured in organic medium. Key: (black bars) ZHN and (grey bars) ZHC. The values are plotted as the mean of duplicate analyses ± the standard errors of the means.

3.3.5 Stability of immobilized LipPS during storage

The stability of LipPS immobilized on ZHN and ZHC was evaluated during 30 days at two temperatures (Fig. 3.8). In both supports, LipPS kept its activity when stored at 4 °C ($p > 0.05$), but at room temperature (average of 21 °C), surprisingly, the activity decreased by 40% only for LipPSZHN. This is probably because the ZHN contains water molecules coordinated with the zinc ions of the layers. These water molecules could promote hydrogen bonds on the support surface which can act as electron density donor, leading to the decrease of interactions between lipases and the ZHN surface, which consequently are leached during the enzymatic assay.

Since there was no loss of activity for the immobilized preparations stored at 4º C, this temperature was defined as the ideal storage temperature.

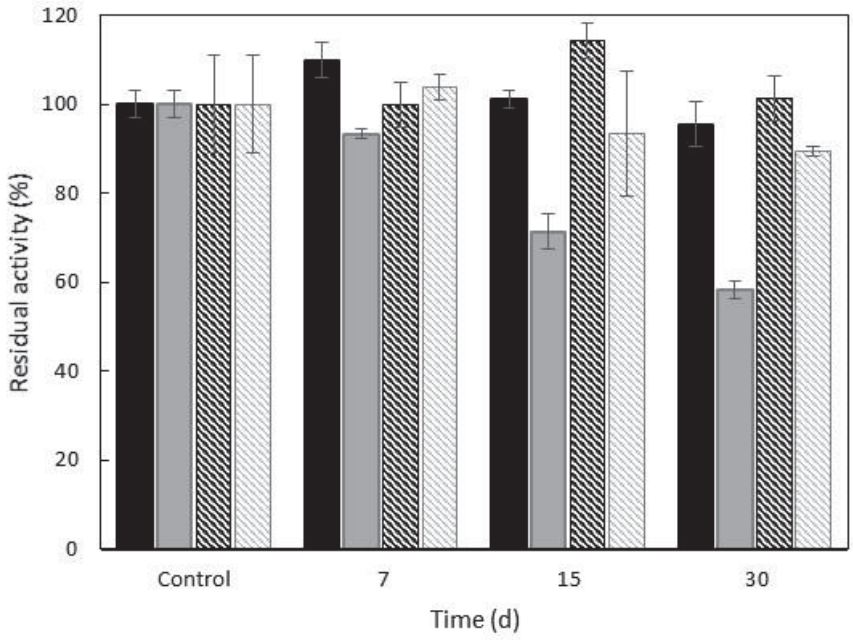

Fig. 3.8 – Stability during storage of *Pseudomonas cepacia* lipase immobilized on zinc hydroxide nitrate (ZHN) and zinc hydroxide chloride (ZHC). Key: (black bars) Storage at 4 °C and room temperature (~ 21 ºC, grey bars). (filled bars) ZHN; (hatched bars) ZHC. Residual triolein-hydrolyzing activity was measured in organic media. Controls refer to initial activity at day one. The values are plotted as the means of triplicate analyses ± the standard errors of the means.

3.3.6 Enzymatic kinetic resolution

LipPSZHN, LipPSZHC and free LipPS were used in the resolution of the racemic secondary alcohol (*R,S*)-1-phenylethanol (Table 3.3), which is a substrate commonly used to determine

the enantioselectivity of lipases [39]. The reaction was followed during 60 min and samples were analyzed by chiral GC analysis (See Section 3.2.9).

Table 3.3 – Resolution of (*R,S*)-1-phenylethanol using the lipases of *Pseudomonas cepacia* in free and immobilized form

Lipase/Support	Time (min)	*c* (%)	ee_s (%)	ee_p (%)	*E*
LipPSZHN	60	45	89	> 99	>200
	90	45	89	> 99	>200
	120	50	> 99	> 99	>200
LipPSZHC	60	33	52	> 99	>200
	90	38	61	> 99	>200
	120	40	73	> 99	>200
Pseudomonas cepacia (free)	60	15	17	> 99	>200
	90	20	22	> 99	>200
	120	27	37	> 99	>200
ZHN or ZHC (control)	60	0	-	-	-
	90	0	-	-	-
	120	0	-	-	-

(*c*) Ester conversion calculated using Eq. (5); (ee_s) enantiomeric excess of substrate and (ee_p) enantiomeric excess of product calculated using Eq. (4); (*E*) enantiomeric ratio calculated using Eq. (6). Reaction conditions: 0.1 mmol of (*R,S*)-1-phenylethanol and 0.4 mmol of vinyl acetate in 2 mL of *n*-hexane. The enzymatic resolution was carried out at 35 °C.

Table 3.3 presents the values obtained in the reactions for the conversion (*c*), the enantiomeric excesses of the substrate (ee_s) and of the product (ee_p) and the enantiomeric ratio (*E*). For LipPSZHN, after 120 min, the conversion reached 50%, with both ee_s and ee_p higher than 99% and *E* >200, but already at 60 min the conversion, *ees* and *eep* were high (45%, 89 and 99%, respectively); whereas, for LipPSZHC, after 120 min, the conversion was 40%, with an

ee_s of 73% (Table 3.2). When free LipPS powder was used, after 120 min, a conversion of 27% was reached, with an ee_s of 37% and *eep* above 99%.

We evaluated the reusability of the LipPSZHN or LipPSZHC in cycles of reaction of 2 h in the resolution of (*R,S*)-1-phenylethanol. During 5 cycles, for both immobilized preparations, no decrease was observed in the percentages of conversion and ee_s obtained in the first cycle (data not shown).

3.4 Discussion

Our work contributes to the area of enzyme immobilization, and shows, for the first time, the viability of zinc hydroxide nitrate and zinc hydroxide chloride for immobilization of lipases. High values of immobilization efficiencies (IE) (>90%) were obtained for all protein loadings studied and the retention of activity parameter was more than 100%.

The results showed that the best LHS for immobilizing LipPS is ZHN. This support has a surface area (10.8 $m^2\ g^{-1}$) and pore volume (3.6 $cm^3\ g^{-1}$) larger than ZHC, in addition to having a higher zeta potential value, which indicates that ZHN has more positive charges on its surface to interact with the lipase molecules. Also, the immobilized preparation LipPSZHN was more effective in the resolution of the racemic secondary alcohol (*R,S*)-1-phenylethanol, with a maximum conversion (50%) reached in 2 h, with ee_s of 99%, while, at the same reaction time, LipPSZHC achieved 40% conversion with 73% ee_s.

Layered double hydroxide (LDH) bio-nanocomposites have been shown to be effective for the immobilization of lipases. Dias et al. [25] used a LDH of Zn/Al-Cl to immobilize the

Pseudomonas cepacia lipase and reported an IE above 90% and R greater than 100% for all protein loading studied. Also, using an HDL of Mg and Al, Dong et al. [40], immobilized the *Candida lipolytica* lipase and reported an activity retention of 69% in the application in the hydrolysis of olive oil in aqueous medium. Thus, in terms of immobilization parameters, our support is compatible or superior to the supports of similar structures used by other authors.

In our work, the LHSs immobilized preparations of LipPS were used for the kinetic resolution of (*R,S*)-1-phenylethanol. High conversion values with high enantioselectivity of LipPS were achieved, without degradation of the support or loss of the catalytic activity, for five cycles of reuse. However, further immobilization studies should be made to improve the productivity of the kinetic resolution, since in similar reaction conditions and using layered double hydroxide of Zn/Al-Cl as a support, Dias et al. [25], achieved in 1 h the same conversion observed in 2 h in our work with LipPSZHN as catalyst.

3.5 Conclusions

The layered hydroxide salts, zinc hydroxide nitrate and zinc hydroxide chloride, can be used to immobilize LipPS with high immobilization efficiency (> 90%) and activity retention (> 171%), showing activation upon immobilization. The best support was ZHN, given that it has a larger surface area and pore volume, beyond having a higher zeta potential. Even though LHSs were not as effective in terms of reaction time in the kinetic resolution of (*R,S*)-1-phenylethanol as the other nanomaterial (LDHs) studied by our group in previous work, they increase the range of possibilities for new materials for immobilizing lipases and foster future studies towards the kinetic resolution of other chiral compounds with other lipases and reaction conditions.

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References

[1] F. Hasan, A.A. Shah, A. Hameed, Industrial applications of microbial lipases, Enzyme Microb. Technol. 39 (2006) 235–251. doi:10.1016/j.enzmictec.2005.10.016.

[2] B. Joseph, P.W. Ramteke, G. Thomas, Cold active microbial lipases: Some hot issues and recent developments, Biotechnol. Adv. 26 (2008) 457–470. doi:10.1016/j.biotechadv.2008.05.003.

[3] L.A. Nguyen, H. He, C. Pham-Huy, Chiral Drugs: An Overview, Int. J. Biomed. Sci. 2 (2016) 85–100. doi:10.1111/deci.12302.

[4] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. 40 (2007) 1451–1463. doi:10.1016/j.enzmictec.2007.01.018.

[5] J.C.S.D. Santos, O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Importance of the Support Properties for Immobilization or Purification of Enzymes, ChemCatChem. 7 (2015) 2413–2432. doi:10.1002/cctc.201500310.

[6] A. Basso, S. Serban, Industrial applications of immobilized enzymes — A review, Mol. Catal. 479 (2019) 110607. doi:10.1016/j.mcat.2019.110607.

[7] B.B. Pinheiro, N.S. Rios, E. Rodríguez Aguado, R. Fernandez-Lafuente, T.M. Freire, P.B.A. Fechine, J.C.S. dos Santos, L.R.B. Gonçalves, Chitosan activated with divinyl

sulfone: a new heterofunctional support for enzyme immobilization. Application in the immobilization of lipase B from *Candida antarctica*, Int. J. Biol. Macromol. 130 (2019) 798–809. doi:10.1016/j.ijbiomac.2019.02.145.

[8] C. Forano, S. Vial, C. Mousty, Nanohybrid enzymes - layered double hydroxides: potential applications, Curr. Nanosci. 2 (2006) 283–294. doi:10.2174/1573413710602030283.

[9] C. Taviot-Guého, V. Prévot, C. Forano, G. Renaudin, C. Mousty, F. Leroux, Tailoring hybrid layered double hydroxides for the development of innovative applications, Adv. Funct. Mater. 28 (2018) 1–33. doi:10.1002/adfm.201703868.

[10] G.G.C. Arizaga, K.G. Satyanarayana, F. Wypych, Layered hydroxide salts: Synthesis, properties and potential applications, Solid State Ionics. 178 (2007) 1143–1162. doi:10.1016/j.ssi.2007.04.016.

[11] G.S. Machado, G.G.C. Arízaga, F. Wypych, S. Nakagaki, Immobilization of anionic metalloporphyrins on zinc hydroxide nitrate and study of an unusual catalytic activity, J. Catal. 274 (2010) 130–141. doi:10.1016/j.jcat.2010.06.012.

[12] G.S. Machado, F. Wypych, S. Nakagaki, Applied Catalysis A: General Anionic iron (III) porphyrins immobilized on zinc hydroxide chloride as catalysts for heterogeneous oxidation reactions, Appl. Catal. A, Gen. 413–414 (2012) 94–102. doi:10.1016/j.apcata.2011.10.046.

[13] M.G. da Rocha, S. Nakagaki, G.M. Ucoski, F. Wypych, G. Sippel Machado, Comparison between catalytic activities of two zinc layered hydroxide salts in brilliant green organic dye bleaching, J. Colloid Interface Sci. 541 (2019) 425–433. doi:10.1016/j.jcis.2019.01.111.

[14] C. Velazquez-Carriles, M.E. Macias-Rodríguez, G.G. Carbajal-Arizaga, J. Silva-Jara, C. Angulo, M. Reyes-Becerril, Immobilizing yeast β-glucan on zinc-layered hydroxide nanoparticle improves innate immune response in fish leukocytes, Fish Shellfish Immunol. 82 (2018) 504–513. doi:10.1016/j.fsi.2018.08.055.

[15] S.R. Tavares, V.S. Vaiss, F. Wypych, A.A. Leitão, Similarities between zinc hydroxide chloride monohydrate and its dehydrated form: A theoretical study of their structures and anionic exchange properties, J. Phys. Chem. C. 118 (2014) 19106–19113. doi:10.1021/jp504051z.

[16] M. Xue, R. Chitrakar, K. Sakane, K. Ooi, S. Kobayashi, M. Ohnishi, A. Doi, Synthesis of ktenasite, a double hydroxide of zinc and copper, and its intercalation reaction, J. Solid

State Chem. 177 (2004) 1624–1630. doi:10.1016/j.jssc.2003.12.026.

[17] R. Marangoni, A. Mikowski, F. Wypych, Effect of adsorbed/intercalated anionic dyes into the mechanical properties of PVA: Layered zinc hydroxide nitrate nanocomposites, J. Colloid Interface Sci. 351 (2010) 384–391. doi:10.1016/j.jcis.2010.08.006.

[18] J.P.E. Machado, R.A. de Freitas, F. Wypych, Layered clay minerals, synthetic layered double hydroxides and hydroxide salts applied as pickering emulsifiers, Appl. Clay Sci. 169 (2019) 10–20. doi:10.1016/j.clay.2018.12.007.

[19] M. Halma, F. Wypych, S.M. Drechsel, S. Nakagaki, Synthesis, characterization and catalytic behavior of iron porphyrins immobilized in layered double hydroxides, J. Porphyr. Phthalocyanines. 6 (2002) 502–513. doi:10.1142/s1088424602000634.

[20] Z. Tong, T. Shichi, K. Takagi, Oxidation catalysis of a manganese(III)porphyrin intercalated in layered double hydroxide clays, Mater. Lett. 57 (2003) 2258–2261. doi:10.1016/S0167-577X(02)01206-5.

[21] S.H.H. Al Ali, M. Al-Qubaisi, M.Z. Hussein, Z. Zainal, M.N. Hakim, Preparation of hippurate-zinc layered hydroxide nanohybrid and its synergistic effect with tamoxifen on HepG2 cell lines, Int. J. Nanomedicine. 6 (2011) 3099–3111. doi:10.2147/ijn.s24510.

[22] M.Z. Hussein, S.H. Al Ali, Z. Zainal, M.N. Hakim, Development of antiproliferative nanohybrid compound with controlled release property using ellagic acid as the active agent, Int. J. Nanomedicine. 6 (2011) 1373–1383. doi:10.2147/ijn.s21567.

[23] H. Nabipour, M. Hosaini Sadr, N. Thomas, Synthesis, characterisation and sustained release properties of layered zinc hydroxide intercalated with amoxicillin trihydrate, J. Exp. Nanosci. 10 (2015) 1269–1284. doi:10.1080/17458080.2014.998301.

[24] N. Boshkov, K. Petrov, S. Vitkova, G. Raichevsky, Galvanic alloys Zn-Mn - Composition of the corrosion products and their protective ability in sulfate containing medium, Surf. Coatings Technol. 194 (2005) 276–282. doi:10.1016/j.surfcoat.2004.09.016.

[25] G. Silva Dias, P.T. Bandeira, S. Jaerger, L. Piovan, D.A. Mitchell, F. Wypych, N. Krieger, Immobilization of Pseudomonas cepacia lipase on layered double hydroxide of Zn/Al-Cl for kinetic resolution of rac-1-phenylethanol, Enzyme Microb. Technol. 130 (2019) 109365. doi:10.1016/j.enzmictec.2019.109365.

[26] C.S. Chen, Y. Fujimoto, G. Girdaukas, C.J. Sih, Quantitative analysis biochemical kinetic resolution of enantiomer, J. Am. Chem. Soc. 104 (1982) 7294–7299. doi:10.1021/ja00389a064.

[27] R.C. Alnoch, V.P. Martini, A. Glogauer, A.C.D.S. Costa, L. Piovan, M. Muller-Santos, E.M. De Souza, F.D.O. Pedrosa, D.A. Mitchell, N. Krieger, Immobilization and characterization of a new regioselective and enantioselective lipase obtained from a metagenomic library, PLoS One. 10 (2015) 1–18. doi:10.1371/journal.pone.0114945.

[28] R.R. Lowry, I.J. Tinsley, Rapid colorimetric determination of free fatty acids., J. Am. Oil Chem. Soc. 53 (1976) 470–472. doi:10.1007/BF02636814.

[29] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Frovenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85. doi:10.1016/0003-2697(85)90442-7.

[30] M.L.N. Da Silva, R. Marangoni, A.C.T. Cursino, W.H. Schreiner, F. Wypych, Colorful and transparent poly(vinyl alcohol) composite films filled with layered zinc hydroxide salts, intercalated with anionic orange azo dyes (methyl orange and orange II), Mater. Chem. Phys. 134 (2012) 392–398. doi:10.1016/j.matchemphys.2012.03.007.

[31] S. Jaerger, A. Zimmermann, S.F. Zawadzki, F. Wypych, S.C. Amico, Zinc layered hydroxide salts: Intercalation and incorporation into low-density polyethylene, Polimeros. 24 (2014) 673–682. doi:10.1590/0104-1428.1733.

[32] S. Majoni, S. Su, J.M. Hossenlopp, The effect of boron-containing layered hydroxy salt (LHS) on the thermal stability and degradation kinetics of poly (methyl methacrylate), Polym. Degrad. Stab. 95 (2010) 1593–1604. doi:10.1016/j.polymdegradstab.2010.05.033.

[33] G.G.C. Arízaga, Intercalation studies of zinc hydroxide chloride: Ammonia and amino acids, J. Solid State Chem. 185 (2012) 150–155. doi:10.1016/j.jssc.2011.11.016.

[34] R. Marangoni, L.P. Ramos, F. Wypych, New multifunctional materials obtained by the intercalation of anionic dyes into layered zinc hydroxide nitrate followed by dispersion into poly(vinyl alcohol) (PVA), J. Colloid Interface Sci. 330 (2009) 303–309. doi:10.1016/j.jcis.2008.10.081.

[35] R. Fernandez-Lafuente, P. Armisén, P. Sabuquillo, G. Fernández-Lorente, J.M. Guisán, Immobilization of lipases by selective adsorption on hydrophobic supports, Chem. Phys. Lipids. 93 (1998) 185–197. doi:10.1016/S0009-3084(98)00042-5.

[36] B. Al-Duri, Y.P. Yong, Characterisation of the equilibrium behaviour of lipase PS (from *Pseudomonas*) and lipolase 1OOL (from *Humicola*) onto Accurel, J. Mol. Catal. B Enzym. 3 (1997) 177–188. doi:10.1016/S1381-1177(96)00052-5.

[37] N. Krieger, T. Bhatnagar, J.C. Baratti, A.M. Baron, V.M. De Lima, D. Mitchell, Non-Aqueous Biocatalysis in Heterogeneous Solvent Systems Nadia, Food Technol. Biotechnol. 42 (2004) 1–8. papers2://publication/uuid/348C88E7-46AB-40C6-A5E2-CA9B20E95A95.

[38] A.M. Klibanov, Why are enzymes less active in organic solvents than in water ?, Trends Biotechnol. 15 (1997) 97–101. doi:10.1016/S0167-7799(97)01013-5.

[39] K.P. Dhake, K.M. Deshmukh, Y.S. Wagh, R.S. Singhal, B.M. Bhanage, Investigation of steapsin lipase for kinetic resolution of secondary alcohols and synthesis of valuable acetates in non-aqueous reaction medium, J. Mol. Catal. B Enzym. 77 (2012) 15–23. doi:10.1016/j.molcatb.2012.01.009.

[40] L. Dong, C. Ge, P. Qin, Y. Chen, Q. Xu, Immobilization and catalytic properties of *Candida lipolytic* lipase on surface of organic intercalated and modified MgAl-LDHs, Solid State Sci. 31 (2014) 8–15. doi:10.1016/j.solidstatesciences.2014.02.006.

Supplementary material

"Layered hydroxide salts as new supports for enzymes immobilization: application in the immobilization of *Pseudomonas cepacia* lipase on zinc hydroxide nitrate and zinc hydroxide chloride"

by

Glauco Silva Dias, Silvia Jaerger, David Alexander Mitchell, Fernando Wypych, Nadia Krieger

1. *Stability of zinc hydroxide nitrate and zinc hydroxide chloride in organic media*

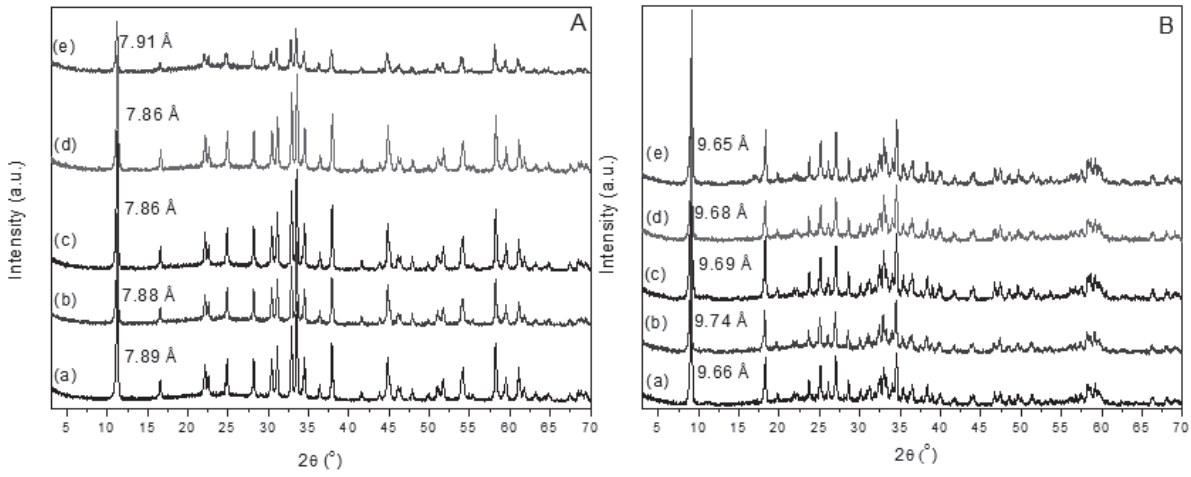

Fig. S 3.1 – X-ray diffractograms (XRD) for A – LipPSZHC and B – LipPSZHN in different organic solvents: methanol (a), ethanol (b), *n*-hexane (c), *n*-heptane (d) and toluene (e).

XRD patterns of the samples obtained after modification with lipase using different solvent are presented in Fig. S1.1 A-B. The X-ray diffractograms present a typical patterns of layered

hydroxide salt compound intercalated with chloride anion (Fig. S1.1-A) and intercalated with nitrate anion (Fig. S1.1-B). It was observed that the basal distance was maintained around 7.88 Å for LipPSZHC and this value is in accordance for the intercalation of chloride anion [1]. The basal distance for the product LipPSZHN was obtained around 9.60 Å, once again proving the intercalation of nitrate anion [2]. Non basal peaks (*002*) and (*021*) were observed in both products. These results show that was not occur some difference in crystallinity and basal stacking peaks when they were incubated with different solvents. Preservation of all reflection peaks after modification with lipase demonstrates that the layer structure maintained and was not occur delamination/ intercalation/ deterioration of the layers [3].

[1] Machado, G. S., Wypych, F., Nakagaki, S. Anionic iron (III) porphyrins mmobilized on zinc hydroxide chloride as catalysts for heterogeneous oxidation reactions. Applied Catalysis A: General 413– 414 (2012) 94– 102.

[2] Majoni, S., Su, S., Hosslopp, J.M. The effect of boron-containing layered hydroxy salt (LHS) on the thermal stability and degradation kinetics of poly (methyl methacrylate). Polymer Degradation and Stability 95 (2010) 1593e1604

[3] Dias, G.S., Bandeira, P.T., Jaerger, S., Piovan, L., Mitchell, D.A., Wypych, F., Krieger, N. Immobilization of *Pseudomonas cepacia* lipase on layered double hydroxide of Zn/Al-Cl for kinetic resolution of rac-1-phenylethanol. Enzyme and Microbial Technology 130 (2019) 109365.

CONCLUSÃO GERAL

Neste trabalho foi estudado o uso dos compostos lamelares sintéticos, hidróxidos duplos lamelares (HDLs) e hidróxidos sais lamelares (HSLs), como suportes para imobilização de lipases visando a aplicação em biocatálise. A enzima modelo escolhida foi a lipase de *Pseudomonas cepacia* (LipPS), resultando em derivados imobilizados com alto desempenho catalítico quando comparados a outros preparados imobilizados descritos na literatura.

O HDL sintetizado na razão molar 4:1 (Zn:Al) foi o mais eficaz para imobilização de LipPS. Após a otimização, a eficiência da imobilização foi de 96% e a retenção de atividade foi de 186% para uma carga de proteína de 162,5 mg g^{-1}. Na transesterificação do *rac*-1-feniletanol com acetato de vinila em *n*-hexano, a preparação imobilizada apresentou alta conversão (50%) em 1 h de reação, com excessos enantioméricos de substratos e produtos superiores a 99% e coeficiente de enantiosseletividade superior a 200. Esse desempenho foi mantido em mais de 30 ciclos de reação.

Os HSLs hidroxinitrato e hidroxicloreto de zinco (HNZ e HCZ, respectivamente) também podem ser utilizados para imobilizar LipPS com alta eficiência de imobilização (> 90%) e retenção de atividade (> 171%), mostrando ativação após imobilização. Entre os HSLs, o melhor suporte foi o HNZ, uma vez que possui uma maior área superficial e maior volume de poros, além de ter um maior potencial Zeta, que indica maior densidade de cargas positivas na superfície do material. Na resolução do álcool (*rac*)-1-feniletanol, obteve-se conversões de 50 e 40% em 2 h~~oras~~ e ee_s de 99 e 73%, para HNZ e HCZ, respectivamente. Embora os HSLs não tenham sido tão eficazes em termos de tempo de reação na resolução cinética do álcool (*rac*)-1-feniletanol, eles aumentam o leque de novos materiais disponíveis para imobilizar lipases.

Na área de novos suportes para imobilização de enzimas, HDLs e HSLs merecem destaque, principalmente pelo baixo custo de produção destes materiais e pela capacidade de imobilizar lipases por adsorção física, via interações eletrostáticas, produzindo materiais nanohíbridos que podem ser utilizados na síntese de compostos quirais de alto valor agregado.

RECOMENDAÇÕES PARA TRABALHOS FUTUROS

Os resultados obtidos neste trabalho abrem perspectivas de utilização dos compostos lamelares, hidróxido duplo lamelar de Zn/Al-Cl e hidróxidos sais lamelares, hidroxinitrato de zinco (HNZ) e hidroxicloreto de zinco (HCZ), para imobilização de lipases visando a aplicação em biocatálise. Portanto, sugerem-se como estudos futuros:

- Sintetizar hidróxidos duplos lamelares e hidróxidos sais lamelares intercalados com ânions orgânicos utilizando, por exemplo, o composto dodecil sulfato de sódio. Isso irá conferir ao suporte características hidrofóbicas e suportes com características hidrofóbicas podem estabilizar lipases, promovendo muitas vezes a ativação da enzima;
- Estudar a modificação de superfície dos compostos lamelares utilizando, por exemplo, do 3-aminopropiltrietoxisilano, um agente de acoplamento de amina, para promover ligações covalentes entre as lipases e o suporte;
- Estudar a imobilização de outras lipases comerciais como, por exemplo, lipases de *Thermomyces lanuginosus* e *Candida antarctica* e não comerciais, como as lipases de metagenômica LipC12 e LipG9, nos compostos lamelares sintetizados (modificados e não modificados) e avaliar os principais parâmetros do processo de imobilização (razão proteína/suporte; eficiência de imobilização; retenção de atividade);
- Estudar a aplicação dos derivados imobilizados em processos ainda mais nobres como, por exemplo, a produção de lactonas e lactamas enantiomericamente puras, que são importantes blocos de construção quirais para a síntese de compostos fungistáticos, bacteriostáticos e citostáticos.
- Estudar a imobilização de lipases nos compostos lamelares por encapsulamento, durante a coprecitpitação dos materiais lamelares e por adição da enzima aos materiais delaminados, para produzir materiais bioinorgânicos com aplicações em biosensores.

REFERÊNCIAS

AGHAEI, H.; GHAVI, M.; HASHEMKHANI G.; KESHAVARZ, M. Utilization of two modified layered doubled hydroxides as supports for immobilization of *Candida rugosa* lipase. **International Journal of Biological Macromolecules**, v. 162, p. 74–83, 2020.

AL-DURI, B.; YONG, Y. P. Characterisation of the equilibrium behaviour of lipase PS (from *Pseudomonas*) and lipolase 1OOL (from *Humicola*) onto Accurel. **Journal of Molecular Catalysis B: Enzymatic**, v. 3, p. 177–188, 1997.

ALI, S. H. H. AL; AL-QUBAISI, M.; HUSSEIN, M. Z.; ZAINAL, Z.; HAKIM, M. N. Preparation of hippurate-zinc layered hydroxide nanohybrid and its synergistic effect with tamoxifen on HepG2 cell lines. **International Journal of Nanomedicine**, v. 6, n. 1, p. 3099–3111, 2011.

ALLOU, N. B.; SAIKIA, P.; BORAH, A.; GOSWAMEE, R. L. Hybrid nanocomposites of layered double hydroxides: an update of their biological applications and future prospects. **Colloid and Polymer Science**, v. 295, n. 5, p. 725–747, 2017.

ALNOCH, R. C.; MARTINI, V. P.; GLOGAUER, A.; COSTA, A. C. D. S.; PIOVAN, L.; MULLER-SANTOS, M.; SOUZA, E. M. DE; PEDROSA, F. D. O.; MITCHELL, D. A.; KRIEGER, N. Immobilization and characterization of a new regioselective and enantioselective lipase obtained from a metagenomic library. **PLoS ONE**, v. 10, n. 2, p. 1–18, 2015.

ARÍZAGA, G. G. C. Intercalation studies of zinc hydroxide chloride: Ammonia and amino acids. **Journal of Solid State Chemistry**, v. 185, p. 150–155, 2012.

ARIZAGA, G. G. C.; SATYANARAYANA, K. G.; WYPYCH, F. Layered hydroxide salts: Synthesis, properties and potential applications. **Solid State Ionics**, v. 178, n. 15–18, p. 1143–1162, 2007.

BARBE, S.; LAFAQUIÈRE, V.; GUIEYSSE, D.; MONSAN, P.; REMAUD-SIMÉON, M.; ANDRÉ, I. Insights into lid movements of *Burkholderia cepacia* lipase inferred from molecular dynamics simulations. **Proteins: Structure, Function and Bioinformatics**, v. 77, n. 3, p. 509–523, 2009.

BASSO, A.; SERBAN, S. Industrial applications of immobilized enzymes — A review. **Molecular Catalysis**, v. 479, n. March, p. 110607, 2019.

BELLEZZA, F.; CIPICIANI, A.; QUOTADAMO, M. A. Immobilization of myoglobin on phosphate and phosphonate grafted-zirconia nanoparticles. **Langmuir**, v. 21, n. 24, p. 11099–11104, 2005.

BI, Y. H.; WANG, Z. Y.; DUAN, Z. Q.; ZHAO, X. J.; CHEN, X. M.; NIE, L. H. An insight into the solvent effect on the positional selectivity of the immobilized lipase from *Burkholderia cepacia* in 1,3-diolein synthesis. **RSC Advances**, v. 5, n. 29, p. 23122–23124, 2015.

BORNSCHEUER, U.; STAMATIS, H.; XENAKIS, A.; YAMANE, T.; KOLISIS, F. N. A comparison of different strategies for lipase-catalyzed synthesis of partial glycerides. **Biotechnology Letters**, v. 16, n. 7, p. 697–702, 1994.

BORNSCHEUER, U. T. Microbial carboxyl esterases: classification, properties and application in biocatalysis. **FEMS Microbiology Reviews**, v. 26, p. 73–81, 2002.

BOSHKOV, N.; PETROV, K.; VITKOVA, S.; RAICHEVSKY, G. Galvanic alloys Zn-Mn - Composition of the corrosion products and their protective ability in sulfate containing medium. **Surface and Coatings Technology**, v. 194, n. 2–3, p. 276–282, 2005.

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

CAO, X.; YANG, J.; SHU, L.; YU, B.; YAN, Y. Improving esterification activity of *Burkholderia cepacia* lipase encapsulated in silica by bioimprinting with substrate analogues. **Process Biochemistry**, v. 44, n. 2, p. 177–182, 2009.

CAVANI, F.; TRIFIRO, F.; VACCARI, A. Hydrotalcite-type anionic clays: preparation, properties and applications. **Catalysis Today**, v. 11, p. 173–301, 1991.

CHANG, Q. L.; LEE, C. H.; PARKIN, K. L. Comparative selectivities of immobilized lipases from *Pseudomonas cepacia* and *Candida antarctica* (fraction B) for esterification reactions with glycerol and glycerol analogues in organic media. **Enzyme and Microbial Technology**, v. 25, n. 3–5, p. 290–297, 1999.

CHEN, C. S.; FUJIMOTO, Y.; GIRDAUKAS, G.; SIH, C. J. Quantitative analysis biochemical kinetic resolution of enantiomer. **Journal of the American Chemical Society**, v. 104, p. 7294–7299, 1982.

COLOMBIÉ, S.; GAUNAND, A.; LINDET, B. Lysozyme inactivation and aggregation in stirred-reactor. **Journal of Molecular Catalysis - B Enzymatic**, v. 11, n. 4–6, p. 559–565, 2001.

CONSTANTINO, V. R. L.; PINNAVAIA, T. J. Basic Properties of Mg2+1−xAl3+x Layered Double Hydroxides Intercalated by Carbonate, Hydroxide, Chloride, and Sulfate Anions. **Inorganic Chemistry**, v. 34, n. 4, p. 883–892, 1995.

CONTEROSITO, E.; GIANOTTI, V.; PALIN, L.; BOCCALERI, E.; VITERBO, D.; MILANESIO, M. Facile preparation methods of hydrotalcite layered materials and their structural characterization by combined techniques. **Inorganica Chimica Acta**, v. 470, p. 36–50, 2018.

CREPALDI, E. L.; VALIM, J. B. Hidróxidos duplos lamelares: síntese, estrutura, propriedades e aplicações. **Química Nova**, v. 21, n. 3, p. 300–311, 1991.

DHAKE, K. P.; DESHMUKH, K. M.; WAGH, Y. S.; SINGHAL, R. S.; BHANAGE, B. M. Investigation of steapsin lipase for kinetic resolution of secondary alcohols and synthesis of valuable acetates in non-aqueous reaction medium. **Journal of Molecular Catalysis B: Enzymatic**, v. 77, p. 15–23, 2012.

DONG, L.; GE, C.; QIN, P.; CHEN, Y.; XU, Q. Immobilization and catalytic properties of *Candida lipolytic* lipase on surface of organic intercalated and modified MgAl-LDHs. **Solid State Sciences**, v. 31, p. 8–15, 2014.

FABER, K. **Biotransformations in Organic Chemistry**. 4. ed. Berlin: Springer, 2000.

FERNÁNDEZ-FERNÁNDEZ, M.; SANROMÁN, M. Á.; MOLDES, D. Recent developments and applications of immobilized laccase. **Biotechnology Advances**, v. 31, n. 8, p. 1808–1825, 2013.

FERNANDEZ-LAFUENTE, R.; ARMISÉN, P.; SABUQUILLO, P.; FERNÁNDEZ-LORENTE, G.; GUISÁN, J. M. Immobilization of lipases by selective adsorption on hydrophobic supports. **Chemistry and Physics of Lipids**, v. 93, n. 1–2, p. 185–197, 1998.

FORANO, C.; VIAL, S.; MOUSTY, C. Nanohybrid enzymes - layered double hydroxides: potential applications. **Current Nanoscience**, v. 2, n. 3, p. 283–294, 2006.

GHANEM, A. Trends in lipase-catalyzed asymmetric access to enantiomerically pure/enriched compounds. **Tetrahedron**, v. 63, n. 8, p. 1721–1754, 2007.

HALMA, M.; WYPYCH, F.; DRECHSEL, S. M.; NAKAGAKI, S. Synthesis, characterization and catalytic behavior of iron porphyrins immobilized in layered double hydroxides. **Journal of Porphyrins and Phthalocyanines**, v. 6, n. 7–8, p. 502–513, 2002.

HANEFELD, U.; GARDOSSI, L.; MAGNER, E. Understanding enzyme immobilisation. **Chemical Society Reviews**, v. 38, n. 2, p. 453–468, 2009.

HARA, P.; HANEFELD, U.; KANERVA, L. T. Sol-gels and cross-linked aggregates of lipase PS from *Burkholderia cepacia* and their application in dry organic solvents. **Journal of Molecular Catalysis B: Enzymatic**, v. 50, n. 2–4, p. 80–86, 2008.

HASAN, F.; SHAH, A. A.; HAMEED, A. Industrial applications of microbial lipases. **Enzyme and Microbial Technology**, v. 39, n. 2, p. 235–251, 2006.

HUSSEIN, M. Z.; ALI, S. H. AL; ZAINAL, Z.; HAKIM, M. N. Development of antiproliferative nanohybrid compound with controlled release property using ellagic acid as the active agent. **International Journal of Nanomedicine**, v. 6, n. 1, p. 1373–1383, 2011.

JAEGER, K.-E.; DIJKSTRA, B. W.; REETZ, M. T. Bacterial Biocatalysts: Molecular Biology, Three-Dimensional Structures, and Biotechnological Applications of Lipases. **Annual Review of Microbiology**, v. 53, n. 1, p. 315–351, 1999.

JAEGER, K. -E; RANSAC, S.; DIJKSTRA, B. W.; COLSON, C.; HEUVEL, M. VAN; ET, O. Bacterial lipases. **FEMS Microbiology Reviews**, v. 15, n. 1, p. 29–63, 1994.

JAEGER, K. E.; REETZ, M. T. Microbial lipases form versatile tools for biotechnology. **Trends in Biotechnology**, v. 16, n. 9, p. 396–403, 1998.

JAERGER, S.; ZIMMERMANN, A.; ZAWADZKI, S. F.; WYPYCH, F.; AMICO, S. C. Zinc layered hydroxide salts: Intercalation and incorporation into low-density polyethylene. **Polimeros**, v. 24, n. 6, p. 673–682, 2014.

JENSEN, R. G.; GALLUZZO, D. R.; BUSH, V. J. Selectivity is an Important Characteristic of Lipases (Acylglycerol Hydrolases). **Biocatalysis**, v. 3, n. 4, p. 307–316, 11 jan. 1990.

JOSEPH, B.; RAMTEKE, P. W.; THOMAS, G. Cold active microbial lipases: Some hot issues and recent developments. **Biotechnology Advances**, v. 26, n. 5, p. 457–470, 2008.

KAMEL, G.; BORDI, F.; CHRONOPOULOU, L.; LUPI, S.; PALOCCI, C.; SENNATO, S.; VERDES, P. V. Adsorption of *Candida rugosa* lipase at water-polymer interface: The case of poly(dl)lactide. **Surface Science**, v. 605, n. 23–24, p. 2017–2024, 2011.

KANG, Y.; HE, J.; GUO, XIAODAN; GUO, XIN; SONG, Z. Influence of pore-diameters on the immobilization of lipase in SBA-15. **Industrial and Engineering Chemistry Research**, v. 46, n. 13, p. 4474–4479, 2007.

KAPOOR, M.; GUPTA, M. N. Lipase promiscuity and its biochemical applications. **Process Biochemistry**, v. 47, n. 4, p. 555–569, 2012.

KAZLAUSKAS, R. J.; WEISSFLOCH, A. N. E.; RAPPAPORT, A. T.; CUCCIA, L. A. A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*. **Journal of Organic Chemistry**, v. 56, n. 8, p. 2656–2665, 1991.

KIM, K. K.; SONG, H. K.; SHIN, D. H.; HWANG, K. Y.; SUH, S. W. Crystallization and preliminary X-ray crystallographic analysis of lipase from *Pseudomonas cepacia*. **Journal of Molecular Biology**, v. 4, n. 227, p. 1258–1262, 1992.

KLIBANOV, A. M. Why are enzymes less active in organic solvents than in water ? **Trends in Biotechnology**, v. 15, n. March, p. 97–101, 1997.

KLIBANOV, A. M. Improving enzymes by using them in organic solvents. **Nature**, v. 409, p. 241–246, 2001.

KNEZEVIC, Z.; SILER-MARINKOVIC, S.; MOJOVIC, L. Immobilized lipases as practical catalysts. **Acta Periodica Technologica**, v. 280, n. 35, p. 151–164, 2004.

KRIEGER, N.; BHATNAGAR, T.; BARATTI, J. C.; BARON, A. M.; LIMA, V. M. DE; MITCHELL, D. Non-Aqueous Biocatalysis in Heterogeneous Solvent Systems Nadia. **Food Technology and Biotechnology**, v. 42, n. 4, p. 1–8, 2004.

KRYSZEWSKI, M. Nanointercalates - novel class of materials with promising properties. **Synthetic Metals**, v. 109, n. 1, p. 47–54, 2000.

LANG, D.; HOFMANN, B.; HAALCK, L.; HECHT, H. J.; SPENER, F.; SCHMID, R. D.; SCHOMBURG, D. Crystal structure of a bacterial lipase from *Chromobacterium viscosum* ATCC 6918 refined at 1.6 Å resolution. **Journal of Molecular Biology**, v. 259, n. 4, p. 704–717, 1996.

LEE, C. H.; PARKIN, K. L. FA selectivity of lipases in acyl-transfer reactions with acetate esters of polyols in organic media. **Journal of the American Oil Chemists' Society**, v. 80, n. 3, p. 231–236, 2003.

LERF, A. Different modes and consequences of electron transfer in intercalation compounds. **Journal of Physics and Chemistry of Solids**, v. 65, n. 2–3, p. 553–563, 2004.

LI, M.; SHAN, H.; ZHOU, L.; YIN, Y.; LI, Z. Novel bioreactor for resolution of (*R,S*)-1-phenylethanol using the functional conducting polymer and ionic liquid with excellent catalytic activity and stability. **Journal of Chemical Technology and Biotechnology**, v. 88, n. 11, p. 2091–2097, 2013.

LIU, J.; BAI, S.; JIN, Q.; ZHONG, H.; LI, C.; YANG, Q. Improved catalytic performance of lipase accommodated in the mesoporous silicas with polymer-modified microenvironment. **Langmuir**, v. 28, n. 25, p. 9788–9796, 2012.

LIU, J.; YANG, Q.; LI, C. Towards efficient chemical synthesis via engineering enzyme catalysis in biomimetic nanoreactors. **Chemical Communications**, v. 51, n. 72, p. 13731–13739, 2015.

LIU, X.; LEI, L.; LI, Y.; ZHU, H.; CUI, Y.; HU, H. Preparation of carriers based on magnetic nanoparticles grafted polymer and immobilization for lipase. **Biochemical Engineering Journal**, v. 56, n. 3, p. 142–149, 2011.

LIU, Y.; GUO, Y. L.; CHEN, D. W.; PENG, C.; YAN, Y. J. Conformation and activity of sol-gels encapsulated cross-linked enzyme aggregates of lipase from *Burkholderia cepacia*. **Advanced Materials Research**, v. 291–294, p. 614–620, 2011.

LOUËR, M.; LOUËR, D.; GRANDJEAN, D. Etude structurale des hydroxynitrates de nickel et de zinc. I. Classification structurale. **Acta Crystallographica**, v. 29, p. 1696–1703, 1973.

LOWRY, R. R.; TINSLEY, I. J. Rapid colorimetric determination of free fatty acids. **Journal of the American Oil Chemists' Society**, v. 53, n. 7, p. 470–472, 1976.

MACHADO, G. S.; ARÍZAGA, G. G. C.; WYPYCH, F.; NAKAGAKI, S. Immobilization of anionic metalloporphyrins on zinc hydroxide nitrate and study of an unusual catalytic activity. **Journal of Catalysis**, v. 274, n. 2, p. 130–141, 2010.

MACHADO, G. S.; WYPYCH, F.; NAKAGAKI, S. Applied Catalysis A: General Anionic iron (III) porphyrins immobilized on zinc hydroxide chloride as catalysts for heterogeneous oxidation reactions. **Applied Catalysis A, General**, v. 413–414, p. 94–102, 2012.

MACHADO, J. P. E.; FREITAS, R. A. DE; WYPYCH, F. Layered clay minerals, synthetic layered double hydroxides and hydroxide salts applied as pickering emulsifiers. **Applied Clay Science**, v. 169, n. October 2018, p. 10–20, 2019.

MAHMOOD, I.; GUO, C.; XIA, H.; JUNHE, M.; JIANGS, Y.; LIU, H. Lipase immobilization on oleic acid-pluronic (L-64) block copolymer coated magnetic nanoparticles, for hydrolysis at the oil/water interface. **Industrial and Engineering Chemistry Research**, v. 47, n. 17, p. 6379–6385, 2008.

MAJONI, S.; SU, S.; HOSSENLOPP, J. M. The effect of boron-containing layered hydroxy salt (LHS) on the thermal stability and degradation kinetics of poly (methyl methacrylate). **Polymer Degradation and Stability**, v. 95, n. 9, p. 1593–1604, 2010.

MANTOVANI, K. M. **Preparação de sólidos por meio da imobilização de metaloporfirinas em hidróxidos duplos lamelares (HDL): estudo sistemático da influência da composição do hdl nos processos de imobilização e na atividade catalítica**. 2015. 95 f. Tese (Doutorado em Química) –: Universidade Federal do Paraná, 2015.

MARANGONI, R.; BUBNIAK, G. A.; CANTÃO, M. P.; ABBATE, M.; SCHREINER, W. H.; WYPYCH, F. Modification of the interlayer surface of layered copper(II) hydroxide acetate with benzoate groups: Submicrometer fiber generation. **Journal of Colloid and Interface Science**, v. 240, n. 1, p. 245–251, 2001.

MARANGONI, R.; MIKOWSKI, A.; WYPYCH, F. Effect of adsorbed/intercalated anionic dyes into the mechanical properties of PVA: Layered zinc hydroxide nitrate nanocomposites. **Journal of Colloid and Interface Science**, v. 351, n. 2, p. 384–391, 2010.

MARANGONI, R.; RAMOS, L. P.; WYPYCH, F. New multifunctional materials obtained by the intercalation of anionic dyes into layered zinc hydroxide nitrate followed by dispersion into poly(vinyl alcohol) (PVA). **Journal of Colloid and Interface Science**, v. 330, n. 2, p. 303–309, 2009.

MARKOV, L.; IONCHEVA, R. Synthesis and thermal decomposition of Cu(II)-Zn(II) hydroxide nitrate mixed crystals. **Materials Chemistry and Physics**, v. 26, p. 493–504, 1990.

MATEO, C.; PALOMO, J. M.; FERNANDEZ-LORENTE, G.; GUISAN, J. M.; FERNANDEZ-LAFUENTE, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. **Enzyme and Microbial Technology**, v. 40, n. 6, p. 1451–1463, 2007.

MELAIS, N.; ARIBI-ZOUIOUECHE, L.; RIANT, O. The effect of the migrating group structure on enantioselectivity in lipase-catalyzed kinetic resolution of 1-phenylethanol. **Comptes Rendus Chimie**, v. 19, n. 8, p. 971–977, 2016.

MENDES, A. A.; GIORDANO, R. C.; GIORDANO, R. D. L. C.; CASTRO, H. F. DE. Immobilization and stabilization of microbial lipases by multipoint covalent attachment on aldehyde-resin affinity: Application of the biocatalysts in biodiesel synthesis. **Journal of Molecular Catalysis B: Enzymatic**, v. 68, n. 1, p. 109–115, 2011.

MEYN, M.; BENEKE, K.; LAGALY, G. Anion-Exchange Reactions of Hydroxy Double Salts. **Inorganic Chemistry**, v. 32, n. 7, p. 1209–1215, 1993.

MOREIRA, A. B. R.; PEREZ, V. H.; ZANIN, G. M.; CASTRO, H. F. DE. Biodiesel synthesis by enzymatic transesterification of palm oil with ethanol using lipases from several sources immobilized on silica-PVA composite. **Energy and Fuels**, v. 21, n. 6, p. 3689–3694, 2007.

MURTY, V. R.; BHAT, J.; MUNISWARAN, P. K. A. Hydrolysis of oils by using immobilized lipase enzyme: A review. **Biotechnology and Bioprocess Engineering**, v. 7, n. 2, p. 57–66, 2002.

NABIPOUR, H.; HOSAINI SADR, M.; THOMAS, N. Synthesis, characterisation and sustained release properties of layered zinc hydroxide intercalated with amoxicillin trihydrate. **Journal of Experimental Nanoscience**, v. 10, n. 16, p. 1269–1284, 2015.

NEWMAN, S. P.; JONES, W. Synthesis , characterization and applications of layered double hydroxides containing organic guests. **New Journal of Chemistry**, p. 105–115, 1998.

NEWMAN, S. P.; JONES, W. Comparative Study of Some Layered Hydroxide Salts Containing Exchangeable Interlayer Anions. **Journal of Solid State Chemistry**, v. 148, n. 1, p. 26–40, 1999.

NGUYEN, L. A.; HE, H.; PHAM-HUY, C. Chiral Drugs: An Overview. **InternatIonal journal of BIomedIcal scIence**, v. 2, n. 2, p. 85–100, 2016.

PALOMO, J. M.; MUOZ, G.; FERNÁNDEZ-LORENTE, G.; MATEO, C.; FERNÁNDEZ-LAFUENTE, R.; GUISÁN, J. M. Interfacial adsorption of lipases on very hydrophobic support (octadecyl-Sepabeads): Immobilization, hyperactivation and stabilization of the open form of lipases. **Journal of Molecular Catalysis B: Enzymatic**, v. 19, n. 20, p. 279–286, 2002.

PENCREAC'H, G.; BARATTI, J. C. Activity of *Pseudomonas cepacia* lipase in organic media is greatly enhanced after immobilization on a polypropylene support. **Applied Microbiology and Biotechnology**, v. 47, n. 6, p. 630–635, 1997.

PETROV, K.; PETKOV, V.; RACHEV, P. X-ray diffraction and electron microscopy studies on the morphology of the Cu0.96CO2.04O4spinel prepared by thermal decomposition of copper (II)-cobalt (II) hydroxonitrates. **Materials Chemistry and Physics**, v. 13, n. 6, p. 577–585, 1985.

PINHEIRO, B. B.; RIOS, N. S.; RODRÍGUEZ AGUADO, E.; FERNANDEZ-LAFUENTE, R.; FREIRE, T. M.; FECHINE, P. B. A.; SANTOS, J. C. S. DOS; GONÇALVES, L. R. B. Chitosan activated with divinyl sulfone: a new heterofunctional support for enzyme immobilization. Application in the immobilization of lipase B from *Candida antarctica*. **International Journal of Biological Macromolecules**, v. 130, p. 798–809, 2019.

POUDEROYEN, G. VAN; EGGERT, T.; JAEGER, K. E.; DIJKSTRA, B. W. The crystal structure of Bacillus subtilis lipase: A minimal α/β hydrolase fold enzyme. **Journal of Molecular Biology**, v. 309, n. 1, p. 215–226, 2001.

RABE, M.; VERDES, D.; SEEGER, S. Understanding protein adsorption phenomena at solid surfaces. **Advances in Colloid and Interface Science**, v. 162, n. 1–2, p. 87–106, 2011.

RAHMAN, MOHD BASYARUDDIN ABDUL; BASRI, M.; HUSSEIN, M. Z.; IDRIS, M. N. H.; RAHMAN, R. N. Z. R. A.; SALLEH, A. B. Immobilisation of lipase from *Candida rugosa* on layered double hydroxides of Mg/Al and its nanocomposite as biocatalyst for the synthesis of ester. **Catalysis Today**, v. 93–95, p. 405–410, 2004.

RAHMAN, M.B.A.; BASRI, M.; HUSSEIN, M. Z.; RAHMAN, R. N. Z. A.; ZAINOL, D. H.; SALLEH, A. B. Immobilization of lipase from Candida rugosa on layered double hydroxides for esterification reaction. **Applied Biochemistry And Biotechnology**, v. 118, p. 313–320, 2004.

RAHMAN, M. B. A.; ZAIDAN, U. H.; BASRI, M.; HUSSEIN, M. Z.; RAHMAN, R. N. Z. R. A.; SALLEH, A. B. Enzymatic synthesis of methyl adipate ester using lipase from *Candida rugosa* immobilised on Mg, Zn and Ni of layered double hydroxides (LDHs). **Journal of Molecular Catalysis B: Enzymatic**, v. 50, n. 1, p. 33–39, 2008.

RIVES, V. **Layered Double Hydroxides: Present and Future**. Huntington: Nova Science Publishers, 2001.

RIVES, V.; ULIBARRI, M. A. Layered double hydroxides (LDH) intercalated with metal coordination compounds and oxometalates. **Coordination Chemistry Reviews**, v. 181, n. 1, p. 61–120, 1999.

ROCHA, M. G. DA; NAKAGAKI, S.; UCOSKI, G. M.; WYPYCH, F.; SIPPEL MACHADO, G. Comparison between catalytic activities of two zinc layered hydroxide salts in brilliant green organic dye bleaching. **Journal of Colloid and Interface Science**, v. 541, p. 425–433, 2019.

SALIS, A.; BHATTACHARYYA, M. S.; MONDUZZI, M.; SOLINAS, V. Role of the support surface on the loading and the activity of *Pseudomonas fluorescens* lipase used for biodiesel synthesis. **Journal of Molecular Catalysis B: Enzymatic**, v. 57, n. 1–4, p. 262–269, 2009.

SALIS, A.; PINNA, M.; MONDUZZI, M.; SOLINAS, V. Comparison among immobilised lipases on macroporous polypropylene toward biodiesel synthesis. **Journal of Molecular Catalysis B: Enzymatic**, v. 54, n. 1–2, p. 19–26, 2008.

SALLEH, A. B.; RAHMAN, R. N. Z. R. A.; BASRI, M. **New lipases and proteases**. New York: Nova Science Publishers, 2006.

SÁNCHEZ, D. A.; TONETTO, G. M.; FERREIRA, M. L. *Burkholderia cepacia* lipase: A versatile catalyst in synthesis reactions. **Biotechnology and Bioengineering**, v. 115, n. 1, p. 6–24, 2018.

SANTOS, J. C. S. D.; BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Importance of the Support Properties for Immobilization or Purification of Enzymes. **ChemCatChem**, v. 7, n. 16, p. 2413–2432, 2015.

SASSO, F.; NATALELLO, A.; CASTOLDI, S.; LOTTI, M.; SANTAMBROGIO, C.; GRANDORI, R. *Burkholderia cepacia* lipase is a promising biocatalyst for biofuel production. **Biotechnology Journal**, v. 11, n. 7, p. 954–960, 2016.

SASSOLAS, A.; BLUM, L. J.; LECA-BOUVIER, B. D. Immobilization strategies to develop enzymatic biosensors. **Biotechnology Advances**, v. 30, n. 3, p. 489–511, 2012.

SCHRAG, J. D. *et al.* The open conformation of a *Pseudomonas* lipase. **Structure**, v. 5, p. 187–202, 1997.

SHELDON, R. A.; PELT, S. VAN. Enzyme immobilisation in biocatalysis: Why, what and how. **Chemical Society Reviews**, v. 42, n. 15, p. 6223–6235, 2013.

SHYLESH, S.; SCHÜNEMANN, V.; THIEL, W. R. Magnetically separable nanocatalysts: Bridges between homogeneous and heterogeneous catalysis. **Angewandte Chemie - International Edition**, v. 49, n. 20, p. 3428–3459, 2010.

SILVA DIAS, G.; BANDEIRA, P. T.; JAERGER, S.; PIOVAN, L.; MITCHELL, D. A.; WYPYCH, F.; KRIEGER, N. Immobilization of Pseudomonas cepacia lipase on layered double hydroxide of Zn/Al-Cl for kinetic resolution of rac-1-phenylethanol. **Enzyme and Microbial Technology**, v. 130, n. March, p. 109365, 2019.

SILVA, M. L. N. DA; MARANGONI, R.; CURSINO, A. C. T.; SCHREINER, W. H.; WYPYCH, F. Colorful and transparent poly(vinyl alcohol) composite films filled with layered zinc hydroxide salts, intercalated with anionic orange azo dyes (methyl orange and orange II). **Materials Chemistry and Physics**, v. 134, n. 1, p. 392–398, 2012.

SMITH, P. K.; KROHN, R. I.; HERMANSON, G. T.; MALLIA, A. K.; GARTNER, F. H.; FROVENZANO, M. D.; FUJIMOTO, E. K.; GOEKE, N. M.; OLSON, B. J.; KLENK, D. C. Measurement of protein using bicinchoninic acid. **Analytical biochemistry**, v. 150, p. 76–85, 1985.

SOARES, C. M. F.; CASTRO, H. F. DE; MORAES, F. F. DE; ZANIN, G. M. Characterization and Utilization of *Candida rugosa* Lipase Immobilized on Controlled Pore Silica. **Applied Biochemistry and Biotechnology**, v. 79, n. 1–3, p. 745–758, 1999.

SOUZA, R. O. M. A. DE; ANTUNES, O. A. C.; KROUTIL, W.; KAPPE, C. O. Kinetic resolution of *rac*-1-phenylethanol with immobilized lipases: A critical comparison of microwave and conventional heating protocols. **Journal of Organic Chemistry**, v. 74, n. 16, p. 6157–6162, 2009.

STÄHLIN, W.; OSWALD, H. R. The topotactic reaction of zinc hydroxide nitrate with aqueous metal chloride solutions. **Journal of Solid State Chemistry**, v. 3, n. 2, p. 256–264, 1971.

SUAN, C.; SARMIDI, M. R. Immobilised lipase-catalysed resolution of (*R,S*)-1-phenylethanol in recirculated packed bed reactor. **Journal of Molecular Catalysis B: Enzymatic**, v. 28, p. 111–119, 2004.

SUGIHARA, A.; UESHIMA, M.; SHIMADA, Y.; TSUNASAWA, S.; TOMINAGA, Y. Purification and characterization of a novel thermostable Lipase from *Pseudomonas cepacia*. **Journal of Biochemistry**, v. 603, p. 598–603, 1992.

TALBERT, J. N.; GODDARD, J. M. Enzymes on material surfaces. **Colloids and Surfaces B: Biointerfaces**, v. 93, p. 8–19, 2012.

TAVARES, S. R.; VAISS, V. S.; WYPYCH, F.; LEITÃO, A. A. Similarities between zinc hydroxide chloride monohydrate and its dehydrated form: A theoretical study of their structures and anionic exchange properties. **Journal of Physical Chemistry C**, v. 118, n. 33, p. 19106–19113, 2014.

TAVIOT-GUÉHO, C.; PRÉVOT, V.; FORANO, C.; RENAUDIN, G.; MOUSTY, C.; LEROUX, F. Tailoring hybrid layered double hydroxides for the development of innovative applications. **Advanced Functional Materials**, v. 28, n. 27, p. 1–33, 2018.

THEISS, F. L.; AYOKO, G. A.; FROST, R. L. Synthesis of layered double hydroxides containing Mg2+, Zn2+, Ca2+and Al3+layer cations by co-precipitation methods - A review. **Applied Surface Science**, v. 383, p. 200–213, 2016.

TONG, Z.; SHICHI, T.; TAKAGI, K. Oxidation catalysis of a manganese(III)porphyrin intercalated in layered double hydroxide clays. **Materials Letters**, v. 57, n. 15, p. 2258–2261, 2003.

TONGBORIBOON, K.; CHEIRSILP, B.; H-KITTIKUN, A. Mixed lipases for efficient enzymatic synthesis of biodiesel from used palm oil and ethanol in a solvent-free system. **Journal of Molecular Catalysis B: Enzymatic**, v. 67, n. 1–2, p. 52–59, 2010.

VELAZQUEZ-CARRILES, C.; MACIAS-RODRÍGUEZ, M. E.; CARBAJAL-ARIZAGA, G. G.; SILVA-JARA, J.; ANGULO, C.; REYES-BECERRIL, M. Immobilizing yeast β-glucan on zinc-layered hydroxide nanoparticle improves innate immune response in fish leukocytes. **Fish and Shellfish Immunology**, v. 82, n. August, p. 504–513, 2018.

VILLENEUVE, P.; MUDERHWA, J. M.; GRAILLE, J.; HAAS, M. J. Customizing lipases for biocatalysis: A survey of chemical, physical and molecular biological approaches. **Journal of Molecular Catalysis - B Enzymatic**, v. 9, n. 4–6, p. 113–148, 2000.

WANG, X.; LIU, X.; ZHAO, C.; DING, Y.; XU, P. Biodiesel production in packed-bed reactors using lipase-nanoparticle biocomposite. **Bioresource Technology**, v. 102, n. 10, p. 6352–6355, 2011.

XUE, M.; CHITRAKAR, R.; SAKANE, K.; OOI, K.; KOBAYASHI, S.; OHNISHI, M.; DOI, A. Synthesis of ktenasite, a double hydroxide of zinc and copper, and its intercalation reaction. **Journal of Solid State Chemistry**, v. 177, n. 4–5, p. 1624–1630, 2004.