UNIVERSIDADE TIRADENTES – UNIT

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE PROCESSOS - PEP

# SISTEMAS AQUOSOS BIFÁSICOS FORMADOS POR CONSTITUINTES NÃO CONVENCIONAIS PARA PURIFICAÇÃO DE ENZIMAS LIPOLÍTICAS

ARACAJU, SE - BRASIL

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Ranyere Lucena de Souza

TESE SUBMETIDA AO PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE PROCESSOS DA UNIVERSIDADE TIRADENTES COMO PARTE DOS REQUISITOS NECESSÁRIOS PARA A OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA DE PROCESSOS

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*"Não desperdice pensamentos puros com enzimas sujas"*

Efraim Racke

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Resumo da Tese apresentada ao Programa de Pós-graduação em Engenharia de Processos da Universidade Tiradentes como parte dos requisitos necessários para a obtenção do grau de Doutor em Engenharia de Processos.

SISTEMAS AQUOSOS BIFÁSICOS FORMADOS POR CONSTITUINTES NÃO CONVENCIONAIS PARA PURIFICAÇÃO DE ENZIMAS LIPOLÍTICAS

As lipases têm atraído uma especial atenção por catalisar com extrema eficiência e especificidade diversas reações de interesse industrial, entretanto a comercialização e a produção em escala industrial destas enzimas dependem das técnicas empregadas na purificação. Sistemas aquosos bifásicos (SABs) é um método de purificação eficiente, importante para o desenvolvimento de processos de separação sustentáveis, "biocompatível", permitindo enzimas com níveis elevados de purificação. Este trabalho apresenta três abordagens que incluem a formação de SABs à base de: solvente orgânico (tetrahidrofurano – THF) + sal; THF + líquidos iônicos (ou sais) à base de colinas; polietilenoglicol (PEG) + sal + líquido iônico (IL) como adjuvante (5 %, m/m). Todos estes sistemas foram aplicados para a purificação da lipase extracelular de *Bacillus* sp. ITP-001, produzida por fermentação submersa. Dois diferentes corantes (rodamina 3G e ácido cloranílico) e outras lipases (*Candida antarctica* B e *Burkholderia cepacia*) foram utilizadas como modelo para a purificação da lipase extracelular ou para a compreensão de alguns efeitos nos diagramas de fases. Após os estudos de otimização, os resultados demonstram que a lipase de *Bacillus* sp. ITP-001 foi purificada ≈ 104 vezes utilizando o SAB à base de THF + tampão fosfato, ao passo que o SAB formado por THF + bitartarato de colina e SAB à base PEG 1500 + tampão fosfato + LI $[C_6mim]Cl$ como adjuvante, a purificação foi de ≈ 130 e 245 (vezes), respectivamente. Os sistemas utilizando líquido como adjuvante foi selecionado como o mais eficaz para a purificação da lipase de *Bacillus* sp. ITP-001 devido as capacidades de interações adicionais. Além disso, os resultados sugerem que estes sistemas podem ser aplicados para a purificação de diferentes lipases, não descartando a aplicação dos sistemas à base de THF ou sais de colinas, pois estes apresentaram boa capacidade para purificação, biocompatibilidade para as lipases estudadas, e são formados por constituintes considerados de baixo custo e de baixa viscosidade.

**Palavras-chave:** lipase, sistemas aquosos bifásicos, purificação, solvente orgânico, líquidos iônicos, colinas.

Abstract of the thesis presented to the Post-graduation Program in Process Engineering of Tiradentes University as part of the requirements for the Doctorate degree in Engineering Processes

AQUEOUS TWO-PHASE SYSTEMS FORMED BY UNCONVENTIONAL CONSTITUENTS FOR PURIFICATION OF LIPOLYTIC ENZYMES

Lipases have attracted special attention for catalyzing with extreme efficiency and specificity various reactions of industrial interest, however the marketing and industrial scale production of these enzymes depend on the techniques used for purification. Aqueous two-phase systems (ATPS) are an efficient purification method, relevant for the development of environmentally friendly and "biocompatible" separation processes, allowing enzymes with high levels of purity. This work presents three strategies that includ the formation of ATPS based on: organic solvent (tetrahydrofuran - THF) + salt; THF + cholinium-based IL; polyethylene glycol (PEG) + salt + IL as adjuvant (5% wt). All of these systems were applied to the purification of extracellular lipase from *Bacillus* sp. PTI-001, produced by submerged fermentation. Two different dyes (rhodamine 3G and chloranilic acid) and other lipases (*Candida antarctica* B and *Burkholderia cepacia*) were used as models for purification of the extracellular lipase or to the understanding some effects on the phase diagrams. After the optimization studies, the results demonstrate that the lipase from *Bacillus* sp. ITP-001, was purified ≈ 104-fold using the system based on THF + $K_2HPO_4$/ $KH_2PO_4$, whereas the ATPS formed by THF + [Ch][Bit] and $KH_2PO_4$ + PEG 1500 + $K_2HPO_4$/$KH_2PO_4$ + [$C_6$mim]Cl as adjuvant leads to a prurification factor of 130 and 245 (fold), respectively. The use of imidazolium-based ILs as adjuvants in ATPS was selected as the most effective for purification of lipase from Bacillus sp. PTI-001 due on the capabilities of additional interactions. Furthermore, the results suggest that these systems may be applied for purification of different lipases, not discarding the use of systems based on cholinium salts or THF, as these offer a good capacity for purification, biocompatibility for lipases studied and are formed of constituents considered to be of low cost and low viscosity.

**Keywords:** lipase, aqueous biphasic systems, purification, organic solvent, ionic liquids, cholinium.

# *SUMÁRIO*

# *LISTA DE FIGURAS*

# *LISTA DE TABELAS*

# *LISTA DE ABREVIATURAS*

| | |
|---|---|
| [mim]Cl | Cloreto de 1-metilimidazólio |
| $[im]^+$ | Íon imidazólio |
| $[C_2mim][C_2SO_4]$ | Etil-sulfato de 1-etil-3-metilimidazólio |
| $[C_2mim][C_4SO_4]$ | Bultil-sulfato de 1-etil-3-metilimidazólio |
| $[C_2mim]Cl$ | Cloreto de 1-etil-3-metilimidazólio |
| $[C_2mim][CH_3CO_2]$ | Acetato de 1-etil-3-metilimidazólio |
| $[C_2mim][N(CN)_2]$ | Dicianamida de1-etil-3-metilimidazólio |
| $[C_4mim][BF_4]$ | Tetrafluoroborato de 1-butil-3-metilimidazólio |
| $[C_4mim][CH_3CO_2]$ | Acetato de 1-butil-3-metilimidazólio |
| $[C_4mim][CF_3SO_3]$ | Trifluorometanosulfonato de 1-butil-3-metil-imidazólio |
| $[C_4mim]Cl$ | Cloreto de 1-butil-3-metilimidazólio |
| $[C_4mim][N(CN)_2]$ | Dicianamida de1-butil-3-metilimidazólio |
| $[C_4mim][TOS]$ | Tosilato de 1-butil-3-metilimidazólio |
| $[C_4mpip][BF_4]$ | Tetrafluoroborato de 1-butil-3-metilpiperidínio |
| $[C_4mpip]Cl$, | Cloreto de 1-butil-3-metilpiperidínio |
| $[C_4mpy]Cl$, | Cloreto de 1-butil-3-metilpiridínio |
| $[C_4mpyr]Cl$, | Cloreto de 1-butil-3-metilpirrolidínio |
| $[C_6mim]Cl$ | Cloreto de 1-hexil-3-metilimidazólio |
| $[C_8mim]Br$ | Brometo de 1-octil-3-metilimidazólio |
| $[C_8mim]Cl$ | Cloreto de 1-octil-3-metilimidazólio |
| $[C_8py][N(CN)_2]$ | Dicianamida de1-octil-3-piridínio |
| $[OHC_2mim]Cl$ | Cloreto de 1-hidroxietil-3-metilimidazólio |
| [BCh]Cl | Cloreto de benzildimetil(2-hidroxietil)amônio |
| $[Ch]^+$ | Íon colina |
| [Ch][Bic] | Bicarbonato de colina |
| [Ch][Bit] | Bitartarato de colina |
| [Ch][But] | Butirato de colina |
| [Ch]Cl | Cloreto de colina |
| [Ch][DHcit] | Dihidrogênio citrato de colina |
| [Ch][DHph] | Dihidrogênio fosfato de colina |
| [Ch][Lac] | Lactato de colina |
| [Ch][Lev] | Levulínato de colina |
| [Ch][Gly] | Glicolato de colina |
| [Ch][Glu] | Glutarato de colina |
| [Ch][Sal] | Salicilato de colina |
| [Ch][Suc] | Succinato de colina |
| $[Ac]^-$ | Íon acetato |

| | |
|---|---|
| $[Br^-]$ | Íon brometo |
| $[But]^-$ | Íon butirato |
| $[Ca_2]^+$ | Íon cálcio |
| $[Cit]^-$ | Íond citrato |
| $[Cl]^-$ | Íon cloreto |
| $[ClO_4]^-$ | Ìon perclorato |
| $[CO_3]^{2-}$ | Íon carbonato |
| $[F]^-$ | Íon floreto |
| $[H_2PO_4]^-$ | Íon dihidrogenofsofato |
| $[HPO_4]^-$ | Íon hidrogenofosfato |
| $[I]^-$ | Íon iodeto |
| $[K]^+$ | Íon fosfato |
| $[Lac]^-$ | Lactato |
| $[Li]^+$ | Ión lítio |
| $[Na]^+$ | Ìon sódio |
| $[Mg_2]^+$ | Íon magnésio |
| $[NH_4]^+$ | Íond amônio |
| $[NO_3]^-$ | Íon nitrato |
| $[Oxe]^-$ | Íon oxilatao |
| $[Pro]^-$ | Íon propionato |
| $[S_2O_3]^{2-}$ | Íon tiossulfato |
| $[SCN]^-$ | Íon tiocianato |
| $[SO_4]^{2-}$ | Íon sulfato |
| $(NH_4)_2SO_4$ | Sulfato de amônio |
| $C_6H_5K_3O_7/C_6H_8O_7$ | Tampão citrato |
| HEPES | Ácido (N-(2-hidroxietil)piperazina-N`-2-etanossulfônico) |
| $K_2HPO_4/KH_2PO_4$ | Tampão fosfato de potássio |
| $K_2CO_3$ | Carbonato de potássio |
| NaCl | Cloreto de sódio |
| $NaH_2PO_4.2H_2O$ | Fosfato monossódico |
| A, B, C, D | Constante de correlação das equações empíricas |
| ABS/ATPS/SAB | Sistema aquoso bifásico |
| *BCL* | Lipase de *Burkholderia cepacia* |
| BSA | Soro de albumina bovina |
| $C^0_p$ | Calor específico a pressão constante |
| $C_{T\ ou\ B}$ | Concentração na fase de topo ou fundo ($g.L^{-1}$ e/ou mg/100g) |
| CA | Ácido cloranílico |
| CaLA | Lipase A de *Candida antárctica* |
| CaLB | Lipase B de *Candida antárctica* |
| DMSO | Dimetilsulfóxido ou sulfóxido de dimetilo |

| | |
|---|---|
| *EE* | Eficiência de extração (%) |
| EO | Polietilenoglicol-polipropilenoglicol |
| $G^0_m$ | Energia livre de Gibbs ($kJ.mol^{-1}$) |
| $H^0_m$ | Entalpia molar ($kJ.mol^{-1}$) |
| *K* | Coeficiente de partição (admensional) |
| $K_{ow}$ | Coeficiente de partição octanol-água |
| LI (IL) | Líquido iônico |
| LIs (ILs) | Líquidos iônicos |
| *Ln* | Logarítmo neperiano |
| *P* | Pressão |
| PEG | Polietilenoglicol |
| $pK_a$ | Constante de dissociação ácida |
| PPG | Polipropilenoglicol |
| PPL | Lipase pancreática suína |
| *R* | Constante universal dos gases ($8{,}31434\ J.K^{-1}.mol^{-1}$) |
| RT ou B | Recuperação na fase de topo ou fundo |
| TEG | Tetraetilenoglicol |
| TL | Linha de amarração |
| TLL | Comprimento da linha de amarração |
| *T* | Temperatura (ºC ou K) |
| $V_T$ | Volume de topo (mL) |
| $V_F$ | Volume de fundo (mL) |
| *X* | Porcentagem mássica |
| *Y* | Porcentagem mássica |

## ESTRUTURA DA TESE

A abordagem da tese cujo o título é S*ISTEMA AQUOSO BIFÁSICO FORMADO POR CONSTITUINTES NÃO CONVENCIONAIS PARA A PURIFICAÇÃO DE ENZIMAS LIPOLÍTICAS*, será dividida em 4 capítulos. Os dois primeiros são apresentados a introdução, objetivo da pesquisa e revisão bibliográfica.

No capítulo seguinte são apresentados os resultados referente aos 3 anos de pesquisa associados à separação e/ou purificação de enzimas lipolíticas. Os dados obtidos da pesquisa foram/serão publicados em revista científicas internacionais. Assim, no capítulo III é apresentado cada artigo que foi/será submetido aos periódicos, adaptados de acordo com as normas de publicação das revistas, sendo os materiais de apoio e as referências bibliográficas adicionadas ao final dos respectivos artigos.

No último capítulo (capítulo IV) são apresentadas as considerações finais, assim como sugestões de trabalhos futuros.

# Capítulo I

## INTRODUÇÃO

As enzimas são uma classe de moléculas sintetizadas pelas células, aptas a catalisar as biotransformações que ocorrem nos organismos vivos. Os benefícios associados ao uso de enzimas em reações de interesse industrial incluem maior qualidade do produto gerado, menor desperdício e reduzido consumo de energia, sendo ainda considerados como "catalisadores ecologicamente corretos", atendendo aos princípios da química verde - *green chemistry*. (CABRAL *et al*., 2003; BINOD *et al*., 2013) De todas as enzimas conhecidas, as lipases (glicerol éster hidrolases, E.C. 3.1.1.3) têm atraído mais atenção para as aplicações industriais, em grande parte devido a sua disponibilidade, estabilidade, grande poder catalítico e por serem altamente específicas (BON *et al*., 2005; LI *et al*., 2014).

Para satisfazer a demanda por lipases, várias são as técnicas de purificação empregadas, dentre as quais podem ser citadas a precipitação por sais, por solventes orgânicos, ultrafiltração, eletroforese, cromatografia, entre outras (SAXENA *et al*., 2003b). Desenvolver técnicas de purificação eficientes e econômicas é uma das preocupações atuais na área de engenharia de processos com o objetivo de viabilizar a produção em escala industrial.

A extração líquido-líquido por meio dos sistemas aquosos bifásicos (SABs) foi orginalmente proposta por Albertson em 1958 (ALBERTSON, 1958). Estes sistemas, sendo estudados há mais de 50 anos, apresentam vantagens como elevada biocompatibilidade com os solutos, baixo custo, processamento rápido e que permitem enzimas com níveis elevados de pureza. Além disso, o elevado conteúdo de água nas fases permite a partição de biomoléculas de diversas origens em condições não-desnaturantes. Tradicionalmente os SABs são compostos de duas fases aquosas imiscíveis, que coexistem em equilíbrio promovido pela adição de sólidos solúveis em água, que podem ser dois polímeros, um polímero e um sal, ou dois sais (ALBERTSON, 1990; ASENJO *et al*., 2012). Por outro lado, uma questão crítica associada aos SABs formados por polímeros e sais é a limitada faixa de polaridade de suas fases coexistentes, e a elevada viscosidade da fase rica em polímero (COLLINS, 1997; SELBER et al., 2001; ESPITIA-SALOMA et al., 2014). Por vezes estes fatores são importantes quando o objetivo é a extração de biomoléculas hidrofóbicas e a ampliação do processo para escala industrial (LI et al., 2005a; DREYER et al., 2008; NEVES et al., 2009; VENTURA et al., 2009).

Algumas abordagens foram desenvolvidas para superar essas limitações, tais como SABs não convencionais formados por diferentes pares de solutos, como os carboidratos, líquidos iônicos (LIs) e solventes orgânicos (ROGERS *et al*., 2003; OOI et al., 2009b). A extração utilizando SABs à base de solventes orgânicos hidrofílicos é uma alternativa vantajosa para os processos de extração de biomoléculas. Suas vantagens incluem a rápida separação de fases, alta eficiência de extração, baixa viscosidade, alta polaridade, baixo custo e fácil reciclagem (OOI et al., 2009b; LI et al., 2011). Porém é importante ressaltar que para o desenvolvimento de um processo eficiente de extração, é necessário encontrar uma combinação adequada entre o solvente orgânico e a biomolécula alvo, uma vez que estes compostos orgânicos podem causar a desnaturação das proteínas.

SABs alternativos a base de LIs hidrofílicos também têm sido propostos para extração de diferentes biomoléculas (FREIRE *et al*., 2012). Ao contrário dos comuns SABs de polímeros e sais, eles não sofrem de alta viscosidade e exibem uma faixa muito mais ampla de polaridade. Uma das principais vantagens da aplicação de LIs em SABs é a possibilidade de manipular suas propriedades físico-químicas (ROGERS *et al*., 2003; FREIRE *et al*., 2012). Por outro lado, utilizando LIs conjugados com diferentes sais inorgânicos ou orgânicos, ou ainda com polímeros, para promover a formação de SAB, normalmente requer a utilização de grandes concentrações de sais ou polímeros e de LIs, tornando o processo de extração mais caro e menos sustentável. Além disso, a maioria dos trabalhos abordam o uso de LIs que apresentam várias limitações no que diz respeito à sua estabilidade térmica e química, alto custo, toxicidade e biodegradabilidade.

A utilização de uma classe de LIs à base de colinas tem sido considerada para a promoção de SABs por compartilhar as excepcionais propriedades dos líquidos iônicos tradicionais, porém apresentam condições apropriadas de sustentabilidade, como baixo custo, baixa toxidade, biocompatibilidade e ainda excelente capacidade de biodegradação (ZASLAVSKY, 1995; RUIZ-RUIZ et al., 2012). Entretanto, ainda são pouco explorados, uma vez que apenas algumas biomoléculas, tais como proteínas, enzimas e fármacos, foram avaliadas em SABs contendo LIs à base de colinas. Uma nova abordagem que tem ganhado destaque é a utilização de LIs tradicionais como adjuvantes em SABs. Neste sentido, uma pequena quantidade de LI (5 %, m/m) é utilizada, assim, reduzindo a necessidade de elevadas concentrações de LIs para promover duas fases aquosas, como ocorre em típicos sistemas à base de IL + sal ou polímero. Além disto, interações adicionais do tipo eletrostáticas, forças de van der Waals e pontes de

hidrogênio desempenham um papel significativo na migração das biomoléculas, conduzindo aos processos de separação mais vantajosos (PEREIRA *et al*., 2010; ALMEIDA et al., 2014).

Portanto, desenvolver processos de extração utilizando sistemas aquosos bifásicos mais eficazes e sustentáveis aplicados para a purificação ou separação de enzimas lipolíticas, ou outras moléculas, ainda é uma questão relevante para o uso desta tecnologia em escala industrial. Para este propósito, nesta tese foram estudados diferentes SABs: sistemas à base de solvente orgânico; sistemas formados por LI à base de colina; e utilizando LI como adjuvante em sistemas comuns polímeros/sal. Este estudo completa uma série de trabalhos desenvolvidos por nosso grupo de pesquisa, envolvendo a aplicação de SABs para a purificação da lipase extracelular de *Bacillus* sp. ITP-001 produzida por fermentação submersa.

# OBJETIVOS

Dentro do contexto referido na seção anterior, o trabalho apresentado nesta tese teve como objetivo geral a separação e/ou purificação de enzimas lipolíticas utilizando sistemas aquosos bifásicos formados por constituintes não convencionais. Para que o objetivo principal fosse conseguido, foram estabelecidos 3 objetivos específicos:

- Formar sistemas aquosos bifásicos:

  Construir diagramas de fases formados por uma série de compostos, incluindo os polímeros (polietilenoglicol, PEG 1500, PEG 4000, PEG 6000 e PEG 8000 g/mol), sais de potássio ($K_3PO_4$, $K_2HPO_4$ e o $K_2HPO_4/KH_2PO_4$ tampão de fosfato de potássio), solvente orgânico (tetrahidrofurano – THF), líquidos iônicos à base de colinas (cloreto de colina – [Ch]Cl, bitartarato de colina – [Ch][Bit] e dihidrogênio citrato de colina – [Ch][DHCit]) e líquidos iônicos à base de imidazólios utilizados como adjuvantes (cloreto de 1-etil-3-metilimidazólio – [$C_2$mim]Cl, cloreto de 1-butil-3-metilimidazólio – [$C_4$mim]Cl, cloreto de 1-hexil-3-metilimidazólio – [$C_6$mim]Cl e cloreto de 1-octil-3-metilimidazólio – [$C_8$mim]Cl) a 5 % m/m). Assim, vários parâmetros foram investigados em termos do seu efeito sobre as características de cada sistema, tais como curva binodal, linha de amarração (TL), comprimento da linha de amarração (TLL) e ponto crítico ($P_C$).

- Explorar a aplicação destes novos sistemas:

  Determinar a partição dos líquidos iônicos, quando utilizados como adjuvantes. Verificar a capacidade dos sistemas em extrair compostos com diferentes cargas elétricas, utilizando dois corantes (Rodamina 6G – R6G e Ácido cloranílico – CA).

- Partição e purificação das lipases:

  Determinar os parâmetros de extração (partição, eficiência de extração, seletividade) e os parâmetros termodinâmicos (entalpia, entropia e energia livre de *Gibbs*), para as lipases utilizadas como modelo – lipase B de *Candida antarctica* e lipase de *Burkholderia cepacia*.

  Purificar a lipase extracelular produzida a partir de um *Bacillus* sp. ITP-001 utilizando estes novos sistemas aquosos bifásicos.

# Capítulo II

## REVISÃO BIBLIOGRÁFICA

Nesta revisão bibliográfica será dado um breve enfoque aos principais temas relacionados com o trabalho desenvolvido, iniciando-se por conceituação de biocatálise, enzimas e suas aplicações industriais, lipases, métodos de purificação e sistemas aquosos bifásicos.

### 2.1. Enzimas e biocatálise

As enzimas são proteínas (com exceção das ribozimas, que não são de natureza protéica) que catalisam com grande eficiência reações químicas que ocorrem nos organismos e em células. Elas desempenham um importante papel funcional em alimentos, medicamentos, assim como em sistemas biológicos. São responsáveis pelo metabolismo, ou seja, pelas reações bioquímicas que resultam no crescimento e desenvolvimento das células. Especialmente impulsionado pelo avanço da biotecnologia nas áreas de genética e de engenharia de proteínas, abriu-se uma nova era de aplicações de enzimas em muitos processos industriais, resultando não somente no desempenho dos vários processos existentes, mas no desenvolvimento de uma série de novos produtos (ROBERTS *et al.,* 1995; HOUDE *et al.,* 2004). Atualmente cerca de 5400 enzimas são listadas na base de dados da *Enzyme Nomenclature Database,* mas apenas 150 a 170 são utilizadas em escala industrial (BINOD *et al.*, 2013).

Estes biocatalizadores são altamente específicos e apresentam grande poder catalítico em sistemas *in vivo* e *in vitro* (WISEMAN, 1995). Estruturalmente, as enzimas possuem todas as características das proteínas, possuindo zonas da sua estrutura responsáveis pela catálise. A região reativa da enzima é denominada centro ativo (ou sítio ativo) e contém os radicais de aminoácidos, conhecidos como grupamentos catalíticos, os quais são responsáveis pela formação e a quebra das ligações. Os reagentes que participam das reações chamam-se substratos, e cada enzima possui um de caráter específico, isto é, atua sobre o substrato e converte-o ao produto (FORGATY, 1990; CABRAL *et al.,* 2003). A velocidade desta reação corresponde à conversão de um certo número de moles de substrato por unidade de tempo. Neste aspecto particular, as enzimas diminuem a energia de ativação durante uma reação, propiciando menor tempo de reação (CABRAL *et al.,* 2003). Uma parte significante dessa energia usada para aumentar a velocidade enzimática é derivada das interações de pontes de

hidrogênio, interações iônicas, forças dispersivas e interações de van der Waals que ocorrem entre o substrato e a enzima (NELSON *et al.,* 2011).

Os biocatalisadores enzimáticos são também eficientes do ponto de vista energético por atuar em condições de temperatura, pressão e pH moderadas (CABRAL *et al.,* 2003). Além disso, a função catalítica de cada enzima é caracterizada por especificações únicas para cada reação, associada aos seus congêneres químicos, dentre as quais podem ser citadas a regiosseletividade, enantiosseletividade, e a quimiosseletividade (JAEGER *et al.,* 2004).

As enzimas podem ser de origem microbiana, vegetal ou animal, obtidas por meio de processos fermentativos ou por trituração de tecidos vegetais e animais (FORGATY, 1990; FUCINOS *et al.,* 2005). Enzimas microbianas podem ser extracelulares dispersas no meio de cultivo, ou intracelulares localizadas no interior celular e portanto obtidas por meio da ruptura da célula (FORGATY, 1990).

Atualmente são conhecidas milhares de enzimas diferentes e não é viável o uso exclusivo de nomes triviais para a sua identificação. Em 1961, a Comissão para Enzimas (*Enzyme Comission* – EC) da União Internacional de Bioquímica (IUB) normatizou uma classificação e nomenclatura de enzimas e de coenzimas (CABRAL *et al.,* 2003). A EC classificou as enzimas por meio das suas propriedades catalíticas, podendo ser: oxidorredutases, transferases, hidrolases, liases, isomerases e ligases, como mostra a Tabela 1. Essas classes são posteriormente divididas em subclasses para especificar o tipo de reação e a natureza química dos reagentes.

Tabela 1: Classificação internacional de enzimas (NELSON e COX, 2011).

| **Nº** | **Classe** | **Tipo de reação catalisada** |
|---|---|---|
| 1 | Oxidorredutases | Transferência de elétrons (íons hidretos ou átomos de H) |
| 2 | Transferases | Reações de transferência de grupos |
| 3 | Hidrolases | Reações de hidrólise (transferência de grupos funcionais da água) |
| 4 | Liases | Adição de grupos a ligações duplas, ou formação de duplas ligações pela remoção de grupos |
| 5 | Isomerases | Transferência de grupos dentro de moléculas para produzir formas isoméricas |
| 6 | Ligases | Formação de ligações C–C, C–S, C–O e C–N |

Os benefícios associados ao uso das enzimas em reações de interesse industrial incluem maior qualidade do produto gerado, menor custo de produção, menos desperdício e reduzido

consumo de energia, sendo ainda considerados como "catalisadores ecologicamente corretos", atendendo aos princípios da química verde - *green chemistry* (JAEGER *et al.,* 2002; LOZANO *et al.,* 2010; PEREZ *et al.,* 2010; BINOD *et al.,* 2013).

Dentre as inúmeras enzimas disponíveis, as lipases são consideradas o terceiro maior grupo de enzimas em volume de vendas, ficando atrás apenas das proteases e carbo-hidrolases (BON *et al.,* 2008). Por seu enorme potencial em catalisar reações de interesse industrial, elas foram selecionadas para a realização dos estudos de extração e/ou purificação aqui propostos. No próximo tópico será descrito o papel das lipases no cenário biotecnológico e suas funções catalíticas.

### 2.1.1. Enzimas lipolíticas

As enzimas lipolíticas ou lipases (EC 3.1.1.3) são hidrolases que atuam na interface orgânico-aquosa, catalisando a hidrólise de ligações éster-carboxílicas e liberando ácidos e álcoois orgânicos (HASAN *et al.,* 2006; BON *et al.,* 2008). Contudo, ao contrário de muitas outras enzimas, as lipases apresentam níveis consideráveis de atividade e estabilidade em ambientes não-aquosos, facilitando a catálise de muitas reações de interesse industrial (HASAN *et al.,* 2006; BON *et al.,* 2008).

As lipases são obtidas na natureza e produzidas por diversas plantas, animais e micro-organismos, mas apenas lipases microbianas são industrialmente aplicadas, pois apresentam grande diversidade de propriedades e especificidade de substrato (RAMAKRISHNAN *et al.,* 2013). As lipases obtidas a partir de animais, principalmente aquelas oriundas de tecido estomacal de bovinos ou cordeiros, ou de tecidos pancreáticos de suínos, apresentam desvantagens quanto à sua utilização devido à presença de tripsina, resultando em aminoácidos com sabor amargo, além da presença de hormônios residuais dos animais (LOTTI *et al.,* 1994; VAKHLU *et al.,* 2006; TREICHEL *et al.,* 2010). Lipases de plantas também estão disponíveis, mas não são exploradas comercialmente em função dos baixos rendimentos de catálise e dos processos envolvidos para extração (SOUISSI *et al.,* 2009; FABISZEWSKA *et al.,* 2014). Assim, lipases microbianas recebem atualmente mais atenção por causa de suas vantagens técnico-econômicas, apresentando relativamente baixo custo de produção, elevado rendimento de conversão de substrato em produto, grande versatilidade em adaptar-se às condições ambientais e facilidade de manipulação genética (SHU *et al.,* 2010).

De uma forma geral, estas enzimas têm aplicações versáteis decorrentes de suas propriedades. Em condições naturais, elas catalisam a hidrólise de ligações ésteres carboxílicos e síntese orgânica. Em condições não aquosas, incluindo solventes orgânicos e em fluidos supercríticos, catalisam reações como esterificação, interesterificação e transesterificação produzindo glicerídeos a partir de glicerol e ácidos graxos (Figura 1) (HARI KRISHNA, 2002; STEPANKOVA *et al.,* 2013; LI *et al.,* 2014). Possuem capacidade de tolerar meios contendo solventes orgânicos, biossintetizados e líquidos iônicos, exibindo uma ampla especificidade ao substrato com elevada régio-, químio- e enantiosseletividade, além de não necessitarem de co-fatores (LI *et al.,* 2014; SHARMA *et al.,* 2014).

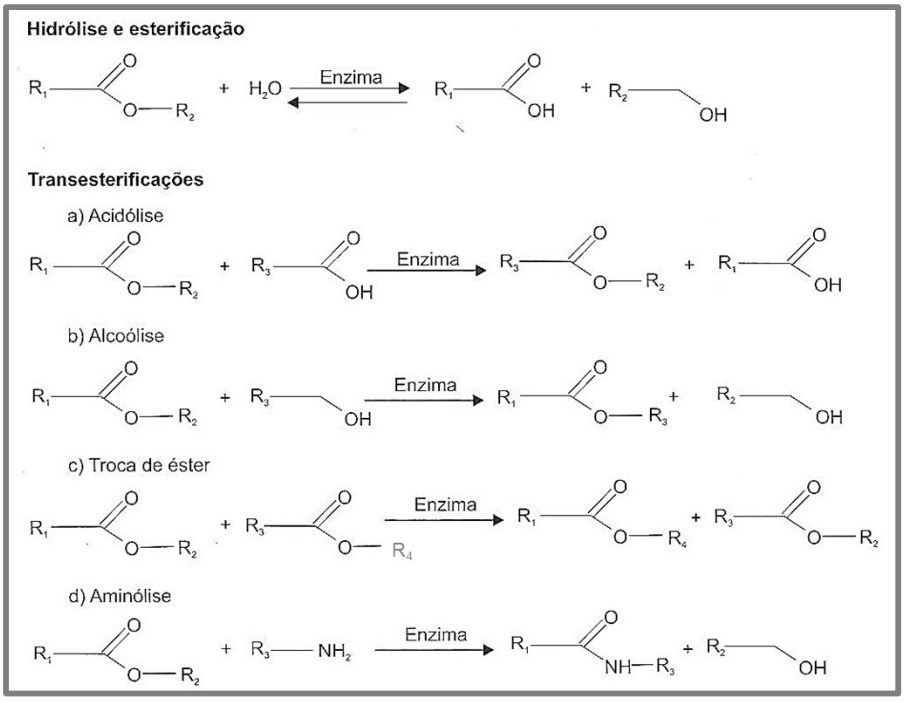


Figura 1: Reações típicas catalisadas por lipases. Adaptado de BON *et al.,* (2008).

Essa versatilidade faz das lipases microbianas uma excelente escolha para aplicações em indústrias de alimentos, detergentes, produtos farmacêuticos, têxteis, couro, cosméticos, biosensor, biorremediação, papel, de tratamento de resíduos e de produção de biodiesel (HASAN *et al.,* 2006; BON *et al.,* 2008). Para alguns desses processos se faz necessário a utilização de lipases puras, demanda que tem aumentado consideravelmente nos últimos anos, principalmente para a produção biocatalítica da chamada química fina, tais como de produtos cosméticos e farmacêuticos, e também para aplicações de diagnósticos avançados (SAXENA *et al.,* 2003b; CIRIMINNA *et al.,* 2013).

Atualmente a síntese de aproximadamente dois terços de produtos quirais produzidos em escala industrial é realizada utilizando biocatalizadores, uma vez que permitem elevados rendimentos e seletividade (DOWNEY, 2013). Existem vários medicamentos quirais de valor agregado que também são produzidos através de biocatálise, incluindo o Januvia (*sitagliptina)*, Crestor (*rosuvastatina)*, Lipitor (*atorvastatina*) e Singulair (*montelukast*) (CIRIMINNA e PAGLIARO, 2013). Em diagnósticos, o uso de lipases pode indicar determinada infeção ou doença em função de sua presença ou dos resultados de suas reações (MAJTAN *et al.,* 2002; REINER *et al.,* 2014). O nível de lipases em soro de sangue pode ser usado como uma ferramenta de diagnóstico para a detecção, por exemplo, de pancreatite aguda e lesão pancreática (WALKER *et al.,* 2013; DURGAMPUDI *et al.,* 2014). Produtos cosméticos sintetizados por lipases também recebem uma grande atenção do mercado industrial (RAHMAN *et al.,* 2011; HORCHANI *et al.,* 2014). A empresa *Unichem International* (Espanha), lançou a produção de hidratantes para a pele, cremes solares, bronzeadores e óleos de banho a partir da produção de miristato de isopropilo, palmitato de isopropilo e palmitato de 2-etilhexil em reações biocatalisadas pela lipase de *Rhizomucor meihei* (HASAN *et al.,* 2006). Os ésteres de ácidos graxos têm aplicações semelhantes em produtos de cuidados pessoais e também estão sendo produzidos via reação enzimática utilizando a lipase de *Candida cylindracea.* De acordo com o fabricante (Croda Universal Ltda.), o custo total de produção é maior comparado ao método convencional, mas o custo é justificado pela melhoria da qualidade do produto final (HASAN *et al.,* 2006).

Entre as principais cepas produtoras de lipases extracelulares comercialmente viáveis, estão as de *Candida, Pseudomonas, Mucor, Rhizopus,* e *Geotrichum* spp. As lipases extracelulares de bactérias, fungos e leveduras facilitam a recuperação da enzima a partir do meio de cultura, que é uma importante característica quando leva-se em consideração o preço final do produto (SAXENA *et al.,* 2003b; TREICHEL *et al.,* 2010). As lipases de *Burkholderia cepacia* (anteriormente *Pseudomonas cepacia*) é uma das lipases mais populares usadas em síntese orgânica e apresentam enorme potencial em reações de hidrólise e transesterificação (KAWAKAMI *et al.,* 2012; ADLERCREUTZ, 2013; TRAN *et al.,* 2014). Outra lipase que merece destaque são as obtidas a partir de *Bacillus* sp. Estas cepas produtoras de lipases também apresentam significativa importância na biocatálise com potencial de síntese reacional, e demonstram geralmente alta especificidade, estabilidade, e tolerância a inúmeros solventes, sais e detergentes (KUMAR *et al.,* 2005; NAGARAJAN, 2012). Podem potencialmente ser aplicadas em diversos ramos industriais como no tratamento de resíduos aquosos, na área de

cosméticos e biossensores, no entanto, seu uso em transformações sintéticas (esterificação, transesterificação, acidólise) é pouco explorado (WANG *et al.,* 2010a; SIVARAMAKRISHNAN *et al.,* 2012; KUMAR *et al.,* 2014b).

Portanto, as exigências para que as indústrias operem seus processos em condições de desenvolvimento sustentável utilizando lipases como biocatalisadores são cada vez mais relevantes para o cenário mundial. Entretanto, é um obstáculo para a indústria superar a disponibilidade de biocatalisadores com elevada pureza e de baixo custo. Para este propósito, são aplicadas técnicas de purificação com o objetivo de fornecer lipases capazes de catalisar reações com elevada eficiência e seletividade.

## 2.2. Extração e purificação de enzimas

Para purificação das lipases de uma forma geral, dois objetivos básicos devem ser considerados: (a) obtenção da enzima pura (homogênea), para melhor estudo de suas características bioquímicas e de sua estrutura e (b) obtenção de um produto com maior atividade específica (unidade de atividade/mg de proteína) para a aplicação nos diversos processos industriais (KOBLITZ *et al.,* 2004).

A viabilidade de comercialização e da produção em escala industrial de substâncias obtidas por meio da biotecnologia depende significativamente das técnicas empregadas na purificação (SAXENA *et al.,* 2003b). Especialmente para as lipases de natureza extracelular, a purificação tende a ser muito difícil, os processos fermentativos geram além do composto desejado, co-produtos que impedem, muitas vezes, a utilização do caldo bruto da fermentação nos procedimentos industriais (LINKE *et al.,* 2011). Por esta razão, são necessárias técnicas de purificação aplicadas para a separação das lipases dos solutos indesejáveis, tais como a presença de outras proteínas (ex. peptidases), sais e inibidores produzidos durante o período de cultura.

Os métodos de separação são geralmente executados com numerosas etapas (ex. precipitação, centrifugação, diálise, cromatográfia de troca iônica, cromatográfia por afinidade, etc) e requerem procedimentos complexos com elevado consumo de energia e de produtos químicos (CAMPERI *et al.,* 1996; LINKE e BERGER, 2011). É importante considerar que para se alcançar a purificação dos produtos até a homogeneidade, estas etapas propiciam elevados custos ao produto final (SAXENA *et al.,* 2003b; NAGARAJAN, 2012). Tipicamente, as estratégias de purificação são responsáveis por 20 a 60 % do custo total do produto

comercializado, no entanto em alguns casos especiais isso pode representar até 80 % (MAESTRO *et al.*, 2008; MARTÍNEZ-ARAGÓN *et al.*, 2009; RUIZ-RUIZ *et al.*, 2012).

Como a maioria das lipases microbianas são extracelulares, o processo de fermentação é geralmente seguido por uma remoção das células a partir do caldo de cultura, utilizando técnicas de centrifugação ou filtração. O caldo de cultura livre de células é, em seguida, concentrado por ultrafiltração, precipitação ou por extração com solventes orgânicos (CAMPERI *et al.,* 1996). A etapa inicial é geralmente chamada de pré-purificação. A maioria das estratégias para a pré-purificação tem utilizado a etapa de precipitação, realizada utilizando sulfato de amônio, etanol, acetona ou um ácido (normalmente ácido clorídrico) (PABAI *et al.,* 1995). A precipitação é descrita como uma tecnologia bastante simples, que pode ser utilizada para remover impurezas ou isolar uma proteína específica de uma mistura. A precipitação com sulfato de amônio é a mais utilizada por não promover reações exotérmicas em solução, como ocorre ao utilizar etanol. Este processo promove o isolamento das proteínas em função da alta molaridade do sal que diminui a solubilidade das proteínas em água (ZHOU, 2005; FISICARO *et al.,* 2011).

Algumas tecnologias aplicadas à purificação de lipases após a pré-purificação, incluem processos com membrana, eletroforese (HURKMAN *et al.,* 1986; WANG *et al.,* 2004; PIERGIOVANNI, 2007), imunopurificação (SAXENA *et al.,* 2003b), micela reversa (NANDINI *et al.,* 2010; GAIKAIWARI *et al.,* 2012), ultrassom (NABARLATZ *et al.,* 2010; NABARLATZ *et al.,* 2012) e cromatografia (AGASØSTER, 1998; ZATLOUKALOVÁ *et al.,* 2004; MASOMIAN *et al.,* 2013). Na maioria dos casos, a etapa de precipitação é seguida por etapas cromatográficas, uma vez que apenas uma etapa não é suficiente para obter o nível requerido de pureza. Alguns tipos de colunas cromatográficas são utilizadas, tais como: colunas de troca iônica, colunas de interação hidrofóbica e colunas de permeação em gel. A destacar também, embora seja aplicada com menos frequência, as colunas de bioafinidade e de adsorção inespecífica – empacotadas por exemplo com hidroxiapatita (SAXENA *et al.,* 2003b).

Como regra geral, a maioria dos métodos, fazendo o uso de técnicas cromatográficas para a purificação de lipases, segue a seguinte sequência: nos primeiros passos de purificação são utilizadas colunas com grande capacidade de troca e de baixo custo. As de troca iônica e as de interação hidrofóbica são exemplos de colunas de baixo custo e com elevada eficiência (TAIPA *et al.,* 1992; NAGARAJAN, 2012). Estes tipos de colunas são aplicadas com sucesso para a purificação de lipases e apresentam vantagens por dispensar etapas de diálise (TAIPA *et al.,* 1992). Nas etapas finais da purificação, geralmente são aplicadas as colunas de permeação em

gel, que garantem a remoção de agregados e produtos da degradação sofrida pela enzima ao longo do processo de purificação (NAGARAJAN, 2012). Entretanto, este tipo de cromatografia conduz a diluição do produto final exigindo uma etapa de concentração posterior, como a liofilização (SAXENA *et al.,* 2003b). Na Tabela 2, são apresentados diferentes trabalhos sobre a purificação de lipases utilizando técnicas cromatográficas.

Tabela 2: Técnicas cromatográficas aplicadas para a purificação de lipases.

| Micro-organismo | Técnica de purificação | $R_E$ (%) | PF (vezes) | Referência |
|---|---|---|---|---|
| *Aneurinibacillus thermoaerophilus HZ* | Permeação em gel (Q-Sepharose);<br>Permeação em gel (Sephadex-G75) | 48,7<br>19,69 | 9,3<br>15,62 | (MASOMIAN *et al.,* 2013) |
| *Burkholderia cepacia* ATCC 25416 | Precipitação com $(NH_4)_2SO_4$;<br>Interação hidrofóbica (Sepharose FF);<br>Troca iônica (DEAE-Sepharose FF) | 45,7<br>5,9<br>4,8 | 1,36<br>3,1<br>4,9 | (WANG *et al.,* 2009) |
| *Bacillus* sp. | Precipitação com $(NH_4)_2SO_4$;<br>Interação hidrofóbica (phenyl Sepharose CL-4B) | 30,0<br>13,0 | 1,54<br>8,6 | (SIVARAMAKRISHNAN e MUTHUKUMAR, 2012) |
| *Bacillus subtilis* EH 37 | Precipitação com $(NH_4)_2SO_4$;<br>Ultrafiltração;<br>Interação hidrofóbica (phenyl Sepharose®) | 23,0<br>20,3<br>16,0 | 4,6<br>11,6<br>17,8 | (AHMED *et al.*, 2010) |
| *Bacillus licheniformes* | Precipitação com $(NH_4)_2SO_4$;<br>Troca iônica (DEAE cellulose);<br>Permeação em gel (Sephadex G-50) | 36,6<br>25,0<br>19,0 | 1,8<br>3,1<br>3,6 | (ANNAMALAI *et al.,* 2011) |
| *Bacillus smithi* BTMS 11 | Precipitação com $(NH_4)_2SO_4$;<br>Troca iônica (DEAE cellulose) | 2,16<br>0,1 | 1,6<br>4,3 | (LAILAJA *et al.,* 2013) |
| *Bacillus coagulans* BTS-3 | Precipitação com $(NH_4)_2SO_4$;<br>Troca iônica (DEAE Sepharose) | 38,0<br>2,5 | 16,0<br>40,0 | (KUMAR *et al.,* 2005) |
| *Burkholderia multivorans* PSU-AH130 | Troca iônica (DEAE-Toyopearl);<br>Permeação em gel (Sephadex G-150) | 2,8<br>12,1 | 9,4<br>21,6 | (CHAIYASO *et al.,* 2012) |
| *Caldanaerobacter subterraneus* DSM 15242 | Interação hidrofóbica (Sepharose®);<br>Permeação em gel (Superdex 200) | 30,0<br>8,1 | 36,3<br>93,6 | (ROYTER *et al.,* 2009) |
| *Candida antártica* (extrato comercial) | Permeação em gel (Sephacryl S-100);<br>Troca iônica (Source 15Q);<br>Troca iônica (Source 15Q) | 59,6<br>47,3<br>20,2 | 1,19<br>1,38<br>0,87 | (LLERENA-SUSTER *et al.,* 2014) |
| *Staphylococcus aureus* | Interação hidrofóbica (phenyl Sepharose CL-4B);<br>Permeação em gel (Superose-12) | 74,91<br>20,0 | 3,62<br>6,76 | (SARKAR *et al.,* 2012) |
| *Streptomyces thermocarboxydus ME168* | Precipitação com acetona;<br>Troca iônica (Resource Q);<br>Permeação em gel (Superdex 200) | 79,6<br>47,0<br>20,3 | 1,1<br>2,5<br>9,6 | (H-KITTIKUN *et al.,* 2012) |
| *Thermoanaerobacter thermohydrosulfuricus* DSM 7021 | Interação hidrofóbica (phenyl Sepharose®)<br>Permeação em gel (Superdex 200) | 48,3<br>13,0 | 49,0<br>108,7 | (ROYTER *et al.,* 2009) |
| *A. carneus* | Precipitação com $(NH_4)_2SO_4$;<br>Interação hidrofóbica (Octyl Sepharose®) | 89,97<br>38,40 | 2,61<br>24,10 | (SAXENA *et al.,* 2003a) |

$R_E$ – rendimento de extração; PF – fator de purificação

Na literatura, é possível observar inúmeros trabalhos que utilizam estas tecnologias para a purificação de lipases. Recentemente, utilizando colunas de bioafinidade foi possível purificar a lipase de CaLB (*Candida Antartica* B) com rendimento de 73 % e um fator de purificação de 91 vezes (YAO *et al.,* 2011). LIU *et al.,* (2008) aplicaram uma sequência de passos para a purificação de lipase de *Aureobasidium pullulans* HN2.3 consistindo de precipitação com sulfato de amônio (80 %), cromatografia por filtração em gel de *Sephadex G-75* e cromatografia de troca iônica (*DEAE-Sepharose*), obtendo ao final do processo um fator de purificação de 3,4 vezes para uma enzima de massa molecular de 63,5 kDa. A partir de uma espécie de levedura (*Malassezi globosa*), a lipase extracelular foi purificada utilizando cromatografia de interação hidrofóbica (coluna de *phenyl Sepharose*) seguido de ultrafiltração. Embora a recuperação tenha sido muito baixa (12,5 %), uma purificação de 20,5 vezes foi conseguida (JUNTACHAI *et al.,* 2011). KOBLITZ e PASTORE (2004), compararam duas técnica cromatográficas para a purificação da lipase de *Rhizopus* sp. A cromatografia de troca iônica purificou 3,9 vezes a lipase e a cromatografia de interação hidrofóbica purificou 6,89 vezes. Os autores associaram a menor purificação devido provavelmente à perda de grupos aminas da proteína.

Portanto, a purificação de lipases microbianas utilizando os métodos cromatográficos depende além das condições iniciais da lipase, tais como características físico-química e caldo de cultura bruto, dos diferentes métodos cromatográficos utilizados durante o processo de purificação (SAXENA *et al.,* 2003b). Os processos tradicionais incluindo o uso de técnicas cromatográficas, embora tenham alta resolução são ocasionalmente problemáticos dificultando a ampliação de escala, pois necessitam de alto investimento, elevado tempo de processamento e resultam normalmente em baixos rendimentos finais (ROSA *et al.,* 2011).

Uma outra abordagem interessante é o isolamento das proteínas pretendidas por extração líquido-líquido. As possíveis vantagens estão relacionadas à alta capacidade, maior seletividade e integração entre a recuperação e purificação. Dentre os processos de separação pode-se destacar a extração líquido-líquido com sistemas aquosos bifásicos. Esta abordagem tecnológica deve ser considerada como alternativa baseando-se em uma visão crítica da tecnologia anterior, uma vez que atualmente são discutidos em âmbito científico e industrial, estratégias de purificação de baixo custo, de processamento rápido, com elevado rendimento e passíveis de operações em grande escala.

## 2.3. Sistemas aquosos bifásicos (SABs)

Os sistemas aquosos bifásicos (SABs) são conhecidos desde o final do século XIX, quando Martinus Beijerinck em 1986 observou a separação espontânea em duas fases líquidas e límpidas ao misturar soluções aquosas de gelatina com ágar ou amido solúvel (BEIJERINK, 1896). A partir desta observação, apenas na década de 50 que Albertsson evidenciou para a comunidade científica a grande potencialidade da aplicação desta técnica para separar biomoléculas em meio aquoso (ALBERTSSON, 1958). O uso destes sistemas tem sido satisfatoriamente estudado há mais de 50 anos, para a separação e purificação de moléculas biológicas, tais como DNA, proteínas, enzimas, alcalóides, antibióticos, drogas entre outros (KESSEL, 1981; LI *et al.,* 2004; BORA *et al.,* 2005; ROSA *et al.,* 2011; ASENJO *et al.,* 2012; MATOS *et al.,* 2014; MOHAMED ALI *et al.,* 2014), e também compostos de origem não orgânica, como os íons metálicos e antibióticos (ROGERS *et al.*, 1993; YIXIN *et al.*, 1994; MANDAL *et al.*, 2014).

Os SABs são compostos por duas fases aquosas imiscíveis que coexistem em equilíbrio promovidas pela adição de compostos solúveis em água. Cada fase do sistema torna-se enriquecido com um dos compostos, originando duas fases aquosas de natureza química e física diferentes, conduzindo a migração das biomoléculas para uma das fases por afinidade. Uma das principais características do sistema é o elevado conteúdo de água nas fases permitindo a separação de biomoléculas de diversas origens em condições não-desnaturantes (ALBERTSSON, 1986; JOHANSSON, 1989; ALBERTSSON, 1990).

Os primeiros sistemas aquosos bifásicos foram do tipo polímero-polímero, e somente em 1986 foi relatado a possibilidade da formação de SAB utilizando um polímero e um sal inorgânico (ALBERTSSON, 1986). Em princípio, a maioria dos polímeros hidrofílicos, naturais ou sintéticos miscíveis com a água, mostram capacidade para separação de fase em uma mistura com um segundo polímero ou com sais. Um SAB ocorre quando dois compostos são misturados acima de uma certa concentração crítica em que o resultado é a formação de duas fases imiscíveis (ALBERTSSON, 1990; ZASLAVSKY, 1995; GLYK *et al.,* 2014).

### 2.3.1. Diagrama de fases

As composições dos SABs são representadas por diagramas de fases que expressam a concentração dos componentes do sistema. Em sistemas com três componentes (sistemas

ternários), as fases são representadas por diagramas de fase triangulares, ou diagramas ternários, onde a composição é indicada por um ponto em um triângulo equilátero como mostrado na Figura 2.

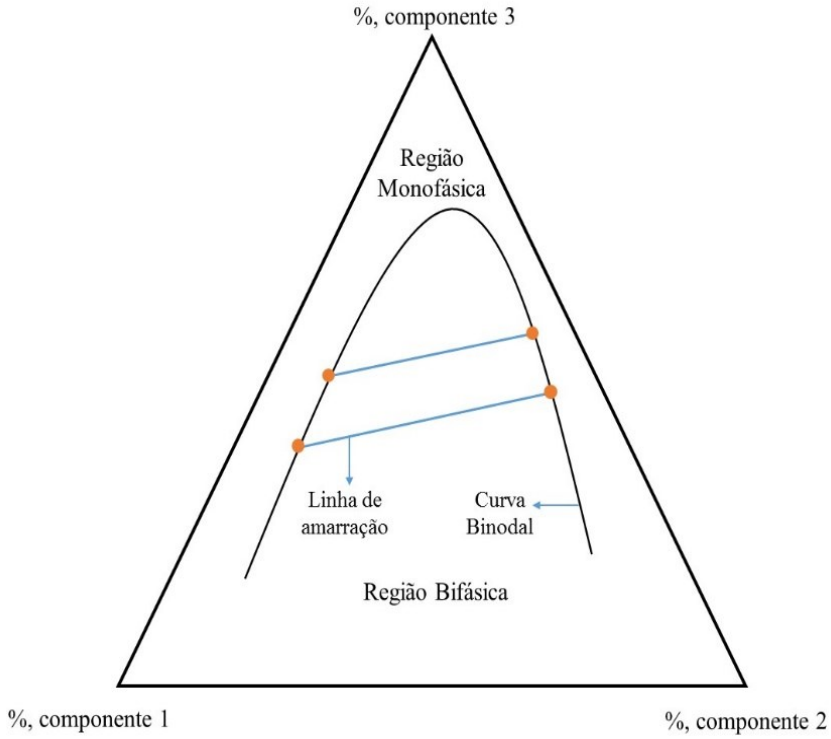


Figura 2: Diagrama de fases em coordenadas triangulares.

Para os sistemas de duas fases aquosas, no qual a concentração de água é bastante elevada (40 – 80 %, m/m) costuma-se utilizar a forma de eixo cartesiano para representá-lo (Figura 3 (a)), excluindo-se a composição de água (ZASLAVSKY, 1995). Nestes diagramas pode ser observada a composição química das duas fases que se encontram em equilíbrio termodinâmico, os quais são expressos em diferentes unidades correspondentes às frações molares ou mássicas. Normalmente unidades de concentração em mol ($mol.Kg^{-1}$) são utilizados para evitar potenciais discrepâncias ocasionadas pelas diferentes massas moleculares dos solutos envolvidos na formação do SAB. Entretanto, para fins de extração ou purificação a maioria dos dados da literatura correspondem à unidade de fração mássica (%, m/m). Os autores geralmente compararam a eficiência ou os rendimentos de extração em função das composições de mistura (em termos mássicos) entre os SABs (FREIRE *et al.*, 2012).

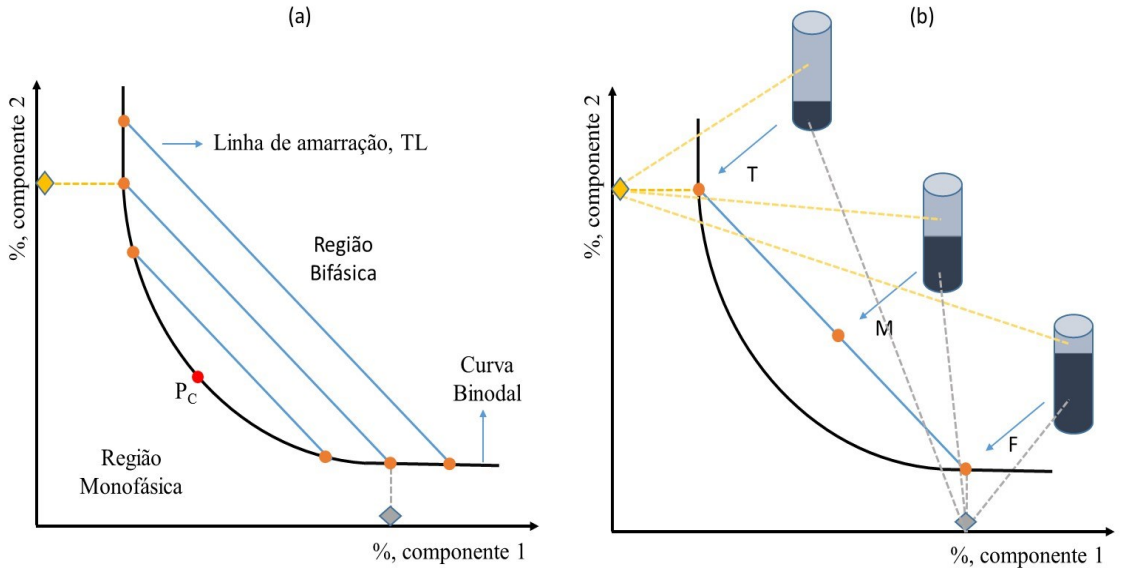


Figura 3: Diagrama de fases para um SAB. (a): ▬, curva binodal; ─, linha de amarração (TL); ●, ponto crítico - $P_C$. Acima da curva binodal o sistema é bifásico abaixo dela o sistema é monofásico; (b): Três sistemas (tubos em azul) pertencentes à mesma linha de amarração. As composições das fases superiores e inferiores são representadas.

Nestes sistemas, a composição total da mistura dos componentes é representada pelo ponto de mistura (M) (Figura 3 (b)). A mistura separa-se em duas fases, que contêm as frações dos constituintes formadores. As composições destas duas fases são representadas pelos pontos da fase de topo (T) e da fase de fundo (F). A união destes pontos forma a linha de amarração, ou *tie-lines* (TL). A partir da união dos pontos extremos de cada linha de amarração forma-se a curva binodal, a qual separa o diagrama em duas regiões, acima da curva binodal tem-se a região bifásica e abaixo a monofásica. O ponto $P_C$ é definido como ponto crítico, neste ponto a composição e o volume das fases coexistentes são iguais e podem fornecer coeficientes de partição igual a 1 (KULA *et al.,* 1982; ZASLAVSKY, 1995; GLYK *et al.,* 2014). O ponto crítico pode ser obtido pela intersecção de uma linha que passa pelo ponto médio de várias TL com a binodal. O ponto médio é representado pelo ponto limite, que é o ponto onde a binodal é tangente à linha que une os segmentos iguais nos eixos do diagrama (Figura 4). A posição relativa do ponto limite e do $P_C$ define a simetria do diagrama de fases (YU *et al.,* 2011; GLYK *et al.,* 2014).

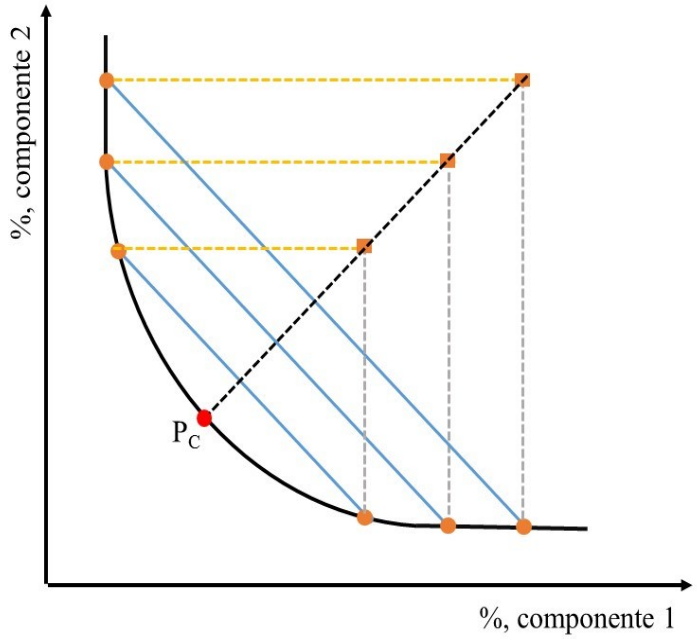


Figura 4: Diagrama de fases para um SAB, onde se tem a curva binodal (▬), a linha de amarração, TL (─), o ponto limite (■) e o $P_C$ (●).

As curvas binodais podem ser determinadas por diferentes métodos. A utilização da cromatografia líquida de alta eficiência (HPLC) é o mecanismo mais preciso para a determinação da composição das fases que formam o SAB (ALBERTSSON, 1986; PLANAS *et al.,* 1997). Por outro lado, um método bastante utilizado devido a simplicidade e rapidez baseia-se em um processo de titulação entre os componentes que formam os sistemas, porém o método baseia-se na observação visual de mudança das fases, o que pode aumentar o grau de incerteza da determinação. Este método é extensivamente o mais utilizado e comumente chamado "*cloud-point tritation"* (CAO *et al.*, 2008; CLAUDIO *et al.*, 2011; VENTURA *et al.*, 2012c; CARDOSO *et al.*, 2014a; GLYK *et al.*, 2014). O procedimento experimental consiste em duas etapas principais: (*i*) adição gota a gota de uma solução aquosa contendo o componente 1 (agente *salting-out*) em uma solução aquosa contendo o componente 2 (ou o componente em seu estado puro) até que uma solução turva e bifásica ou vice-versa seja observada (Figura 5 (a)); (*ii*) adição gota a gota de água, até a formação de uma solução transparente e límpida que corresponde ao regime monofásico (Figura 5 (b)). As composições dos sistemas são determinadas pela quantificação da massa (g).

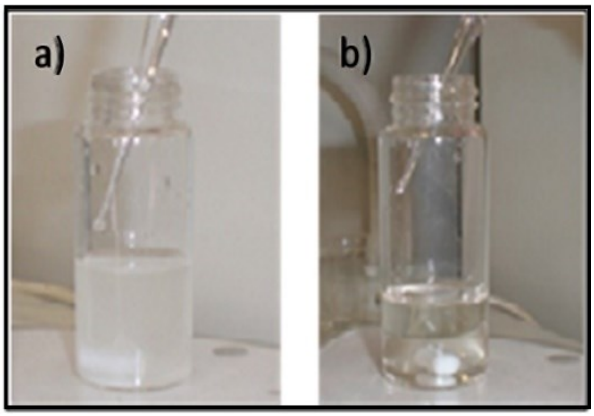


Figura 5: Ilustração da determinação experimental da curva binodal para os sistemas aquosos PEG – sal, a 25 ºC: a) adição de uma solução aquosa de sal para a formação de uma mistura turva; b) adição de água para a formação de uma mistura límpida.

Para correlacionar os pontos experimentais que descrevem os sistemas à base de polímeros, Merchuk *et al.,* (1998) propôs um modelo matemático com três parâmetros ajustáveis (Equação 1). Além deste, outros modelos empíricos (Equação 2) também são utilizados para correlacionar os dados binodais de sistemas polímero-sal (REGUPATHI *et al.,* 2009; RAJA *et al.,* 2013). Para o ajuste de curvas binodais formadas por solventes orgânicos como etanol e metanol com a presença de sais, geralmente são considerados os ajustes descrito pela Equação 3 (KATAYAMA *et al.,* 2008; WANG *et al.,* 2010b).

$$w_1 = A\,exp[(Bw_2^{0,5}) - (Cw_2^3)] \quad (1)$$

$$w_1 = A + Bw_2^{0,5} + Cw_2 \quad (2)$$

$$w_1 = A + Bw_2^{0,5} + Cw_2 + Dw_2^2 \quad (3)$$

onde: $w_1$ e $w_2$ são as percentagens em fração de massa do componente 1 e do componente 2, respectivamente. Os parâmetros de ajuste *A*, *B*, *C* e *D* são obtidos pela regressão dos mínimos quadrados.

As *tie-lines* (TL) são comumente determinadas pelo método de Merchuck e colaboradores (1998), que consiste na seleção de um ponto dentro da região bifásica (ponto M, na Figura 6,b). A mistura é pesada e misturada cuidadosamente, após atingir o equilíbrio as fases de topo e fundo são separadas e pesadas. Cada TL individual será determinada pela aplicação de regra da alavanca na relação entre a composição mássica da fase de topo e do sistema global. A

determinação da TL é acompanhada pela resolução das seguintes Equações 4 a 7 (MERCHUK *et al.,* 1998).

$$Y_T = (Y_M/\alpha) - ((1-\alpha)/\alpha)Y_F \quad (4)$$

$$X_T = (X_M/\alpha) - ((1-\alpha)/\alpha)X_F \quad (5)$$

$$Y_T = f(X_T) \quad (6)$$

$$Y_F = f(X_F) \quad (7)$$

onde: $f(X)$ é a função que representa a binodal, o subscrito M, T e F denotam a mistura, fase de topo e fase de fundo, respectivamente. O valor de α é a razão entre a massa de topo e da massa total da mistura.

O comprimento da linha de amarração (usualmente referido como TLL, do inglês *Tie Line Length*) é um importante parâmetro termodinâmico, geralmente utilizado como variável determinante dos processos de partição (SARAVANAN *et al.,* 2008; SOUZA *et al.,* 2010; PEREZ *et al.,* 2013). O comprimento da linha de amarração pode ser calculado aplicando a Equação 8 (SILVA, 2006).

$$TLL = [(\Delta w_1)^2 + (\Delta w_2)^2]^{0,5} \quad (8)$$

onde: $\Delta w_1$ e $\Delta w_2$ são as diferenças de concentração do componente 1 e 2 entre as fases, respectivamente.

A deterimação do ponto crítico ($P_C$) para sistemas ternários é estimada aplicando a Equação 9 (FREIRE *et al.*, 2012).

$$Y = f + gX \quad (9)$$

onde: $Y$ e $X$ são as composições do componente 1 e 2, respectivamente. $f$ e $g$ são parâmetros de ajuste.

Os parâmetros termodinâmicos como calor específico ($C_P$), entalpia ($H$), entropia ($S$) e energia livre de Gibbs ($G$), também são importantes para descrever as propriedades de qualquer sistema em equilíbrio (COOPER, 1999). A determinação da partição de uma biomolécula em SAB é determinada pela teoria de Flory-Huggins, que descreve a energia necessária para a obtenção da energia livre de Gibbs ($\Delta G_m^0$). Para este propósito, a variação do calor específico

*(*$\Delta C_P$) é o principal parâmetro termodinâmico utilizado para se obter as variações da entalpia ($\Delta H_m^0$) e da entropia ($\Delta S_m^0$) (Equações 10 e 11, respectivamente) (COOPER, 1999; PESSOA *et al.,* 2004; JOHANSSON *et al.,* 2011).

$$\Delta H_m^0 = \int_0^T \Delta C_P dT + \Delta H\ (0) \tag{10}$$

$$\Delta S_m^0 = \int_0^T \left(\frac{\Delta C_P}{T}\right) dT \tag{11}$$

onde: $\Delta H_m^0$ é a variação da entalpia (máxima energia de um sistema termodinâmico) e o $\Delta S_m^0$ é a variação da entropia (grau de irreversibilidade de um sistema termodinâmico).

A energia livre de Gibbs é o parâmetro que expressa o equilíbrio molecular, ela indica a direção dos processos, bem como a quantidade de trabalho necessária para que ele ocorra (JOHANSSON *et al.,* 2000; DE SOUSA *et al.,* 2009; LU *et al.,* 2011). Pode ser expressa em função da energia livre à pressão constante (Equação 12) ou a nível molecular (equação 13).

$$\Delta G_m^0 = \Delta H_m^0 - T\Delta S_m^0 \tag{12}$$

$$\Delta G_m^0 = -RT \ln K \tag{13}$$

onde: *R* é a constante dos gases e *K* é a constante de equilíbrio.

Geralmente, estes parâmetros são obtidos pelo ajuste linear da equação que correlaciona o coeficiente de partição (*K*) e o inverso da temperatura (em Kelvin) (JOHANSSON, 1985).

O coeficiente de partição (*K*) é uma grandeza adimensional que descreve a migração das biomoléculas entres as fases coexistentes de um SAB*,* no qual assume o valor numérico da proporção entre as concentrações das biomoléculas na fase superior (topo) e fase inferior (fundo) (Equação 14). A determinação das recuperações dos solutos (também associado à eficiência de extração – *EE*, %) nas fases de topo ($R_T$) e fundo ($R_F$), também são comumente utilizados para avaliar a eficiência dos SABs (Equação 15 e 16). Já o fator de purificação (*PF*) dos solutos é calculado pela razão da atividade específica depois (*SA*) e antes ($SA_i$) do procedimento de extração (Equação 17). Estes parâmetros são os principais mecanismos para avaliar a capacidade em particionar, recuperar ou purificar os solutos em SABs (JOHANSSON, 1985; BASSANI *et al.,* 2007; SOUZA *et al.,* 2010; VENTURA *et al.,* 2012a; ZHOU *et al.,* 2013; PRINZ *et al.,* 2014).

$$K = \frac{[C_T]}{[C_F]} \quad (14)$$

$$R_T = 100/(1 + \left(\frac{1}{R_V K}\right)) \quad (15)$$

$$R_B = 100/(1 + R_V K) \quad (16)$$

$$PF = \frac{SA}{SA_i} \quad (17)$$

onde: $R_V$ é a razão volumétrica entre as fases ($V_T$ é o volume da fase de topo – mL e $V_F$ é o volume da fase de fundo – mL).

### 2.3.2. Fatores que influenciam a partição em SABs

Nos SABs diversos fatores influenciam a distribuição desigual das biomoléculas entre as duas fases a qual é resultante de um complicado e delicado balanço de interações entre a biomolécula e as outras espécies presentes nas duas fases que coexistem em equilíbrio (JOHANSSON, 1989). As propriedades físico-químicas inerentes às composições das fases, tais como, hidrofobicidade, cargas na superfície, concentração dos constituintes e entre outros, são normalmente responsáveis pela partição em SABs (KULA *et al.,* 1982; ALBERTSSON, 1986; ANDREWS *et al.,* 2005; ASENJO *et al.,* 2011). Entretanto, nem todas essas propriedades são igualmente importantes, mas estas podem ser manipuladas para se obter um melhor desempenho de separação (ASENJO e ANDREWS, 2011). A seguir será apresentado especificamente alguns dos principais fatores que podem influenciar na partição ou migração das proteínas em um SAB.

*Efeito da hidrofobicidade*

As interações hidrofóbicas desempenham um importante efeito na migração de proteínas em SABs, as quais promovem efeitos conhecidos envolvidos neste tipo de interação: o efeito da hidrofobicidade das fases e o efeito *salting-out* (ANDREWS *et al.,* 2010).

O efeito hidrofóbico das fases está diretamente relacionado com a identidade química dos componentes do sistema, bem como as suas concentrações. Embora a princípio ambas as fases do sistema são hidrofílicas devido à grande quantidade de água, a fase rica em polímero é geralmente mais hidrofóbica. A influência desta característica favorece a separação das

proteínas hidrofóbicas para esta fase em particular. Em sistemas polímero-sal a hidrofobicidade de fase pode ser manipulada variando a TLL, a massa molecular do polímero e/ou por adição de um sal (ALBERTSSON, 1986; ANDREWS *et al.,* 2005; ASENJO e ANDREWS, 2012; PRINZ *et al.,* 2014).

O aumento da massa molecular do polímero, diminui a razão entre as áreas dos grupos hidrofílicos/hidrofóbicos promovendo o aumento da hidrofobicidade da fase polimérica, principalmente devido a disponibilidade de água nas fases coexistentes. Especialmente sobre a TLL, uma redução intrínseca do teor de água é alcançada quando a TLL é aumentada, tornando as fases mais hidrofóbicas (com menos grupos hidroxilas disponíveis) (ASENJO e ANDREWS, 2012). O deslocamento de proteínas por adição de sais neutros, como o cloreto de sódio, reduz a hidrofobicidade das fases induzindo o aumento dos coeficientes de partição (BRADOO *et al.,* 1999; ANDREWS *et al.,* 2005; PRINZ *et al.,* 2014).

Além disso, para uma discussão do efeito hidrofóbico para a separação de proteínas, a estrutura das proteínas contém resíduos de aminoácidos (tirosina, histidina, triptofano, fenilalanina) que constituem aproximadamente 10% dos resíduos totais (LINS *et al.,* 2003). O teor destes resíduos apresenta anéis aromáticos com grupos hidrofóbicos, e consequentemente o coeficiente de partição das proteínas para a fase mais hidrofóbica deve aumentar (PEI *et al.,* 2009).

O efeito *salting-out* também está relacionado com a partição hidrofóbica das biomoléculas em SABs. Este efeito é observado em sistemas com pelo menos uma fase altamente iônica (por exemplo, SAB à base de sal). Neste caso, uma vez que a quantidade de água necessária para dissolver os sais no sistema é elevada, a biomolécula é apenas parcialmente hidratada. Em tais circunstâncias, a migração das biomoléculas para a fase menos hidrofílica é favorecido (HACHEM *et al.,* 1996).

Na Figura 6 podemos observar a representação esquemática deste efeito sobre a partição de biomoléculas em um SAB à base de polímero-sal. A solubilidade das macromoléculas biológicas na fase rica em sal (fase de fundo) diminui com o aumento da concentração de sal, o que resulta em um aumento da migração das biomoléculas para a fase oposta (fase de topo, rica em polímero), entendido como efeito *salting-out* (Figura 6 (b)) (BABU *et al.,* 2008). O aumento da solubilidade de proteínas em meios aquosos ricos em sais (efeito *salting-in*) é também conseguido em função da presença de sais em baixas concentrações no SAB. Desta forma, os íons salinos interagem com as cargas iônicas das proteínas aumentando assim o

número efetivo de cargas e a quantidade de moléculas de água fixadas à ionosfera protéica (RUCKENSTEIN *et al.,* 2006).

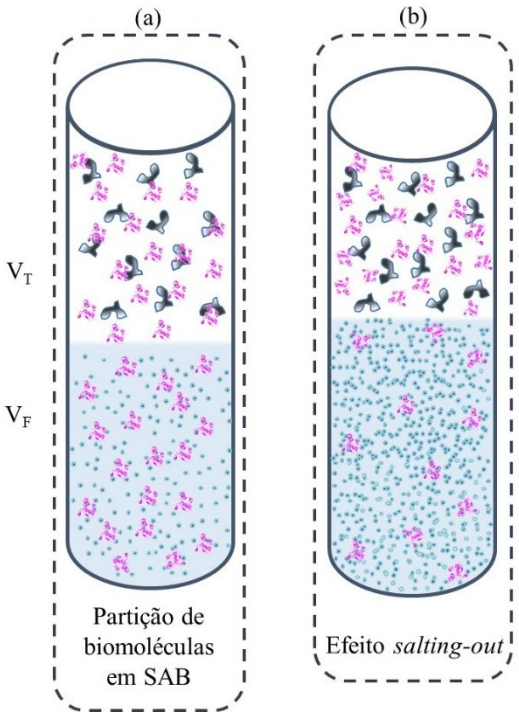


Figura 6: Representação esquemática do comportamento da partição das biomoléculas em SAB: (a), sistema típico polímero/sal; (b), efeito do aumento da concentração de sal; (), polímero; (), biomolécula; (), sal; $V_T$: volume de fase de topo; $V_F$: volume de fase de fundo.

*Efeito de carga*

As interações eletroquímicas podem ter um papel importante no comportamento de migração das biomoléculas, especialmente as proteínas por apresentarem grupamentos iônicos. Como cargas opostas se atraem, a presença de constituintes carregados podem gerar um separação seletiva das proteínas para uma das fases do sistema (COLLINS, 1997; KOWACZ *et al.,* 2012).

As proteínas são moléculas anfóteras cuja carga é determinada pelo pH do meio onde estão suspensas. Portanto, a influência do pH sobre as interações eletroquímicas é fundamental para ajustar a migração de proteínas, visto que o pH do sistema pode ser manipulado a fim de promover uma separação mais seletiva. A utilização de meios com valores de pH abaixo do

ponto isoelétrico (p*I*) da proteína pode induzir afinidades adicionais para a fase rica em polímero (COLLINS, 1995,1997). O efeito contrário também pode ocorrer, neste caso, valores de pH acima do ponto isoelétrico, as biomoléculas são carregadas negativamente resultando no aumento da afinidade para fase rica em sal (FORCINITI *et al.,* 1991b; BASSANI *et al.,* 2007; BARBOSA *et al.,* 2011; VENTURA *et al.,* 2011).

O efeito da densidade de carga do sal também pode determinar a eficiência de uma extração. O aumento da força iônica diminui a capacidade de hidratação das biomoléculas, em tais condições, é observado uma suspensão causada pela agregação das macromoléculas biológicas e consequentemente a eficiência do sistema é comprometida (FORCINITI *et al.,* 1992; COLLINS, 1995).

*Efeito do tamanho (massa molar)*

Uma vez que as biomoléculas a serem particionadas no sistema têm um tamanho definido (massa molar e diâmetro hidrodinâmico), bem como geometria (conformação tridimensional), estas são submetidas aos efeitos estéricos impostos pelos componentes do sistema. Estes efeitos são tipicamente relacionados com o volume disponível para a biomolécula migrar para uma das fases, e são geralmente conhecidos como o efeito de volume de exclusão (FORCINITI *et al.,* 1991b; ANDREWS *et al.,* 2005). Em sistemas PEG/sal é claramente observado este efeito, é comum que a partição das biomoléculas dependa do efeito de volume de exclusão em função do polímero em sua fase rica (fase de topo) e/ou, como visto anteriormente, do efeito *salting-out* do sal em sua fase rica (fase de fundo). Para facilitar a compreensão, a representação esquemática deste efeito pode ser vista na Figura 7. O volume ocupado pelo polímero aumenta com o aumento da concentração do polímero (Figura 7 (b)), que resulta em um espaço reduzido para biomoléculas na fase superior e consequentemente forçando uma migração para a fase oposta (fase de fundo rica em sal). Este efeito também pode ser originado pelo aumento do comprimento da cadeia polimérica ou da massa molar do polímero, como mostrado na Figura 7(c) (BABU *et al.,* 2008). Na Figura 7(d), pode-se observar a representação esquemática dos efeitos combinados de volume de exclusão e efeito *salting-out* em SAB formado por uma fase rica em polímero e outra em sal para partição de biomoléculas. Para este caso específico, as biomoléculas migram para a interface, ou seja, no sistema polímero/sal, a fase polimérica está com elevada concentração ou massa molar, e a fase fundo com elevada concentração de sal (FORCINITI *et al.,* 1991a; BABU *et al.,* 2008).

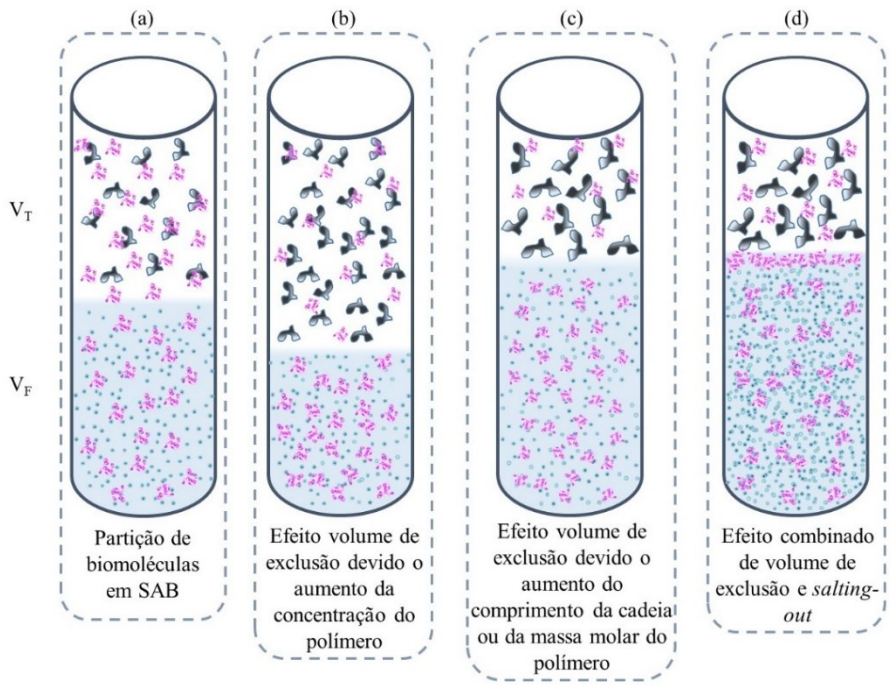


Figura 7: Representação esquemática do comportamento da partição das biomoléculas em SAB: (a), sistema típico polímero/sal; (b), o efeito de aumento da concentração de polímero; (c), o efeito do aumento do comprimento de cadeia ou peso molecular do polímero; (d), efeito combinado do volume de exclusão e *salting-out*; ( ), polímero; ( ), biomolécula; ( ), sal; $V_T$: volume de fase de topo; $V_F$: volume de fase de fundo.

*Efeito da concentração*

O efeito da concentração de biomoléculas na partição em SAB é dependente das suas propriedades físico-químicas. Para análise deste efeito foram considerados sistemas à base de polímero e sal. Na fase superior, a concentração máxima de uma biomolécula é determinada principalmente pelo efeito de volume de exclusão do polímero, e de interações hidrofóbicas entre o polímero e as biomoléculas. Na fase de fundo (rica em sal), a concentração máxima é determinada principalmente pelo efeito *salting-out* do sal presente no SAB. O comportamento do coeficiente de partição, independente da concentração da proteína, ocorre apenas a uma concentração relativamente baixa de biomoléculas (FORCINITI *et al.,* 1991b; ANDREWS *et al.,* 2005). Entretanto, quando a concentração da macromolécula biológica na fase de topo é relativamente alta, uma terceira fase é formada o que representa uma fase de agregação sólida. Este tipo de efeito também pode justificar precipitações na interface do SAB ocasionado pela concentração das biomoléculas (Figura 8) (ASENJO e ANDREWS, 2011).

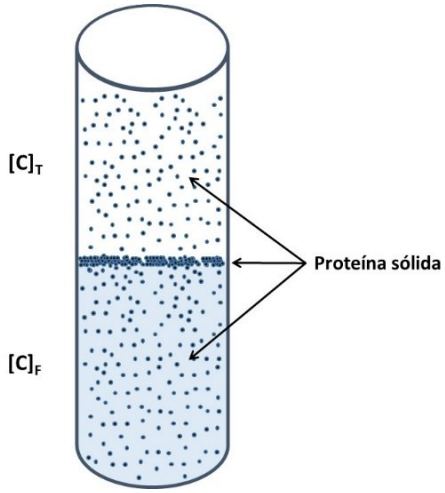


Figura 8: Diagrama que mostra um SAB com uma única proteína presente formando uma fase sólida, onde: [C], concentração; os índices T e F, são fase de topo e fundo, respectivamente.

### 2.3.3. Composição dos SABs

Devido às suas propriedades favoráveis os SABs têm sido considerados um método de processamento eficaz, que pode ser satisfatoriamente utilizado para a recuperação e purificação de diferentes tipos de solutos (KESSEL, 1981; ROGERS *et al.,* 1993; LI *et al.,* 2004; BORA *et al.,* 2005; ROSA *et al.,* 2011; ASENJO e ANDREWS, 2012; MANDAL e MANDAL, 2014; MATOS *et al.,* 2014; MOHAMED ALI *et al.,* 2014). Aplicações eficientes de SABs para estes fins dependem além das propriedades dos compostos a serem extraídos ou purificados, dos constituintes que formam os SABs. A capacidade em manipular as propriedades das fases é dependente dos constiuintes, pelo qual é determinante para se obter alta seletividade e coeficientes de partição adequados. Para este propósito, os constituintes devem ser cuidadosamente selecionados considerando a capacidade em formar duas fases aquosas imiscíveis, promovida pela adição de compostos solúveis em água. Atualmente uma série de compostos estão disponíveis comercialmente, tais como os polímeros, sais orgânicos e inorgânicos, solventes orgânicos, açúcares ou líquidos iônicos (CARDOSO *et al.*, 2014a; FERREIRA *et al.*, 2014; GLYK *et al.*, 2014; TAN *et al.*, 2014).

Além das propriedades físico-químicas relacionadas aos constituintes do SAB (que será abordada nos próximos tópicos), uma vez que o objetivo deste processo é aplicado à purificação e/ou separação de compostos de interesse industrial, é razoável considerar o custo comercial dos constituintes para diminuir os custos totais gastos na purificação. A Tabela 3, apresenta os

principais constituintes disponíveis atualmente para formar SABs e o seu respectivo valor de mercado.

Tabela 3: Principais constituintes disponíveis atualmente para formar sistemas aquosos bifásicos e o seu respectivo valor de mercado.

| Composto | Pureza | Quantidade | R$ | Empresa |
|---|---|---|---|---|
| PEG 300 | | 1 L | 326,00 | Fluka |
| PEG 600 | PA | 1 Kg | 215,00 | Sigma Aldrich |
| PEG 1000 à 50 % | - | 100 mL | 130,00 | Sigma Aldrich |
| PEG 1500 | PA | 1 Kg | 189.03 | Fluka |
| PEG 4000 | PA | 1 Kg | 105,70 | Sigma Aldrich |
| PEG 6000 | PA | 1 Kg | 105,50 | Sigma Aldrich |
| PEG 8000 | PA | 1 Kg | 203.70 | Sigma Aldrich |
| PPG 750 | PA | 500 g | 395,00 | Sigma Aldrich |
| PPG 1000 | PA | 500 g | 377,70 | Sigma Aldrich |
| PPG 2000 | PA | 500 g | 345,90 | Sigma Aldrich |
| Dextran 1000 | PA | 500 g | 663,70 | Fluka |
| $K_2HPO_4$ | ≥ 98 % | 1 Kg | 170,50 | Sigma Aldrich |
| $KH_2PO_4$ | ≥ 99 % | 1 Kg | 355,00 | Sigma Aldrich |
| $K_3PO_4$ | ≥ 98 % | 1 Kg | 210,00 | Sigma Aldrich |
| $Na_2SO_4$ | ≥ 99 % | 1 Kg | 95,00 | Sigma Aldrich |
| $K_2CO_3$ | ≥ 99 % | 1 Kg | 203,50 | Sigma Aldrich |
| $(NH_4)_2SO_4$ | ≥ 99 % | 1 Kg | 382,00 | Sigma Aldrich |
| NaCl | ≥ 99 % | 1 Kg | 173,00 | Sigma Aldrich |
| HEPES | ≥ 99,5 % | 25 g | 162,00 | Sigma Aldrich |
| Etanol | ≥ 99,5 % | 500 mL | 50,00 | SIAL |
| Metanol | ≥ 99,8 % | 1 L | 164,30 | Sigma Aldrich |
| *n*-propanol | ≥ 99,5 % | 1 L | 162,20 | ACS reagent |
| THF | PA | 1 L | 67,00 | SIAL |
| $[C_2mim][CH_3CO_2]$ | ≥ 90 % | 100 g | 740,00 | BASF |
| $[C_2mim][BF_4]$ | ≥ 98 % | 100 g | 620.00 | BASF |
| $[C_4mim][CH_3CO_2]$ | ≥ 95 % | 100 g | 705,50 | BASF |
| $[C_4mim]Cl$ | ≥ 98 % | 50 g | 578,00 | Sigma Aldrich |
| $[C_4mpip][BF_4]$ | ≥ 99 % | 50 g | 985.00 | Sigma Aldrich |
| $[C_4mpy]Cl$, | ≥ 97 % | 5g | 126,50 | Sigma Aldrich |
| $[C_4mpyr]Cl$, | ≥ 99 % | 50 g | 720.50 | Fluka |
| [Ch]Cl | ≥ 98 % | 1 Kg | 510,00 | Sigma Aldrich |
| [Ch][Bit] | ≥ 98 % | 500 g | 504,00 | Sigma Aldrich |
| [Ch][DHCit] | ≥ 98 % | 1 Kg | 509,00 | Sigma Aldrich |

** todos estes compostos foram cotados no dia 25/08/2014 pelo Sigma Aldrich.*

*Polímeros e sais*

A maioria dos processos de extração exploraram sistemas de duas fases do tipo polímero-sal, polímero-polímero e sal-sal, chamados de SABs convencionais (ou tradicionais). Nos sistemas de polímero-sal, o polietilenoglicol (PEG) é o mais utilizado, embora há relatos de SABs com dextrana, maltodextrana entre outros. Para a classe dos sais, os fosfatos são os mais comumente utilizados, seguidos do sulfato de sódio, citrato de sódio ou sulfato de magnésio (ZASLAVSKY, 1995; RUIZ-RUIZ *et al.,* 2012).

O polietilenoglicol, ($HO–(CH_2CH_2O)_n– CH_2CH_2OH$), é um poliéter neutro, solúvel em água e na maioria dos solventes orgânicos, de grande importância comercial (GUAN *et al.,* 1992), e produzido em grandes quantidades e com massas molares variando de poucas centenas a milhares de Daltons. Esta molécula, apesar de simples, é foco de grande interesse para a comunidade científica, biotécnica e biomédica. Isto porque o polietilenoglicol (PEG) é eficiente na exclusão de outros polímeros quando presente em ambientes aquosos. Além disso, PEG não é tóxico e em certas concentrações, ou de massa molelucar mais baixo, não desnatura proteínas ou células ativas, embora interaja com membranas celulares (GUAN *et al.,* 1992). Dentre as principais propriedades têm-se que o PEG é solúvel em água, tolueno, cloreto de metileno, e muitos outros solventes orgânicos; é insolúvel em etiléter, hexano, e etilenoglicol; insolúvel em água a elevadas temperaturas; forma complexos com cátions metálicos; facilmente sujeito a modificações químicas; possui um grande volume de exclusão em água; pode ser usado para precipitar proteínas e ácidos nucléicos; e é atóxico (FORCINITI *et al.,* 1991b; LI *et al.,* 2003).

A composição dos sais nos SAB é de grande relevância para o sucesso de separação das biomoléculas. Usualmente são utilizadas soluções tampão com a finalidade de manter o pH no valor desejado, uma vez que o pH altera o comportamento de partição. Entretanto, quando é desejável a partição de determinados compostos presentes, outros tipos de sais podem ser adicionados visando uma boa partição, sem a necessidade de utilização de um ligante específico (RITO-PALOMARES, 2004). Sistemas com diferentes sais podem apresentar significativas mudanças nos coeficientes de partição para um mesmo soluto. Isso significa que os íons têm diferentes afinidades pelas duas fases (FRANCO *et al.,* 1990). Os sais de fosfato possuem maior afinidade pela fase inferior do SAB à base de polímero + sal, já o sal de lítio tem maior afinidade pela fase superior e o NaCl tem afinidade similar entre as duas fases. Portanto, é de grande importância a seleção ideal do sal a ser adicionado ao sistema, pois eles terão grande influência na diferença de potencial elétrico entre as fases (ALBERTSSON, 1990).

Normalmente a escolha do sal é auxiliada pela série de Hofmeister (ou série liotrópica), que classifica os íons (cátions e ânions) com a capacidade em induzir *salting-in* ou *salting-out* de proteínas, ou seja, a posição de um íon na série Hofmeister é determinada essencialmente pelo seu grau de hidratação (Figura 9) (HOFMEISTER *et al.,* 1988). Entretanto, estes sais também podem interagir diretamente com a estrutura proteica alterando o equilíbrio químico das proteínas. Desta forma, sais mais caotrópicos podem desnaturar as proteínas, ao passo que sais mais cosmotrópicos podem estabilizar as proteínas (ZHOU, 2005; ZHANG *et al.,* 2006). Assim, a série de Hofmeister é geralmente utilizada para explicar a capacidade dos íons em formar duas fases aquosas, resultado de sua capacidade de hidratação (SILVÉRIO *et al.*, 2013).

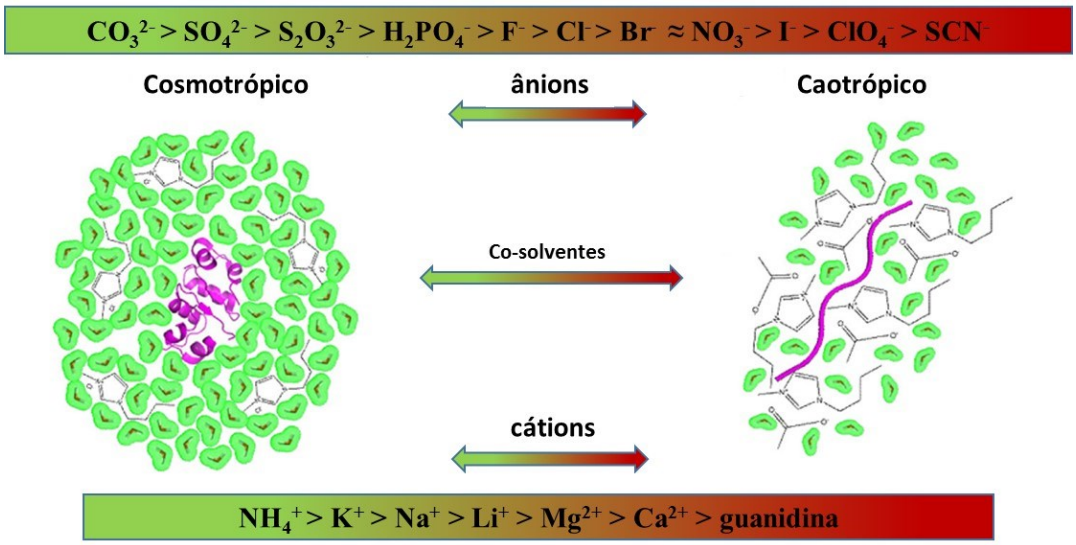


Figura 9: Estabilização das proteínas com base na série de Holfmeister. Adaptado de: http://tinyurl.com/ed5gj

Portanto, SABs convencionais formados pela mistura de polímeros e sais são reconhecidos desde 1980 como sistemas biocompatíveis às células, organelas e substâncias biologicamente ativas, por apresentar propriedades físico-químicas que os tornan bons sistemas a serem aplicados na recuperação e purificação de biomoléculas (STURESSON *et al.,* 1990; ANDREWS *et al.,* 2005). As vantagens associadas ao uso destes constituintes incluem baixa tensão interfacial, taxa de separação rápida e baixo custo (ASENJO e ANDREWS, 2012; RUIZ-RUIZ *et al.,* 2012). Foram plicados para a extração e purificação de diferentes enzimas incluindo celulases (HERCULANO *et al.*, 2012), colagenases (ROSSO *et al.*, 2012; LIMA *et al.*, 2013), poligalacturonases (MACIEL *et al.*, 2014), α-galactosidases (NAGANAGOUDA *et*

*al.*, 2008), lacases (SILVÉRIO *et al.*, 2013; PRINZ *et al.*, 2014), pectinases (LIMA *et al.*, 2002; MACIEL *et al.*, 2014), fitases (NEVES *et al.*, 2012), proteases (DE MEDEIROS E SILVA *et al.*, 2013) e tanase (RODRÍGUEZ-DURÁN *et al.*, 2013) e lipases (BRADOO *et al.*, 1999; BASSANI *et al.*, 2010; SOUZA *et al.*, 2010; BARBOSA *et al.*, 2011; ZHOU *et al.*, 2013).

SABs formados por PEG de diferentes massas moleculares (400 à 10000 $g.mol^{-1}$) e sal fosfato de potássio foram aplicados para a separação de enzimas pectinolíticas; após etapas de otimização, considerando o efeito da massa molecular do polímero, os fatores de purificação foram mais elevados para os sistemas com PEG de elevada massa molecular: PEG 6000 para a exo-poligalacturonase (5,49 vezes) e de PEG 10000 para a endo-poligalacturonase (16,28 vezes), pectinesterase (16,64 vezes) e pectina liase (14,27 vezes) (LIMA *et al.,* 2002). BIM e FRANCO (2000) observaram que o melhor sistema para a purificação de xilanase foi PEG 6000/fosfato de potássio (22 – 13 %, m/m), com incorporação de 12 % de NaCl. Os valores dos coeficientes de partição da enzima e das proteínas totais foram de 47 e 0,1, respectivamente. O fator de purificação e rendimento da enzima na fase de topo foi de 33 e 98 %, respectivamente. BRANDO *et al.,* (1999) aplicaram o sistema aquoso bifásico na separação de uma mistura de lipases em sistemas PEG 600/fosfato de potássio (70-40%, m/m) com utilização de 3 % (m/m) de NaCl a pH 7,0 e obtiveram rendimento de 5,27 e 15,35 para a lipase ácida e neutra, respectivamente. OOI *et al.,* (2009a), estudou o comportamento de partição da lipase de *Burkholderia pseudomallei* em SABs convencionais; as melhores condições para a purificação de lipase foram obtidas em um sistema com PEG 6000/fosfato de potássio utilizando uma TLL de 42,2% (v/v) com adição de 1% (v/v) de NaCl, a pH 7. Nestas condições, o fator de purificação da lipase foi aumentado para 12,42 vezes, com um elevado rendimento de 93%.

Nosso grupo de pesquisa realizou um trabalho pioneiro na separação e purificação de uma enzima lipolítica extracelular produzido por *Bacillus* sp. ITP-001 a partir do caldo de fermentação, utilizando SAB formado por PEG 8000 + fosfato de potássio (20 – 18 %, m/m) e onde a lipase foi purificada 201,53 vezes (BARBOSA *et al.,* 2011). Anteriormente SOUZA *et al.,* (2010) avaliou a influência da massa molecular e da concentração de PEG, na purificação da lipase pancreática suína (comercial). A enzima foi purificada de forma mais eficiente em PEG 8000 a 20 % (m/m) com um fator de purificação de 3,89 vezes; os autores consideraram os SABs extremamente eficientes por se tratar da purificação de uma lipase comercial pré-purificada. Pesquisas mais recentes também demonstraram a capacidade dos SAB à base de polímeros e sais para a purificação de outras enzimas. A tanase de *Aspergillus niger* foi purificada em sistemas aquosos em duas fases compostos por PEG com diversas massas

moleculares (400, 600 e 1000 g.mol$^{-1}$) e fosfato de potássio, e onde foi encontrado um bom desempenho uma vez que a recuperação da enzima na fase inferior do sistema composto por PEG 1000 foi cerca de 96% com um aumento de 7,0 vezes na sua pureza (RODRÍGUEZ-DURÁN *et al.,* 2013). SAB a 25 ºC e pH 7 formado por PEG 3000 e fosfato de sódio foi utilizado com sucesso para a purificação e separação da enzima lacase, resultando em um fator de purificação e rendimento na recuperação de atividade enzimática de 2,74 e 96%, respectivamente (PRINZ *et al.,* 2014).

Padillha *et al.,* (2012) estudou a partição da lipase produzida por *Burkholderia cepacia* em sistemas PEG/fosfato de potássio, e o fator de purificação foi de 209 vezes utilizando o PEG 1500. Sistema convencionais de PEG/fosfato de potássio (17-13 %, m/m) também foram aplicados à purificação da lipase pancreática suína (PPL), uma eficiência de extração de 94,7 % e um fator de purificação de cerca 4 vezes demonstram que SAB é um método altamente eficiente para a purificação de PPL (ZHOU *et al.,* 2013). SALES *et al.,* (2013) estudaram a melhor condição para a extração da lipase de *Bacillus* sp. UFPEDA 485 utilizando um SAB formado por PEG 8000/$Na_2SO_4$ (18-13 %, m/m). A partição da enzima foi para a fase de fundo (rica em sal) com valor do coeficiente de partição de 0.3. A lipase de *Rhodotorula glutinis* foi purificada em SAB à base de PEG 4000/oxalato de potássio (17.5-12.5 %,m/m), com um fator de purificação de 13,9 e rendimento da enzima para a fase de topo de 71.2% (KHAYATI *et al.,* 2013).

Apesar das vantagens intrínsecas destes sistemas mostrada por diversos pesquisadores, o seu desempenho é contudo significativamente afetado pela limitada faixa de polaridades das fases coexistentes, que é uma questão importante quando o objetivo é a extração de biomoléculas mais hidrofóbicas (LI *et al.,* 2005a; DREYER *et al.,* 2008; NEVES *et al.,* 2009; VENTURA *et al.,* 2009). Algumas abordagens foram desenvolvidas para superar esta limitação, tais como a funcionalização de polietilenoglicol (PEG) (ZALIPSKY, 1995; WU *et al.,* 2008) ou a inclusão de sal (HACHEM *et al.,* 1996; FERREIRA *et al.,* 2011). A primeira abordagem é focada na modificação da estrutura química do PEG, enquanto que a segunda é baseado na inclusão de um sal inorgânico adicional, manipulando a partição do soluto entre as duas fases aquosas. No entanto, deve-se observar o elevado custo associado com a funcionalização do polímero, limitando assim sua aplicação em escala industrial.

Outra dificuldade na utilização de sistemas convencionais em escala industrial, está relacionado ao uso de polímeros de alta viscosidade, ocasionalmente é formado uma solução

opaca interferindo na análise do composto extraído (COLLINS, 1997; SELBER *et al.,* 2001; ESPITIA-SALOMA *et al.,* 2014). Com uma abordagem interessante, recentemente um grande número de pesquisas foram desenvolvidas com o objetivo de melhorar a eficiência catalítica de biocatalisadores utilizando líquidos iônicos (KRAGL *et al.,* 2002; KLEMBT, 2007; NAUSHAD *et al.,* 2012; SINTRA *et al.,* 2014) e/ou solventes orgânicos em reações com enzimas (BOSE *et al.,* 2013; SHARMA e KANWAR, 2014).

*Solventes orgânicos*

A extração de compostos utilizando SABs à base de solventes orgânicos hidrofílicos (solúveis em água) pode ser uma alternativa vantajosa. Uma razão importante para isto está relacionada as propriedades físicas, como a alta viscosidade dos sistemas à base de polímeros (COLLINS, 1997; SELBER *et al.,* 2001; ESPITIA-SALOMA *et al.,* 2014). Atualmente na literatura, os trabalhos que envolvem solventes orgânicos para a formação de SABs incluem principalmente o etanol, *n*-propanol e acetonitrila (Tabela 4). A Figura 10, ilustra a capacidade de SABs à base de solventes orgânicos em concentrar compostos orgânicos para a fase de topo (rico em solvente orgânico) (TAHA *et al.,* 2012a).

Figura 10: Sistemas ternários formados por tampão biológico HEPES + diferentes solventes orgânicos: THF, 1,3-dioxolano, 1,4-dioxano, 1-propanol, 2-propanol, terc-butanol, acetonitrila e acetona da esquerda para a direita, respectivamente. Adaptado de TAHA *et al.* (2012a).

Tabela 4: Sistemas aquosos bifásicos à base de solventes orgânicos.

| SAB | | Extração | Referência |
|---|---|---|---|
| Etanol; 2-propanol | $K_2HPO_4$; $Na_2SO_4$ | Glicirrizina de *Glycyrrhiza uralensis Fisch* (Glicosídeo) | (TAN *et al.,* 2002) |
| Etanol; *n*-propanol | $(NH_4)_2SO_4$; NaCl; $NaH_2PO_4$ | Ácido B de Salvianolic | (ZHI *et al.*, 2006) |
| Etanol; 1-propanol; 2-propanol | $(NH_4)_2SO_4$; citrato de sódio; $K_3PO_4$ | Lipase de *Burkholderia pseudomallei* | (OOI *et al.,* 2009b) |
| Etanol; 1-propanol; 2-propanol | $(NH_4)_2SO_4$ | Antraquinonas de *Aloe vera L.* | (TAN *et al.,* 2013) |
| Metanol; etanol; 1-propanol; 2-propanol | $K_3PO_4$; $K_2HPO_4$; $KH_2PO_4/K_2HPO_4$ | Vanilina; Rutina | (REIS *et al.,* 2012; REIS *et al.,* 2013) |
| 1-propanol | $(NH_4)_2SO_4$ | Allin de alho em pó | (JIANG *et al.,* 2014) |
| Etanol | $(NH_4)_2SO_4$ | Lignana de *Schisandra chinensis* | (GUO *et al.,* 2013) |
| Etanol; | $(NH_4)_2SO_4$; $NaH_2PO_4$; $K_3PO_4$ | Ácido clorogênico (ACG) | (TAN *et al.,* 2014) |
| Acetonitrila | D-glicose; sacarose; D-frutose | Ácido ferúlico; Ácido siríngico; Ácido p-cumárico | (WANG *et al.,* 2008) |
| Acetonitrila | D-glicose; D-manose; D-galactose; D-xilose; D-frutose; L-arabinose; D-maltose; Sacarose | Vanilina; | (CARDOSO *et al.*, 2013) |
| Acetonitrila | Glicerol; Eritritol; Xilitol; Sorbitol; Maltitol | Vanilina | (CARDOSO *et al.*, 2014a) |
| Acetonitrila | Dextranas (Dx-100; Dx-40; Dx-6) | Vanilina | (CARDOSO *et al.*, 2014b) |
| THF; 1,3-dioxolano; 1,4-dioxano; 1-propanol; 2-propanol; Terc-butanol; acetonitrila; acetona | HEPES | - | (TAHA *et al.,* 2012a) |
| Acetonitrila; Acetona | EPPS | - | (TAHA *et al.,* 2012c) |
| 1-propanol; 2-propanol; Terc-butanol | EPPS | - | (TAHA *et al.,* 2012b) |

Recentemente, o solvente tetrahidrofurano (THF) foi mostrado capaz de formar SABs com adição do tampão biológico HEPES (TAHA *et al.,* 2012a). Cabe ressaltar que esta foi a primeira vez que o THF foi utilizado para formar SAB. Mais recentemente, HIRAYAMA *et al.,* (2014) avaliou a capacidade do THF em induzir SAB com o LI $[C_4mim]Cl$ aplicado para a separação e extração de metais Fe (III) e Zn (II). O THF ganhou destaque nos últimos anos,

sendo amplamente utilizado pela indústria na fabricação de materiais para embalagem, transporte e armazenamento de alimentos (MÜLLER, 2000). O THF é um éter cíclico que apresenta excelente poder de solvatação para numerosas substâncias orgânicas, também é utilizado para a extração de biocompostos hortícolas, incluindo comercialmente valiosos como os carotenóides (SU *et al.,* 2002). Possui prótons acptores de oxigênio que formam pontes de hidrogênio com água, como resultado, este composto é completamente miscível em água a qualquer composição (PURKAYASTHA *et al.,* 2013).

SABs formados por solventes orgânicos hidrofílicos conjugado com uma solução de sal, têm sido propostos e utilizados para o estudo de partição de diferentes biomoléculas, como proteínas, aminoácidos e outros produtos naturais (LOUWRIER, 1998; TAN *et al.*, 2002; ZHI e DENG, 2006; OOI *et al.*, 2009b; REIS *et al.*, 2013; TAN *et al.*, 2013; CARDOSO *et al.*, 2014a). A Tabela 4 apresenta alguns outros solventes orgânicos conjugados com outros compostos para formar SAB, e em alguns casos, também é mostrado sua aplicação para extração de biomoléculas.

Por vezes, estes SABs apresentam vantagens que incluem a rápida separação de fases, alta eficiência de extração, baixa viscosidade, alta polaridade, baixo custo e fácil reciclagem (OOI *et al.,* 2009b; LI *et al.,* 2011). No entanto, para o desenvolvimento de um processo eficiente de extração, é necessário encontrar uma combinação adequada entre o solvente orgânico e a biomolécula alvo, uma vez que estes compostos orgânicos podem causar tanto a desnaturação de uma proteína, como a ativação em outras (JOHNSON, 1986; MARTÍNEZ-ARAGÓN *et al.,* 2009; SHARMA e KANWAR, 2014).

A partir de estudos de proteínas em sistemas monofásicos (por exemplo, catálise enzimática em meios não-aquosos e em água com misturas de solventes), como regra geral, os solventes mais polares podem causar maior desnaturação. O indicador de polaridade mais indicado é o log *P* (LAANE *et al.,* 1987; KHMELNITSKY *et al.,* 1991; GUPTA, 1992). O log *P* é um logaritmo com base no coeficiente de distribuição (*P*) de um soluto entre um solvente e a água em um sistema bifásico. O mais comum é o log $P_{ow}$, baseia-se no solvente 1-octanol, o qual indica a medida da hidrofobicidade de um soluto (KHMELNITSKY *et al.,* 1991). De acordo com KLIBANOV (2001), solventes menos hidrofóbicos (log $P < 2$) causam maior desnaturação em enzimas, devido às fortes ligações com água, que retira as moléculas de água essenciais para as enzimas. É bem conhecido que a água atua como um lubrificante que permite uma elevada flexibilidade conformacional das moléculas proteicas. Por outro lado, quando os

solventes com valores de log *P* elevados (log *P* > 4) são utilizados, há menos impacto sobre a atividade da proteína (MARTÍNEZ-ARAGÓN *et al.,* 2009). Entretanto em alguns casos, como o demonstrado por RAHMAN *et al.,* (2005) ao avaliar a estabilidade da lipase *Pseudomonas* sp. cepa S5, observou que os solventes orgânicos não seguiram as tendências de log *P*, a atividade mais elevada foi obtida com ciclo-hexano (log *P* 3,2), benzeno (log *P* 2,0), *n*-hexano (log *P* 3,6), 1-decanol (log *P* 4,0) e n-decano (log *P* 5,6), com o aumento das atividades relativas de 31,8, 30,6, 18,9, 3,48 e 4,51 %, respectivamente, após 30 min de incubação. OGINO *et al.,* (2000) relataram que a protease de *Pseudomonas aeruginosa* H-01 é muito estável na presença de etanol ou metanol com valor de log *P* inferior a 2,0. Atividade da lipase de *Streptomyces* sp. CS133 foi significativamente aumentada na presença de solventes orgânicos (a 25 %, v/v) com log *P* ≥ 0,87 (exceto para o caso de decano, em que a atividade foi minimamente inibida), para os casos particulares do éter etílico (log *P* 0,87), diclorometano (log *P* 1,25) e hexano (log *P* 3,5), as atividades relativas foram 123, 129 e 179 %, respectivamente (MANDER *et al.,* 2012).

De uma forma geral é possível encontrar vários relatos na literatura relacionados com a estabilidade de proteínas em meios aquosos contendo solventes orgânicos, como o demonstrado por OOI *et al.,* (2009b) em que a lipase de *Burkholderia* pode manter a atividade catalítica altamente estável em solventes orgânicos, como o etanol, 1-propanol e 2-propanol, com atividade relativa superior a 99,5 %, em soluções a 40 % (v/v). A estabilidade da lipase de *Pseudomonas aeruginosa* LST-03 em meios aquosos de *n*-decano, *n*-octano, dimetilsulfóxido (DMSO) e N,N-dimetilformamida (DMF), mostraram em média atividade relativa da lipase superior a 90 % (OGINO *et al.,* 2000). As lipases de *Bacillus* também foram reportadas como tolerantes a alguns solventes orgânicos. SULONG *et al.,* (2006), observou uma boa tolerância da lipase de *Bacillus sphaericus* 205y em 25 % (v/v) de DMSO, metanol, etanol, p-xileno, *n*-hexano e *n*-decano, com valores de atividade relativa sempre superiores a 96 %. BARBOSA *et al.,* (2012), verificou que a atividade relativa da lipase de *Bacillus* sp. ITP-001 foi sempre superior 94,5 % em isopropanol, piridína, etanol, metanol, acetona e acetonitrila a 30 % (v/v).

Portanto, devido à grande variedade de solventes orgânicos hidrofílicos é essencial avaliar sua capacidade de interagir com as proteínas, visto que o efeito de solventes orgânicos sobre a atividade da enzima é único para cada proteína (RAHMAN *et al.,* 2005; SHARMA e KANWAR, 2014). Outrossim, é mostrado que os SAB são uma técnica de extração eficiente para uma grade variedade de solutos, e que a compreensão dos fenômenos de interação entre os constituintes das fases e as biomoléculas de interesse são os principais desafios para uma boa extração.

*Líquidos Iônicos (LIs)*

Os líquidos iônicos (LIs) são solventes orgânicos inteiramente compostos por íons, sendo definidos como sais de cátions orgânicos de grandes dimensões e com baixo ponto de fusão (geralmente inferiores a 100 ºC). Uma grande quantidade de LIs são líquidos à temperatura ambiente permitindo combinações mútuas das propriedades apresentadas tanto dos sais, como dos solventes orgânicos (WILKES, 2002). Os LIs ganharam uma grande atenção da academia e da indústria desde a década de 1990, devido a uma série de propriedades interessantes que incluem baixa pressão de vapor, não inflamabilidade, boa estabilidade química e térmica, e boa capacidade de solvatação (KULKARNI *et al.,* 2007). O custo é o principal fator negativo na utilização dos líquidos iônicos, embora possam ser reciclados e reutilizados dependendo do processo aplicado (BATCHELOR *et al.,* 2009; ALVAREZ-GUERRA *et al.,* 2014; CLAUDIO *et al.,* 2014).

Suas propriedades físico-químicas podem ser ajustáveis para uma determinada aplicação pela variação da combinação dos cátions e ânions ou pela junção de grupos funcionais na constituição dos íons. O número estimado de combinações disponíveis de ânions e cátions é maior do que 1 milhão (ROGERS *et al.*, 2003). A Figura 11 mostra alguns exemplos de cátions e ânions utilizados para formar os LIs. Em virtude destas propriedades, os LIs cobrem praticamente todo o intervalo de hidrofilicidade e hidrofobicidade (NAUSHAD *et al.,* 2012). Entre eles, os cátions mais comuns são os imidazólios, piridínios, piperidínios, pirrolidínios, amônios, fosfônios e colinas que podem ser combinados com uma grande variedade de ânions orgânicos ou inorgânicos. Na maioria dos cátions, os grupos substituintes são cadeias alquílicas de tamanho variável que também podem adicionadas (HUDDLESTON *et al.,* 2001; HOLBREY *et al.,* 2002).

Os LIs vêm sendo pesquisados para uma variedade de aplicações biotecnológicas incluindo reações e biocatálise, em biossensores e em tecnologias de separação, como por exemplo na purificação de aminoácidos e enzimas do tipo álcool desidrogenase (PARK *et al.,* 2003; VAN RANTWIJK *et al.,* 2003; DREYER e KRAGL, 2008; KAPOOR *et al.,* 2012; NAUSHAD *et al.,* 2012). Além disso, o uso de líquidos iônicos como solventes para reações químicas também revela suas excelentes propriedades físico-químicas, como elevada solubilidade em solventes orgânicos polares e apolares, solventes inorgânicos e materiais poliméricos (WILKES, 2002; FUKAYA *et al.*, 2007; FREIRE *et al.*, 2010; GARCIA *et al.*, 2010; HALLETT *et al.*, 2011; FREIRE *et al.*, 2012; STEPANKOVA *et al.*, 2013).

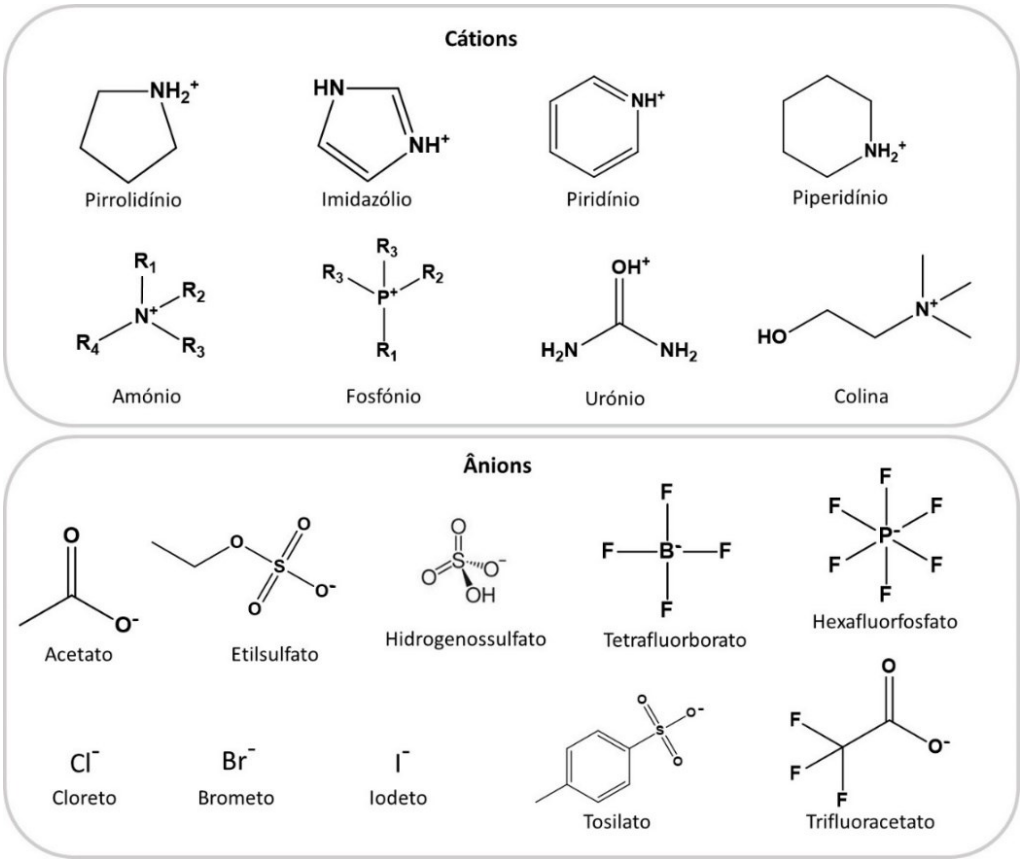


Figura 11: Estrutura química dos cátions e ânions utilizados para formar LIs.

Neste sentido, ROGERS e colaboradores (2003) foram os primeiros a mostrar que uma mistura de uma solução aquosa de cloreto de 1-butil-3-metilimidazólio ([$C_4$mim]Cl – Figura 12) e $K_3PO_4$ poderia formar SAB com uma fase superior rica em LI e uma fase inferior rica em sal. Em sistemas aquosos compostos de LIs, sal inorgânico e água, a força motriz para a separação de fases é a competição entre as moléculas de LI e sal para as moléculas de água. A maior afinidade do sal inorgânico com a água induz a migração da água para "longe" dos íons do IL, diminuindo sua hidratação e reduzindo a solubilidade do LI em água (BRIDGES *et al.*, 2007; NEVES *et al.*, 2009; VENTURA *et al.*, 2009; FREIRE *et al.*, 2012). Os sais inorgânicos mais utilizados, e classificados como fortes indutores de *salting-out* com ânions de cargas múltiplas como fosfatos, sulfatos, carbonatos ou citratos (BRIDGES *et al.,* 2007; ZAFARANI-MOATTAR *et al.,* 2009; VENTURA *et al.,* 2011; MARQUES *et al.,* 2013).

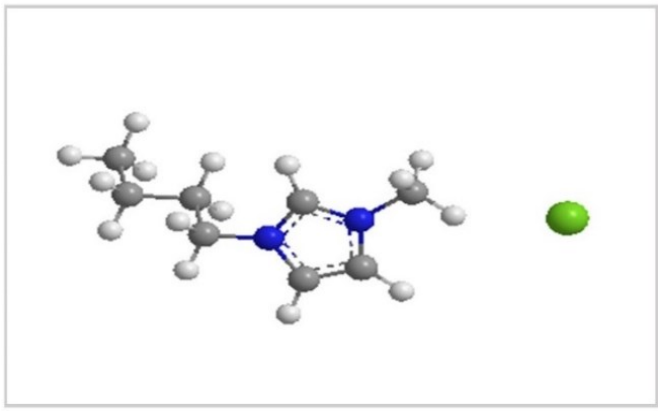

Figura 12: Estrutura molecular do primeiro líquido iônico utilizado para formar SAB – [$C_4$mim][Cl], cloreto de 1-butil-3-metilimidazólio.

A grande variedade de produtos extraídos utilizando SABs à base de LIs vão desde simples compostos como os aminoácidos (PEREIRA *et al*., 2013) a compostos mais complexos como é o caso das proteínas e enzimas (PEI et al., 2010; VENTURA et al., 2012a). Outros exemplos de compostos incluem a extração de drogas, compostos fenólicos, alcalóides, antibióticos, compostos anti-inflamatórios e corantes naturais (LI *et al.*, 2005b; DREYER e KRAGL, 2008; FREIRE *et al.*, 2012; SHIRI *et al.*, 2013; YUE *et al.*, 2014). A elevada seletividade das fases e os altos rendimentos na recuperação dos produtos são possíveis em função das diferentes combinações entre cátions e ânions, capazes de controlar as propriedades físico-químicas das fases coexistentes (NEVES *et al.,* 2009; VENTURA *et al.,* 2009). O grande número trabalhos utilizando SABs à base de LIs para extração de diferentes biomoléculas, revelou ser uma estratégia interessante para superar as limitações relacionadas aos SABs convencionais formados por polímeros ou sais (LI *et al.*, 2005a; DREYER e KRAGL, 2008; NEVES *et al.*, 2009; VENTURA *et al.*, 2009; FREIRE *et al.*, 2012). Ao contrário dos SABs à base de polímeros e sais, eles não sofrem de alta viscosidade e exibem uma faixa muito mais ampla de polaridade (COLLINS, 1997; SELBER *et al.,* 2001; NAUSHAD *et al.,* 2012).

PEI *et al.,* (2010) estudaram o comportamento de partição da albumina de soro bovino (BSA) a diferentes temperaturas em sistemas de [$C_4$mim]Br + $K_2HPO_4$ e [$C_4$mim][$N(CN)_2$] + $K_2HPO_4$, o fator de purificação foi de 6,96 e com uma eficiência de extração de 82,7 – 100,7%, respectivamente. DU *et al.,* (2007) mostrou que o fator de purificação da BSA em SAB composto de [$C_4$mim]Cl + $K_2HPO_4$ foi de 20 vezes, e com eficiência de extração superior a 90%. A proteína penicilina G foi utilizada por LIU *et al.,* (2005; 2006), em dois diferentes

trabalhos, com rendimentos de extração na ordem de 90,8 – 93,7% utilizando SAB composto de $[C_4mim][BF_4]$ e $NaH_2PO_4.2H_2O$ (pH 4-5).

Para tentar descrever os fenômenos que governam a migração das proteínas em SABs à base de LI de cátion imidazólio, DREYER et al. (2009) e DU *et al.,* (2007) indicaram que interações eletrostáticas que ocorrem entre os resíduos aminoácidos carregados negativamente na superfície das proteínas com o cátion imidazólio do líquido iônico é a principal força motriz para a extração de proteínas. Além disso, PEI *et al.,* (2009) sugeriu que a migração das proteínas em sistemas com líquidos iônicos é favorecida por interações hidrofóbicas, em função de interações π-π entre os resíduos aromáticos dos aminoácidos e o cátion imidazólio do líquido iônico. LIN *et al.,* (2013) investigou sistematicamente o coeficiente de partição e a eficiência de extração de quatro proteínas (albumina sérica bovina (BSA), hemoglobina (Hb), tripsina (Try) e lisozima (Lys) em SAB formado por $[C_8mim]Br + K_2HPO_4$. Os autores concluíram que o processo de migração das proteínas foi determinado por interações hidrofóbicas (efeito *salting-out*), mas que a interação eletrostática também desempenhou um papel importante relacionado com a eficiência de extração.

RUIZ-ANGEL *et al.,* (2007) compararam a eficiência de extração de quatro proteínas (citocromo c, mioglobina, ovalbumina e hemoglobina) usando SAB à base em LIs com os SAB à base de PEG. Os coeficientes de partição dos sistemas compostos de LIs foram de 2 a 3 ordens de grandeza maior do que os obtidos com sistemas baseados em PEG, e atribuiu estes resultados de partição há diferença de polaridade entre as duas fases dos SABs à base de LIs. CAO *et al.,* (2008) descreveram a eficácia de extração (superior a 80 %) da peroxidase de raiz forte utilizando o sistema $[C_4mim]Cl + K_2HPO_4$. Além disso, os autores concluíram que em comparação com SABs convencionais, o sistema à base de $[C_4mim]Cl$ possui viscosidade muito mais baixa.

Especialmente para as lipases, que representam a maioria das enzimas utilizadas na síntese orgânica, é essencial que a atividade catalítica deva ser preservada, ou até mesmo aumentada para uma melhor eficiência do biocatalizador, portanto é importante que as interações entre as soluções aquosas de LIs e de enzimas sejam equilibradas, o suficiente para interagir com as enzimas e extrai-las, mas não forte o suficiente para desconfigurar a sua estrutura e/ou interagir com seu sítio ativo (FREIRE *et al.*, 2012). Para isto, o uso de SABs com LIs mais hidrofílicos são susceptíveis a serem mais adequados para o isolamento de proteínas (LIU *et al.,* 2005; DU *et al.,* 2007; RUIZ-ANGEL *et al.,* 2007; CAO *et al.,* 2008; DREYER e

KRAGL, 2008; DREYER *et al.,* 2009; PEI *et al.,* 2009; PEI *et al.,* 2010), uma vez que a toxicidade destes LIs está diretamente relacionada com a sua hidrofobicidade (COULING *et al.,* 2006; VENTURA *et al.,* 2012b; VENTURA *et al.,* 2013). O que significa que LIs hidrofílicos possuem pelo menos, toxicidades mais baixas do que os seus homólogos mais hidrofóbicos (WEBACESSO, 2014). Além disto, o uso de LIs hidrofóbicos em processos de extração em grande escala será limitado devido aos custos financeiros e ambientais, uma vez que geralmente contêm ânions fluorados com elevado custo e não estáveis. Diante dessa ressalva, o potencial para aplicar líquidos iônicos hidrofílicos em processos de separação é muito maior devido à disponibilidade de ânions mais "verdes" (FREIRE *et al.*, 2012).

Assim, LIs hidrofílicos foram aplicados em SABs para a separação da lipase de *Thermomyces lanuginosus* (TL), lipase A de *Candida antarctica* (CaLA), lipase B de *Candida antarctica* (CaLB), e lipase de *Bacillus* sp. ITP-001 (DEIVE *et al.,* 2011; VENTURA *et al.,* 2011; DEIVE *et al.,* 2012; VENTURA *et al.,* 2012a). DEIVE *et al.,* (2011) estudou SABs formados por LIs de diferentes cátions e ânions para a separação da lipase A de *Candida antarctica*; a eficiência de extração da lipase foi quase que completamente alcançada (($EE$ = 99 %) utilizando o sistema de $[C_2mim][C_4SO_4]$ combinado com sal $K_2CO_3$. Estudos posteriores de DEIVE et al. (2012), para a extração da lipase CaLA em SABs formados por LIs à base de cátions imidazólios e envolvendo diferentes ânions (alquilsulfato e alquilsulfonato) foram avaliados e os melhores resultados de recuperação da enzima ($EE$ = 99 %) foram conseguidos com o sistema $[C_2mim][C_4SO_4] + (NH_4)_2SO_4$. A lipase comercial CaLB foi também purificada em SAB à base de LIs, avaliando diferentes cátions, ânions e o comprimento da cadeia alquílica lateral do cátion imidazólio o melhor fator de purificação (2,6 vezes) foi encontrado para o sistema formado com o LI $[C_8mim]Cl$ (VENTURA *et al.,* 2011). Em todos estes casos, o LI com cátion imidazólio provou ser o mais eficiente para a extração ou purificação das lipases. Além disso, DABIRMANESH *et al.,* (2012) observou que a presença do ânion $Cl^-$, conjugado com cátion imidazólio $[mim]^+$, levou a uma maior estabilidade da enzima desidrogenase *Thermoanaerobacter brockii* (TBADH). Os autores concluíram que as moléculas proteicas desta enzima são eletroforeticamente estáveis em presença de LIs com ânion cloreto (RAWAT *et al.,* 2012).

Mais recentemente, nosso grupo de pesquisa utilizou SAB à base de LI/sal para a purificação de uma enzima lipolítica extracelular produzido por *Bacillus* sp. ITP-001. Diferentes LIs foram estudados como o $[C_4mim][N(CN)_2]$, $[C_4mpyr]Cl$, $[C_4mim]Cl$ e $[C_8mim]Cl$ com adição do tampão fosfato de potássio (pH 7). Ao final do processo de

otimização a lipase foi purificada 51 vezes utilizando o SAB formado por [$C_8$mim]Cl + sal (VENTURA *et al.,* 2012a). A lipase de *Bacillus* sp. ITP-001 vem sendo estudada por nós desde o ano de 2008, e a primeira etapa do estudo foi direcionada às condições de estabilidade e à capacidade do micro-organismo em produzir lipases, descritas por CARVALHO *et al.,* (2008). Em 2011 a lipase de *Bacillus* sp. ITP-001 foi pela primeira vez purificada utilizando SABs convencionais PEG/sal, com um fator de purificação de 201,53 vezes (BARBOSA *et al.,* 2011).

Apesar da maior capacidade de extração dos SABs formados por LIs comparados aos SABs convencionais à base de polímeros, como relatado por RUIZ-ANGEL *et al.,* (2007), a casos em que esta verdade não é absoluta, como o mostrado por nós sobre a purificação da lipase de *Bacillus* sp. ITP-001. Em condições otimizadas a purificação foi maior utilizando SAB convencionais à base de polímeros (PF ≈ 201 vezes) (BARBOSA *et al.,* 2011), que em SABs à base de LIs (PF ≈ 51 vezes) (VENTURA *et al.,* 2012a). Portanto, é evidente que as vantagens oferecidas pelos sistemas convencionais para processos de purificação de biomoléculas, apresentadas no tópico anterior, são importantes a nível de interações soluto-solvente e devem também ser consideradas.

*LI como adjuvante*

Uma nova abordagem para processos de extração e/ou purificação utilizando SABs à base de polímeros e sais foi a introdução adicional de um composto iônico, demonstrado pela primeira vez por PEREIRA *et al.,* (2010). Estes sistemas quaternários são formados por polímero, sal, água e também por líquidos iônicos, no entanto em quantidades relativamente menores [5 % (m/m) da massa total do SAB] que os outros constituintes do sistema (comumente entre 20-60 %, m/m). A utilização dos SABs com LIs como adjuvantes é uma alternativa aos problemas relacionados ao uso de LI hidrofílicos, visto que são necessárias grandes concentrações dos constituintes (LIs, polímeros e/ou sais) para promover a formação de fases, tornando o processo de extração mais dispendioso e menos sustentável. Além disso como visto anteriormente, sistemas à base de polímeros apresentam uma limitada faixa de polaridade o que impede a extração de determinadas biomoléculas para a fase rica em polímero (LI *et al.,* 2005a; DREYER e KRAGL, 2008; NEVES *et al.,* 2009; VENTURA *et al.,* 2009).

Sistemas PEG/sal utilizando diferentes LIs como adjuvantes apresentaram em sua grande maioria coeficiente de partição acima de 1 (Tabela 5), indicando uma preferencial migração do LI para a fase rica em PEG. Desta forma, a adição de LI no SAB forma um complexo PEG +

LI alterando as propriedades físico-químicas da fase, podendo ser ajustada para se obter elevados rendimentos de extração (WU *et al.,* 2008; JIANG *et al.,* 2009; PEREIRA *et al.,* 2010).

Tabela 5: Coeficiente de partição dos LIs utilizados como adjuvantes em sistemas PEG/sal.

| Sistema PEG/sal | LI (5 %, m/m) | $K_{LI}$ | Referência |
|---|---|---|---|
| PEG 600/$Na_2SO_4$ | [im]Cl | 0,48 | (PEREIRA *et al.,* 2010) |
| | [$C_1$mim]Cl | 2,23 | |
| | [$C_2$mim]Cl | 4,80 | |
| | [$C_4$mim]Cl | 7,04 | |
| | [$C_7H_7$mim]Cl | 15,02 | |
| | [$C_4$C1mim]Cl | 13,80 | |
| | [amim]Cl | 2,91 | |
| | [$OHC_2$mim]Cl | 2,21 | |
| | [$C_4$mim][$HSO_4$] | 6,64 | |
| | [$C_4$mim][$CH_3$CO2] | 7,34 | |
| | [$C_4$mim][$MeSO_4$] | 8,74 | |
| PEG 300/$Na_2SO_4$ | [$C_4$mim][TOS] | 9,4 ± 0,5 | (ALMEIDA *et al.,* 2014) |
| | [$C_4$mim][SCN] | 7,6 ± 0,2 | |
| | [$C_4$mim][$N(CN)_2$] | 4,6 ± 0,3 | |
| | [$C_4$mim][$CH_3CO_2$] | 4,4 ± 0,4 | |
| | [$C_4$mim]Cl | 7,1 ± 0.9 | |
| | [$C_4$mpyr]Cl | 0,9 ± 0,2 | |
| | [$C_4$mpip]Cl | 1,9 ± 0,1 | |

Os LIs que apresentam ânions aromáticos adicionais, como o [$C_4$mim][TOS], ou que apresentem π elétrons do cátion imidazólio, são mais fortemente migrados para a fase rica em PEG comparado aos LIs não aromáticos (ALMEIDA *et al.,* 2014). SARKAR et al., (2008) ao estudar um sistema binário composto por tetraetilenoglicol (TEG) e o LI 1-butil-3-metilimidazólio ([$C_4$mim][$PF_6$]) propôs a "hiperpolarização" do polímero devido a formação de complexos envolvendo o cátion [$mim^+$] e o átomo de oxigênio do TEG, assim como interações entre o ânion [$PF_6^-$] e os grupos hidroxilas terminais, desta forma exibindo polaridades aparentes acima das próprias fases puras. Além disso, o aumento da cadeia alquílica lateral do LI aumenta seu caráter hidrofóbico, promovendo maior miscibilidade com a fase mais hidrofóbica rica em PEG.

Atualmente apenas dois trabalhos demonstram a aplicação destes SABs para a extração de biomoléculas, apesar de serem compostos mais simples, como aminoácidos e compostos fenólicos (PEREIRA *et al.,* 2010; ALMEIDA *et al.,* 2014). PEREIRA *et al.,* (2010) demonstrou que a adição de 5% (m/m) em SAB formado por polímero + sal é capaz de modificar as polaridades das duas fases aquosas, conduzindo a processos de separação do L-triptofano mais vantajosos, com a melhoria dos parâmetros de extração (coeficiente de partição e eficiência de extração). SABs à base de PEG + $Na_2SO_4$ utilizando ILs como adjuvantes (5 ou 10%, m/m) foram estudados para a extração dos ácidos gálico, vanílico e siríngico. Os melhores resultados foram observados com adição de apenas 5% de IL, com eficiências de extração que variaram entre 80% e 99% (ALMEIDA *et al.,* 2014). Estes resultados demonstram claramente a capacidade do IL em ajustar a polaridade da fase rica em PEG, sendo o principal responsável por controlar a extração dos antioxidantes (ALMEIDA *et al.,* 2014). Outrossim, a adição de pequenas quantidades de LI foi favorável a formação de sistemas líquido-líquido de duas fases, ou seja, o LI como adjuvante minimiza a concentração dos constituintes necessário para formar duas fases aquosas (PEREIRA *et al.,* 2010; ALMEIDA *et al.,* 2014).

*LIs à base de colinas*

Tradicionalmente SABs são formados por combinações de soluções aquosas de dois polímeros hidrofílicos ou, em alternativa, um polímero e um sal inorgânico (ZASLAVSKY, 1995; RUIZ-RUIZ *et al.,* 2012). Recentemente, como visto anteriormente outras espécies também ganharam destaque como é o caso dos LIs (GUTOWSKI *et al.,* 2003). Embora bons resultados tenham sido relatados utilizando SABs à base de LIs para extração de diferentes compostos, por outro lado a maioria LIs utilizados nestes trabalhos apresentam agumas limitações no que diz respeito a sua estabilidade térmica e química, custo financeiro, toxicidade e biodegradabilidade (YU *et al.,* 2008; LI *et al.,* 2009; THUY PHAM *et al.,* 2010; STOLTE *et al.,* 2012; VENTURA *et al.,* 2013).

Para superar estes desafios, a busca por LIs de baixo custo e mais seguros, do ponto de vista toxicológico e ambientalmente sustentável, ainda é uma questão fundamental. Para este propósito os LIs à base de colinas têm ganhado destaque para aplicações em SABs. Esta classe de LIs é derivado a partir de sais quaternários de amônio, descritos como estruturas importantes nos processos biológicos e utilizados como precursores para a síntese de vitaminas (vitamina B - tiamina) e enzimas que participam do metabolismo de glicídios (MECK *et al.,* 1999). O cloreto

de colina foi oficialmente reconhecida em 1998 pelo Instituto de Medicina (*Institute of Medicene - IOM*) como um nutriente essencial devido à sua ampla gama de funções no metabolismo humano (FOOD AND NUTRITION BOARD, 1998). É encontrado numa grande variedade de alimentos, entre as fontes mais concentradas estão os ovos, carnes e salmão (ZEISEL *et al.,* 2009).

Dada sua importância, recentemente alguns trabalhos têm demonstrado a síntese de novos LIs com o cátion colina ($[Ch]^+$) combinado com diferentes ânions (FUKAYA *et al.,* 2007; NOCKEMANN *et al.,* 2007; PERNAK *et al.,* 2007; COSTA *et al.,* 2012a). A Figura 13 mostra algumas das possíveis combinações de ânions com o cátion $[Ch]^+$. Esta classe de LI apresenta excepcionais propriedades compartilhadas dos LIs tradicionais (por exemplo com cátions imidazólios, piridínios e pirrolidínios), apresentando pressão de vapor despresível em condições ambientais e não-inflamabilidade, entretanto são considerados de baixo custo (uma vez que podem ser obtidos a partir de matérias-primas mais baratas), com baixa toxidade e ainda com excelente capacidade de biodegradação (FUKAYA *et al.,* 2007; VIJAYARAGHAVAN *et al.,* 2010; COSTA *et al.,* 2012a; SEKAR *et al.,* 2012; VENTURA *et al.,* 2014).

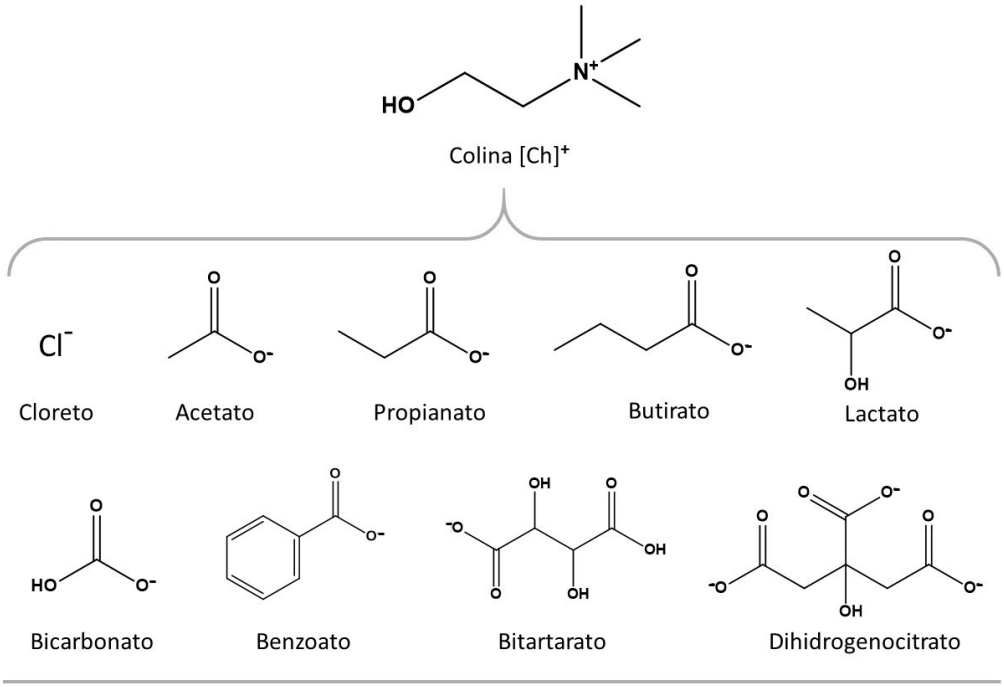


Figura 13: Principais ânions utilizados para formar líquidos iônicos com o cátion de colina.

Portanto, não surpreendentemente, o uso de LIs à base de colinas como alternativa para substituir os LIs tradicionais tem atraído o interesse científico para diferentes aplicações como, solvente para a absorção de amônia e $CO_2$, no pré-tratamento de material lignocelulósico, como co-substrato para micro-organismos na degradação de corantes, e no isolamento de suberina da cortiça (PALOMAR *et al.,* 2011; APARICIO *et al.,* 2012; BEDIA *et al.,* 2012; FERREIRA *et al.,* 2012; HOU *et al.,* 2012; SEKAR *et al.,* 2012). Além disso, contrário a alguns líquidos iônicos tradicionais que podem causar a desnaturação de proteínas, e a compreensão destes efeitos ainda é limitado (KUMAR *et al.,* 2014a), estudos demonstraram que LIs à base de colinas mostram boa capacidade em manter a estrutura catalítica ativa, e em alguns casos até melhoram a atividade de enzimas (FUJITA *et al.,* 2005; WEAVER *et al.,* 2010; LI *et al.,* 2012).

O interesse por estes compostos aplicados a processos de extração de biomoléculas utilizando SABs é visto como uma alternativa promissora e ainda pouco explorada (LI *et al.,* 2012; PEREIRA *et al.,* 2013; SHARIARI *et al.,* 2013; PEREIRA *et al.,* 2014). Além disso, misturas de LI à base de colinas com água ou solventes orgânicos ainda é pouco caracterizada o que dificulta a compreensão destes sistemas (CONSTANTINESCU *et al.,* 2007; ZHOU *et al.,* 2009; COSTA *et al.,* 2012b; KHAN *et al.,* 2014). Recentemente foi demonstrado a possibilidade de formar SABs pela combinação de LIs à base de colinas com polímeros ou sais inorgânicos (Tabela 6).

Tabela 6: Sistemas aquosos bifásicos formados por LIs à base de colinas.

| **Componente 1 / Líquido iônico** | **Componente 2** | **Referência** |
|---|---|---|
| [TCh][Cit] > [DCh][Oxe] > [Ch][Gly] > [Ch][Pro] ≈ [Ch][Lac] ≈ [Ch][Ac] > [Ch][But] | PPG 400 | (LI *et al.,* 2012) |
| [Ch][Lac] ≥ [Ch][Gly] > [Ch][Pro] > [Ch]Cl | PPG 1000 | (LIU *et al.,* 2013) |
| [Ch][Lac] | PPG 400 < PPG 1000 < $EO_{10}PO_{90}$ | |
| [Ch][Sal] > [BCh]Cl > [Ch][Lev] > [Ch][Glu] ≈ [Ch][Suc] ≈ [Ch][Ac] > [Ch]Cl | $K_3PO_4$ | (SHARIARI *et al.,* 2013) |
| [Ch][DHph] > [Ch][Bit] > [Ch][Bic] > [Ch][DHcit] ≈ [Ch][Ac] ≈ [Ch][Lac] ≈ [Ch][Gly] > [Ch]Cl | PEG 400; PEG 600 e PEG 1000 | (PEREIRA *et al.,* 2014) |
| [Ch][Sal]; [BCh]Cl | $K_2CO_3$; $KH_2PO_4/K_2HPO_4$; $C_6H_5K_3O_7/C_6H_8O_7$ | (SINTRA *et al.,* 2014) |

Assim, para avaliar a capacidade de extração destes novos sistemas LI *et al.,* (2012) estudou SABs formados por PPG 400 + LI à base de colina conjugado com diferentes ânions. Os resultados de eficiência de extração seguiram a seguinte tendência para as proteínas: BSA < tripsina < papaína < lisozima, os valores de recuperação foram entre 86.4 – 99.9 %. Um estudo complementar sobre o efeito da atividade da tripsina em meio aquoso com $[Ch]^+$ conjugado com diferentes ânions ($[Ac]^-$, $[Pro]^-$, $[But]^-$, $[Gly]^-$, $[Lac]^-$, $[Oxe]^-$, $[Cit]^-$) foi também investigado. Os resultados demonstraram que não houve perda da atividade enzimática, provavelmente devido à capacidade destes LIs em realizar ligações por pontes de hidrogênio com as proteínas (LI *et al.,* 2012). Estes resultados são importantes também para comparar a estabilidade da tripsina, em soluções aquosas de LIs à base de cátions imidazólios. Em sistemas IL+ sais apenas 88-90% de atividade enzimática é recuperada (PEI *et al.,* 2009). PEREIRA *et al.,* (2013) comparou SABs formados por PEG 600/$Na_2SO_4$, PEG 600/[Ch]Cl, PEG 600/[Ch][Bic] e [Ch]Cl/$K_3PO_4$. A maior capacidade em extrair a tetraciclina a partir do caldo de fermentação ocorreu com o sistema [Ch]Cl/$K_3PO_4$, com uma eficiência de extração de 92 %. Os autores ainda sugeriram uma etapa de pré-purificação utilizando o sistema com PEG 600/$Na_2SO_4$, por se considerar economicamente viável, uma vez que a recuperação da tetraciclina para a fase rica em PEG foi de 80 % (PEREIRA *et al.,* 2013).

A aplicação destes novos sistemas considerados não tóxicos, de baixo custo, biodegradáveis e biocompatíveis para a separação/purificação de compostos de interesse industrial ainda não foi totalmente explorada. E a possibilidade de combinações com outros compostos para induzir duas fases aquosas, como por exemplo açúcares, solventes orgânicos, ou até mesmo outros sais inorgânicos, polímeros e LIs, é uma possibilidade para ajustar as propriedades das fases a fim de uma ótima separação e/ou purificação de um composto de interesse.

# Capítulo III

## INTRODUÇÃO AO CAPÍTULO III

O capítulo III será apresentado em forma de artigos científicos (ARTIGO I, II, III e IV) os quais correspondem aos resultados referentes aos 3 anos de pesquisa associado a três diferentes abordagens para a formação de SABs aplicados para a separação e/ou purificação de enzimas lipolíticas. Os artigos foram organizados conforme as normas propostas pelo periódico de publicação, dos quais os materiais de apoio e as referências bibliográficas foram adicionadas ao final dos respectivos artigos.

ARTIGO I – *Novel aqueous two-phase systems based on tetrahydrofuran and potassium phosphate buffer for purification of lipase*. Artigo submetido ao periódico *Process Biochemistry*.

ARTIGO II – *Aqueous two-phase systems based on cholinium salts and tetrahydrofuran and their use for lipase purification.* Artigo será submetido ao periódico *Separation and Purification Technology*.

ARTIGO III – *Effect of ionic liquids as adjuvants on PEG-based ABS formation and the extraction of two probe dyes.* Artigo publicado no periódico *Fluid Phase Equilibria* 375 (2014) 30–36.

ARTIGO IV – *Lipase purification using ionic liquids as adjuvants in aqueous two-phase systems.* Artigo será submetido ao periódico *Biotechnology and Bioengineering*.

Estes trabalhos foram direcionados para a aplicação de novos SABs para a separação e/ou purificação de diferentes lipases, em especial, para a lipase produzida a partir de uma cepa de *Bacillus* sp. ITP-001 isolada por nosso grupo de pesquisa em um solo, com histórico de contaminação com petróleo, localizado no município de Carmópolis – SE. Esta lipase foi avaliada quanto a sua estabilidade, em processos de imobilização e em reações de interesse industrial (BARBOSA *et al.,* 2012; SOUZA *et al.,* 2012; CABRERA-PADILLA *et al.,* 2013; CARVALHO *et al.,* 2013; CARVALHO *et al.,* 2014). Com o objetivo de melhorar a eficiência catalítica destas lipases de *Bacillus* sp. ITP-001 por meio de sua purificação, foram estudadas por nós diferentes estratégias utilizando SABs. BARBOSA *et al.,* (2011) pela primeira vez utilizou SABs formados por PEG/sal. Neste trabalho foram avaliados PEGs com diferentes

massas molares, e os melhores resultados foram associados aos PEGs mais viscosos como o PEG 8.000 e 20.000 ($g.mol^{-1}$). Posteriormente, a purificação destas lipases foi avaliada utilizando SABs à base de LI formado por diferentes ânions e cátions. Os melhores resultados foram com LIs à base de cátions imidazólios conjugados com o ânion cloreto (VENTURA *et al.,* 2012a).

Os resultados destas investigações foram motivadores para o desenvolvimento do presente estudo, uma vez que a homogeneidade destas lipases não foi completa e que estes sistemas podem servir como plataforma para a purificação de outros compostos. Portanto, três novas abordagens foram aplicadas: (a) formação de SAB à base de solvente orgânico (tetrahidrofurano – THF) + sal com o objetivo de substituir a fase rica em polímero por um composto de fácil recuperação e de baixo custo (apresentado no ARTIGO I). Além disto, nesta abordagem a fase salina é composta por tampão fosfato de potássio a pH 7. Estes sais são comumente utilizados como tampão para formar SABs e foram empregados com sucesso para a purificação de diferentes lipases. Além disso o pH 7 foi escolhido devido à estabilidade da lipase de *Bacillus* sp. ITP-001 investigado anteriormente por nosso grupo (BARBOSA *et al*., 2012); (b) No ARTIGO II é apresentado SABs formados por THF + LI à base de colinas, uma vez que estes LIs são considerados biocompatíveis, de baixo custo e toxidade. (c) Por fim, SABs à base de polietilenoglicol (PEG) + sal + LI como adjuvante (5 %, m/m) foram estudados com o objetivo de ajustar a polaridade da fase rica em polímero e diminuir a dependência por grandes concentrações de constituintes que são necessárias para promover fases em SAB à base de LI + sal ou polímero (ARTIGO III e IV). Portanto, este trabalho completa uma série de estudos desenvolvido pelo nosso grupo de pesquisa envolvendo a aplicação de SABs para a purificação da lipase de *Bacillus* sp. ITP-001.

## ARTIGO I

# Novel aqueous two-phase systems based on tetrahydrofuran and potassium phosphate buffer for purification of lipase

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**Abstract**

Aqueous two-phase systems (ATPS) based on tetrahydrofuran (THF) + potassium phosphate buffer (pH 7) were used in this work for the purification of lipase produced by fermentation. Binodal curve, tie lines and critical point were obtained for the new THF−salt ATPS at 25 °C. To optimize the extraction capability of these ATPS the effects of the concentration of components and temperature of equilibrium on the partition coefficients and extraction efficiencies were investigated using lipase from *Burkholderia cepacia* (commercially obtained) as a model compound. The optimum conditions were then applied to the purification of lipase extracellular from *Bacillus* sp. ITP-001 produced by submerged fermentation. The results show that the experimental binodal data were successfully correlated with the empirical nonlinear equation proposed by Merchuck or Hu. The best extraction point was 20 wt% of THF and 20 wt% of potassium phosphate buffer (pH 7) for which a purification factor of 103.9 ± 0.9 and a enzyme recovery of 95.2 ± 1.1 % were achieved using this process.

*Keywords*: aqueous biphasic systems, tetrahydrofuran, phosphate buffer, purification, lipase

## 1. Introduction

Lipases, triacylglycerol ester hydrolases (EC 3.1.1.3), occupy a place of prominence among biocatalysts because of their potential applications in various industries such as food, dairy, pharmaceutical, detergents, textile, biodiesel, and cosmetic, besides participating in the synthesis of fine chemicals and agrochemicals [1-5]. The demand for these biocatalysts has been increasing, and the advantages include a high degree of specificity, mild reaction conditions and a lower probability of occurring side reactions [6]. However, lipases especially of microbial origin, are produced through a fermentation process that, besides the desired components, also generates secondary or intermediate products that frequently hinder the use of fermented broth in industrial procedures [7, 8].

Efficient downstream processing techniques are essential for the success of commercial production of enzymes and proteins. When these processes are applied to biological or pharmaceutical materials, these purification steps must be delicate enough to preserve the activity of these biomolecules [9]. The most common techniques used for purification are the ammonium sulphate precipitation [10], dialysis, filtration, electrophoresis [11-14], reverse micelles [15, 16] and, ionic and affinity chromatography [17, 18]. Nevertheless, the main constraints to the production of highly pure enzymes are the several steps necessary for purification, that in general, cause losses of enzyme activity and require high consumption of energy and chemicals [19, 20]. In this sense, aqueous two-phase systems (ATPS) can be foreseen as an alternative technique for extraction and/or purification of biocompounds since they have a low cost and lead to a high product purity, while maintaining the biological activity of the molecules due to their water-rich environment [21]. Conventional ATPS are formed by two water-soluble polymer–polymer, polymer–salt or salt–salt combinations that phase separate above given concentrations [22]. These systems have been used to the separation and purification of a great number of biological products, such as proteins, genetic material,

bionanoparticles, cells and organelles [23, 24]. Despite all these advantages, the limited range of polarity of the polymer-based systems, limits their applicability in the purification of biomolecules [25]. Currently, the number of systems capable of forming two aqueous phases is increasing and some alternatives include the use of alcohol/salt [26-29], acetonitrile/sugars [30-32], acetonitrile/polyols [33] and ionic liquids (ILs) [34, 35].

ATPS composed of a hydrophilic organic solvent and an inorganic salt solution have many advantages, which include rapid phase-separation, high extraction efficiency, low viscosity, high polarity differences between the phases, a gentle aqueous environment, and may be formed by inexpensive chemicals easy to recycle [27, 36]. These systems, formed by adding a salt solution to an aqueous solution of an organic compound, have been recently proposed and used for the partition of different biomolecules, such as proteins, amino acids and other natural products [27-29, 37-39].

For being widely used by industry in the fabrication of materials for packaging, transporting, and storing foods, the tetrahydrofuran (THF) gained prominence in recent years [40]. This cyclic eter, with excellent solvent power for numerous substances, is also used for the extraction of biocompounds from plants, including commercially valuable compounds such as carotenoids [41]. Taha et al. [42] have demonstrated the ability of THF to form ATPS with the biological buffer 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES). More recently Hirayama et al. [43] evaluated the ability of THF to form ATPS with the IL [$C_4$mim]Cl). To the best of our knowledge these are the only works available in the literature that use THF in the formation of ATPS.

In the present work, the phase diagrams of novel ATPS formed by tetrahydrofuran (THF) + potassium phosphate buffer (pH 7) were studied at 25 °C. The binodal curve, tie-lines and critical points were determined for the studied system. Additionally, the binodal data were correlated using the Merchuk [44] or Hu [45] equations. Aiming at exploring the applicability

of those novel ATPS for the purification of lipase, the enzyme produced by *Burkholderia cepacia* (commercially obtained) was here used as a model to evaluate the profile of the enzymatic partition, namely considering the overall system composition and temperature of equilibrium, optimizing the conditions for its extraction. Subsequently, the best extraction conditions selected based on the model lipase were employed with the objective to purify/separate lipase from *Bacillus* sp. ITP-001 produced by submerged fermentation.

## 2. Materials and methods

### *2.1 Materials*

The chemicals used in the present study, tetrahydrofuran (THF, ≥ 99.9 wt% purity) and the potassium phosphate salts that compose the buffer $K_2HPO_4/KH_2PO_4$ (≥ 99.9 wt% purity) were purchased from Sigma-Aldrich. The lipase from *Burkholderia cepacia – BCL* (≥ 30,000 $U.g^{-1}$, pH 7.0, 50 °C - optimum pH and temperature) was purchased from Sigma-Aldrich, and the extracellular lipolytic enzyme from *Bacillus* sp. ITP-001 was produced by submerged fermentation in this work. The ammonium sulphate (P.A.) was obtained from Synth (Brazil) and coconut oil was purchased at a local market. The bovine serum albumin (BSA, ≥ 97 wt% purity) was obtained from Merck.

### *2.2 Production of lipase by Bacillus sp.ITP-001*

#### *2.2.1 Fermentation conditions*

The microorganism of *Bacillus* sp. ITP-001 was isolated from petroleum-contaminated soil by the *Instituto de Tecnologia e Pesquisa – ITP* (Aracaju-Sergipe, Brazil). The bacterial strain was maintained on nutrient agar slants at4 ºC. The strain was cultivated in 500 mL erlenmeyer flasks

containing 200 mL medium with the following composition (%, w/v): $KH_2PO_4$ (0.1), $MgSO_4 \cdot 7H_2O$ (0.05), $NaNO_3$ (0.3), yeast extract (0.6), peptone (0.13), and starch (2.0) as the carbon source. The fermentation conditions were: initial pH 7; incubation temperature of 37 ºC, and stirring speed of 170 rpm. After 72 h of cultivation, coconut oil (4%, v/v) and Triton X-100 were added as inductors as described by Feitosa et al. [46].

*2.2.2 Pre-purification steps*

The fermented broth was centrifuged at 3,000 rpm for 15 min, so that the bottom phase was discharged (biomass) and the supernatant was used to determine the enzymatic activity and the total protein. Protein contaminants in the cell-free fermented broth were precipitated using ammonium sulphate at 80% (w/v) saturation. The solution was prepared at room temperature and the broth was subsequently centrifuged at 3,000 rpm for 30 min, separating the aqueous solution and precipitate. The aqueous phase was dialyzed using MD 25 (cut-off: 12,000-16,000 Da) against ultra-pure water for 24 h at 4 ºC. The dialyzed solution containing the enzyme was then used to prepare the ATPS. These pre-purification steps were previously described by our group [8].

*2.3 Phase diagrams and tie-lines*

The binodal curves of the ATPS were determined through the cloud point titration method at 25 ± 1ºC and at atmospheric pressure. In a test vial, a THF solution of known concentration was added, and a potassium phosphate buffer ($K_2HPO_4/KH_2PO_4$) solution of known mass fraction was then added dropwise until the mixture became turbid, then a known mass of water was added to make the mixture clear again. The potassium phosphate buffer was a mixture of potassium phosphate monobasic and bibasic at a ratio of 1.087 (w/w) and pH 7. This procedure was repeated to obtain sufficient data for the construction of a liquid–liquid equilibrium binodal curve. The compositions were determined by the weight quantification of all components added

within an uncertainty of ± $10^{-5}$ g. The experimental tie-lines (TLs), were measured with the procedure outlined in our previous work [33] and their respective length (tie line length - TLL) were determined through the application of Eq. (1), based on the concentrations of THF and salt ($K_2HPO_4/KH_2PO_4$, at pH 7) in the two phases.

$$\mathrm{TLL} = \sqrt{([\mathrm{THF}]_T - [\mathrm{THF}]_B)^2 + ([\mathrm{salt}]_T - [salt]_B)^2} \quad (1)$$

where the indexes *T* and *B* are of top and bottom phases, respectively.

The location of the critical point of the ternary systems was estimated by extrapolation of the TLs compositions applying Eq. (2) [35].

$$[\mathrm{THF}] = f + \mathrm{g}[\mathrm{salt}] \quad (2)$$

where *f* and *g* are fitting parameters.

*2.4 Preparation of the ATPS and Lipase partition studies*

The biphasic systems were prepared in graduated centrifuge tubes (15 mL) by weighing the appropriate amounts of THF (10 – 20 wt%) and salt (10 – 25 wt%) All systems contained approximately 2 wt% of *BCL* (≈ 20 mg.mL$^{-1}$). For the partition studies of lipase from *Bacillus* sp. ITP-001 the THF and salt ($K_2HPO_4/KH_2PO_4$ at pH 7) aqueous solutions were prepared with the dialysate solution obtained from the pre-concentration of the lipase from *Bacillus* sp. ITP-001.

Each mixture was prepared gravimetrically within ±$10^{-5}$ g. After 2 min of gentle stirring, the systems were centrifuged at 3,000 rpm for 20 min. The tubes were brought to equilibrium in a thermostatic bath at 25 ± 1ºC and local atmospheric pressure (1 atm) overnight (for at least 12 h). After this treatment, the two phases became clear and transparent and the interface was well defined. The phases were carefully withdrawn using a pipette for the top phase and a syringe with a long needle for the bottom phase. During the equilibration period, to avoid contamination

and/or evaporation of THF (boiling point at 66 °C) the vials were kept closed. The volumes and weights were determined in graduated test tubes (the total mass of the extraction systems prepared is 5.0 g). At least three independent replicates were made and the average partition coefficients and associated standard deviations were therefore determined.

The partition coefficient was defined as the protein concentration ($K_P$) or enzyme activity ($K_E$) in the top phase, divided by the corresponding value in the bottom phase, as describe of by Eqs. (3) and (4).

$$K_P = \frac{C_T}{C_B} \tag{3}$$

$$K_E = \frac{EA_T}{EA_B} \tag{4}$$

where $C$ is the total protein concentration (mg.mL$^{-1}$), $EA$ is the enzyme activity (U.mL$^{-1}$), and the subscript $T$ and $B$ are top and bottom phases, respectively. Selectivity ($S$) was defined as the ratio of the lipase enzyme partition coefficient ($K_E$) to the protein partition coefficient ($K_P$).

In order to evaluate the purification process, the enzyme specific activity ($SA$, U.mg$^{-1}$ protein) was calculated using Eq. (5), the volume ratio between the volumes of top and bottom phases ($R_V$), the contaminant proteins recovered in the top phase ($R_{PT}$, %), the enzyme recovered in the bottom phase ($R_{EB}$, %), and the purification factor ($PF$ - fold) were calculated according to Eqs. (6) - (8).

$$SA = \frac{EA}{C} \tag{5}$$

$$R_{PT} = \frac{100}{1 + \left(1/R_V K_P\right)} \tag{6}$$

$$R_{EB} = \frac{100}{1 + R_V K_E} \tag{7}$$

$$PF = \frac{SA}{SA_i} \tag{8}$$

The purification factor (*PF*) was calculated as the ratio between the *SA* in the bottom phase and the initial specific activity (*SAi*).

*2.5 Enzymatic activity*

Lipolytic activity was assayed using the modified oil emulsion method proposed by Soares *et al*. [47]. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of Arabic gum solution (7%, w/v). The reaction mixture containing 5 mL of the oil emulsion, 2 mL of 100 mM sodium phosphate buffer (pH 7) and enzyme extract (1 mL) was incubated in a thermostated batch reactor for 5 min at 37 °C. A blank titration was done on a sample where the enzyme was replaced with distilled water. The reaction was stopped by the addition of approximately 0.33 g of sample in 2 mL of acetone–ethanol–water solution (1:1:1). The liberated fatty acids were titrated with 40 mM potassium hydroxide solution in presence of phenolphthalein as indicator. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1μmol of free fatty acid per min ($\mu mol.min^{-1}$) under the assay conditions (37 °C, pH 7, 120 rpm).

*2.6 Protein Assay*

The total concentration of protein in each aqueous phase was quantified through Bradford's method [48], using a Varian Cary 50 Bio UV-Vis Spectrophotometer at 595 nm, and a calibration curve previously established for the standard protein bovine serum albumin (BSA).

*2.7 SDS-PAGE electrophoresis*

Electrophoresis was performed with the Mini-PROTEAN Tetra System (BioRad, Brazil) using 12 % resolving gels and 5% stacking gels as described by Laemmli [49]. Proteins were visualized by staining with silver stain [8, 50]. Protein markers used were trypsin inhibitor (21.5kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovinealbumin (66.2 kDa), and phosphorylase (97.4 kDa) purchased from BioRad (Brazil).

## 3. Results and Discussion

### *3.1. Binodal curve and correlation*

The development of novel, more performant, and economic ATPS, with low viscosity and high polarity difference between the two phases to recovery or purify enzymes is a priority issue in this work, the choice of salt (potassium phosphate buffer, pH 7) was due their ability to form ATPS with less polar compounds, due to its hydration capacity [51] and because it was previously used with success for the purification of lipases by ATPS [8, 9, 50, 52, 53]. Aqueous solutions of THF (80 wt%) and of salt (40 wt%) were initially prepared and used for the determination of the binodal curve at 25 ± 1 ºC and atmospheric pressure. Using the cloud point titration method [25], it was possible to observe the ability of THF to form two phases with the salt. This is a direct consequence the formation of hydration complexes between the water and salt, reducing the ability to hydrogen bond between the salting water and THF. The binodal curve, shown in Fig. 1, was fitted using the Merchuk equation (Eq. (9)) [44].

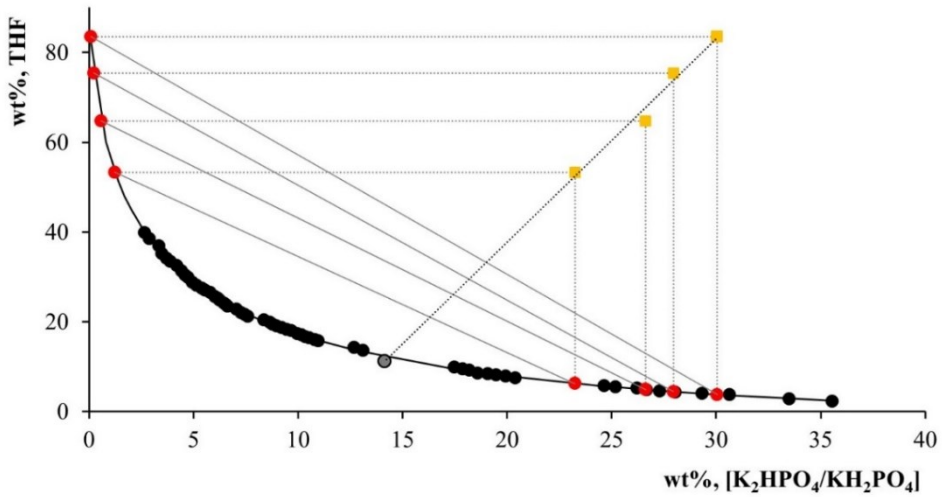


**Fig. 1:** Binodal curve for the ternary system composed of THF + potassium phosphate buffer (pH 7) + water, at 25 ± 1 ºC and atmospheric pressure. ▬, calculated binodal from Eq. 9; ●, experimental solubility data; ●, tie-line data; –, tie-lines; ■, auxiliary curve data; ●, critical point.

$$[\mathrm{THF}] = A \times \exp\{(B \times [\mathrm{salt}]^{0.5}) - (C \times [\mathrm{salt}]^3)\} \quad (9)$$

In order to get a more accurate fitting, we also used a non liner empirical expression (Eq. (10)) [45], with four fitting parameters to correlate the binodal data. This equation has been successfully used for the correlation of binodal data of alcohols + salt ATPS [54, 55].

$$[\mathrm{THF}] = \exp(A + B \times [\mathrm{salt}]^{0.5} + C \times [salt] + D \times [salt^2] \quad (10)$$

The regression parameters were estimated by least-squares regression using Eqs. 9 and 10, and their values with the respective standard deviations (*std*) and correlation factors ($R^2$) along with the weight fraction experimental data (*w*) for the system are given in Table 1. On the basis of the obtained $R^2$ and *std* in Table 1, in general, good correlation were obtained for the two equations used, indicating that these fittings can be used to predict data in a given region of the phase diagram where no experimental results are available.

A series of tie-lines in the two-phase region of the binodal curve were investigated and are reported in Table 2 and shown in Fig. 1, together with the overall composition, TLL, and critical point. The tie-lines are approximately parallel to each other, thus allowing easily to estimate the coexisting phase compositions for any system. The critical point (*cp*) for the studied systems was estimated by extrapolation from the TLs compositions applying Eq. (2).

After the complete characterization of the studied ATPS by the determination of the phase diagram, TLs, TLLs and critical point, the effect of THF on the stability of the lipase and the ability of the system composed of THF + salt to purify the enzyme was investigated.

**Table 1:** Parameters obtained through Equations 9 and 10 with the respective standard deviations (*std*) and correlation factors ($R^2$) along with the weight fraction experimental data ($w$) for the systems composed of THF (1) + potassium phosphate buffer (2) + $H_2O$, at 25 ± 1 ºC.

| **Binodal parameters** | | | | | |
|---|---|---|---|---|---|
| | **Eq. (9)** | | | **Eq. (10)** | |
| **A** | 97.7 ± 0.8 | | | 4.48 ± 0.06 | |
| **B** | -0.545 ± 0.004 | | | -0.456 ± 0.053 | |
| **C** | $9.9 \times 10^{-6} \pm 1.1 \times 10^{-6}$ | | | $-0.0198 \pm 1.3 \times 10^{-2}$ | |
| **D** | | | | $1.9 \times 10^{-13} \pm 2.0 \times 10^{-4}$ | |
| $R^2$ | 0.9997 | | | 0.9995 | |
| **Experimental data** | | | | | |
| 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ |
| 39.84 | 2.66 | 23.42 | 6.63 | 9.39 | 17.87 |
| 38.44 | 2.89 | 22.72 | 7.07 | 9.16 | 18.19 |
| 36.91 | 3.36 | 21.97 | 7.29 | 8.56 | 18.58 |
| 35.11 | 3.50 | 21.56 | 7.46 | 8.35 | 19.09 |
| 34.15 | 3.72 | 21.15 | 7.61 | 8.06 | 19.49 |
| 33.41 | 3.89 | 19.82 | 8.66 | 7.50 | 20.38 |
| 32.51 | 4.21 | 19.49 | 8.77 | 5.78 | 24.64 |
| 31.33 | 4.45 | 19.06 | 8.99 | 5.51 | 25.19 |
| 30.36 | 4.60 | 18.69 | 9.24 | 5.22 | 26.23 |
| 29.78 | 4.75 | 18.29 | 9.47 | 4.83 | 26.69 |
| 28.78 | 4.96 | 17.92 | 9.70 | 4.52 | 27.32 |
| 28.08 | 5.16 | 17.35 | 9.97 | 4.22 | 28.05 |
| 27.50 | 5.40 | 17.02 | 10.17 | 3.99 | 29.32 |
| 27.01 | 5.54 | 16.68 | 10.34 | 3.70 | 30.65 |
| 26.52 | 5.80 | 16.36 | 10.54 | 2.75 | 33.51 |
| 25.55 | 6.04 | 16.03 | 10.79 | 2.20 | 35.54 |
| 25.11 | 6.17 | 15.74 | 10.98 | | |
| 24.71 | 6.25 | 14.25 | 12.68 | | |
| 24.16 | 6.45 | 13.54 | 13.13 | | |
| 23.79 | 6.53 | 9.80 | 17.49 | | |

**Table 2:** Experimental data of TLs, TLLs and critical point values of the coexisting phases for the THF + potassium phosphate buffer (pH 7) system at 25 ± 1 ºC.

| Weight fraction/(wt%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $[THF]_M$ | $[salt]_M$ | $[THF]_T$ | $[salt]_T$ | $[THF]_B$ | $[salt]_B$ | TLL | $[Y]_{Critical}^*$ | $[X]_{Critical}^*$ |
| 13.22 | 19.98 | 53.34 | 1.23 | 6.23 | 23.24 | 51.99 | 11.23 | 14.15 |
| 20.14 | 19.98 | 64.76 | 0.57 | 4.87 | 26.62 | 65.32 | | |
| 29.99 | 17.97 | 75.44 | 0.23 | 4.41 | 27.95 | 76.52 | | |
| 19.99 | 23.96 | 83.53 | 0.08 | 3.76 | 30.06 | 85.22 | | |

** in the critical point: f = 52.84; g = 4:53; and $R^2$ = 0.9877.*

### *3.2. Effect of THF in lipase activity*

In the traditional purification processes of proteins, several steps of manipulation are required and the enzyme activity unavoidably decreases in each step of the purification. In order to examine the effect of composition in THF-rich phase on the lipase-stability, solutions were prepared at different concentrations by dissolving the pure THF in distilled water, and then crude *BCL* was mixed with THF solutions. For this study a wide range of concentrations of the THF (10 – 80 wt%), and times of the incubation (up to 24 hours) were considered. The study was carried at 25 ± (0.1) ºC.

The results are shown in Fig. 2, from which it is surprising to find that the activity of lipase was not decreased up to 18 hours in contact with THF. In general there is an increase of enzymatic activity with the use of THF, regardless of its concentration. The THF is an organic solvent of hydrophilic character (log $P$ = 0.53) [56]. Although it is known that hydrophobic organic solvents (log $P$ > 4) may improve the stability of enzymes by stimulating the open conformation of the active site of the lipase [57], some studies have also reported increased stability of lipases using hydrophilic organic solvents. Activity of lipase from *Streptomyces* sp. CS133 was significantly increased in presence of organic solvents with log $P$ = 0.87 (diethyl ether), log $P$

= 1.25 (dichloromethane) and log *P* = 2.0 (benzene) while the relative activities were 123, 129 and 161 %, respectively [58]. In addition, previous studies have shown that lipase from *Burkholderia* can maintain highly stable catalytic activity in organic solvents, such as ethanol, 1-propanol and 2-propanol, all leading to a relative activity above 99.5 % in organic solvent solutions at 40% (v/v) [27, 59]. Lipase from *Aspergillus carneus* was also investigated in various organic solvents, and it was found stable in iso-octane, benzene, toluene and xylene [4]. It is believed that the organic solvent contributes to maintain the enzymes open conformation by exposing its active site and thereby stimulating lipase activity. Therefore, at the present stage it is appropriate to admit that the ATPS formed with THF are promising systems for the efficient and high activity extraction and purification of lipases.

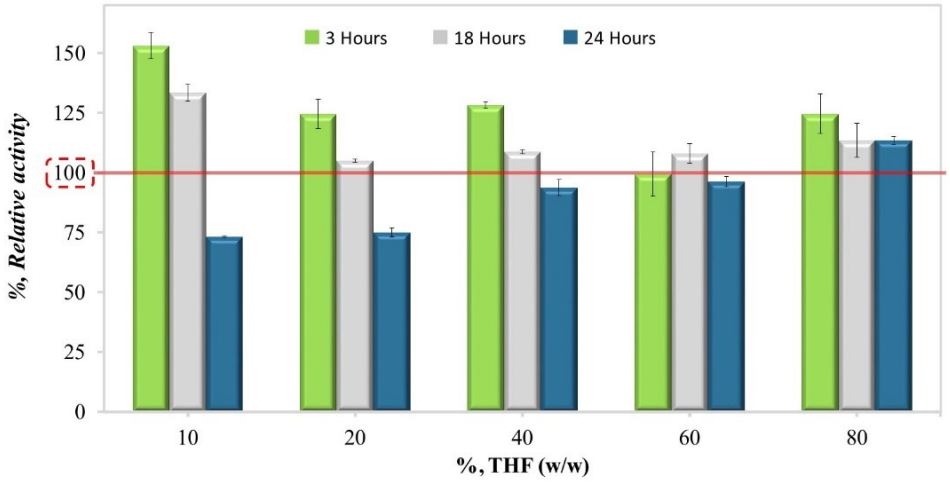


**Fig. 2:** Effect of concentration of THF on the stability of lipase from *Burkholderia cepacia.* The crude lipase feedstock was incubated at room temperature up to 24 h. The relative activity was measured using a lipase assay. The lipase activity of phosphate buffer (pH 7.0) was used as the control. The THF concentrations were expressed as (wt%).

### *3.3. Partition of the model lipase*

To optimize the lipase partition in a THF + potassium phosphate buffer (pH 7), eight systems (each in triplicate) were evaluated using the *BCL* as model lipase. These systems systematically

varied the THF and potassium phosphate concentrations and temperature of formation of the system. It should be remarked that for the systems tested, the bottom phase is the salt-rich phase whereas the top phase corresponds to the THF-rich phase. These systems were selected so that the liquid–liquid systems could be formed taking into account their phase diagram.

According to Fig. 3, the optimal condition for partitioning lipase was observed in the THF 20 wt% and potassium phosphate buffer 20 wt% system, which has a $K_E$ of 0.42 ± 0.01. For the extraction efficiency it was observed for THF 10 wt% and potassium phosphate buffer 20 wt% ($R_{EB}$ = 91.8 ± 0.3 %).

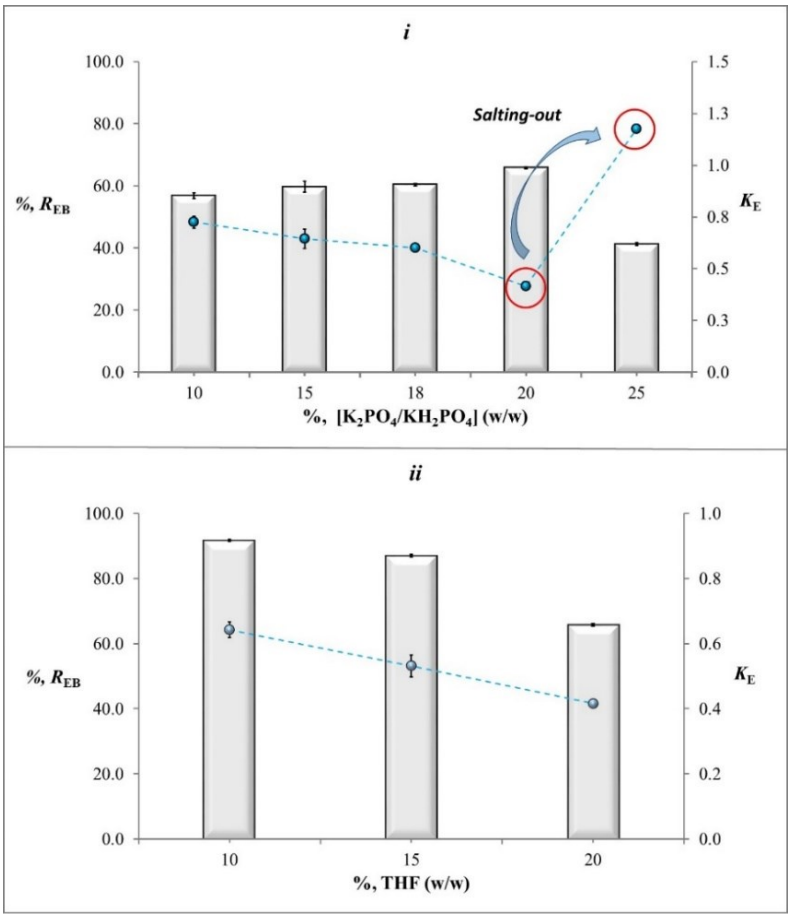


**Fig. 3:** Enzyme recovered in the bottom phase (%, $R_{EB}$ - represented by bars) and partition coefficients ($K_E$ - represented by symbols) of lipase from *Burkholderia cepacia*, for systems based in THF + potassium phosphate buffer (pH 7) + water, at 25 ± (0.1) ºC and atmospheric pressure, as a function of concentration: (*i*), 20 wt% THF + wt% salt; (*ii*), wt% THF + 20 wt% salt.

In Fig. 3 (*i*), the effect of potassium phosphate at concentrations ranging between 10 and 20 wt% was investigated. The concentration of THF was fixed at 20 wt% and the system was operated at 25 ºC and pH7. A gradual increase in salt concentration, favored the partitioning and the enzyme recovery for the bottom phase (salt-rich) as the value of $K_E$ decreased from 0.73 to 0.42, and the value of $R_{EB}$ increase from 56.8 to 65.8 %. This trend is observed up to concentrations of 20 wt%, after this optimal concentration other effects lead to the migration of lipase for the opposite phase, THF-rich ($K_E$> 1). According to Babu et al.[60], increasing the concentration of salt decreases the solubility of biomolecules in the salt-rich phase (bottom), which results in increased partitioning of biomolecules to the top phase, due to a salting out effect. Souza et al. [9] observed the same trend, where the partition efficiency of porcine pancreatic lipase is negatively affected, for phosphate concentrations above of 18 wt% in ATPS with PEG 1500 g.mol$^{-1}$ and above 20 wt% in ATPS with PEG 8000 g.mol$^{-1}$. In all these works, the lipase preferentially migrated to the salt-rich phase. This fact is due to the pH of the saline phase (pH = 7.0) being above the isoelectric point (p*I* = 6.0) of the enzyme, resulting in increased affinity for the more hydrophilic salt-rich phase [8, 61].

Following the study of optimization, the concentration of the potassium phosphate was fixed in 20 wt% and the concentration of THF ranged from 10 to 20 wt% (Fig. 3 (*ii*)). The increase of the THF concentration leads to lower partition coefficients of lipase, that is, to a higher ability of lipase to migrate for the salt-rich phase. The values of $K_E$ decrease from 0.64 (with 10 wt% THF) to 0.42 (with 20 wt% THF). This effect is due to the enrichment of the most hydrophobic region (THF-rich phase, with log *P* 0.53), which favors the migration of lipases for the salt-rich phase. However, the enzyme recovery in the bottom phase has a tendency opposite to the partition coefficient. The maximum value of recovery is achieved with 10 wt% THF ($R_{EB}$ = 91.8 ± 0.3 %). At low concentrations of THF in the top phase, the volume ratio of the phases is much smaller ($R_V$ = 0.14) than in higher concentrations, for example with 20 wt% of THF ($R_v$

= 1.25). This is a result of the gradual increase in the ability of THF to conduct interactions with water via hydrogen bonds [62]. For the following steps of this work, data of enzyme partition ($K_E$) are taken into account due to the higher selectivity of lipase for the salt-rich phase, which may allow the increase of the purification factor when the objective is to apply these conditions for the purification of lipase from *Bacillus* sp. ITP-001. Data of enzyme recovery in the bottom phase ($R_{EB}$), partition coefficients ($K_E$) and volumetric ratio ($R_V$) for ATPS with different compositions of THF and potassium phosphate buffer in (wt%) are shown in Table A.1.

To assess the effect of temperature on lipase partitioning, the system composed of THF at 20 wt% and potassium phosphate buffer at 20 wt%, was chosen because it represents the best condition of partitioning of the lipase. The thermodynamic functions calculated for the transfer of lipase, namely the molar Gibbs energy ($\Delta G_m^o$), the molar enthalpy ($\Delta H_m^o$) and the molar entropy of transfer ($\Delta S_m^o$), Eqs. (10) - (12) were used according to,

$$\ln K = -\frac{\Delta H_m^{\mathrm{O}}}{R} \times \frac{1}{T} + \frac{\Delta S_m^{\mathrm{O}}}{R} \tag{10}$$

$$\Delta G_m^{\mathrm{O}} = \Delta H_m^{\mathrm{O}} - T\Delta S_m^{\mathrm{O}} \tag{11}$$

$$\Delta G_m^{\mathrm{O}} = -RT \ln(K) \tag{12}$$

Fig. 4 shows the profile of $K_E$ as a function of equilibrium temperature, adjusted from 5 to 25 ºC. An increase in temperature slightly favors the extraction of lipase for the salt-rich phase. The partitioning of lipase from the THF-rich phase to the salt-rich phase is spontaneous, as shown by the calculated negative value for $\Delta G_m^o$ (-1.67 KJ/mol) and endothermic ($\Delta H_m^o$ = 10.80 KJ/mol). The main forces that govern the migration of biomolecules are entropic ($\Delta S_m^o$= 43.51 J/mol.K), since $T \times \Delta S_m^o > \Delta H_m^o$.

In summary, the optimal condition for partitioning of the *BCL* was obtained in ATPS composed of THF at 20 wt% and potassium phosphate buffer at 20 wt%, at an equilibrium temperature of 25 °C. Therefore, these conditions were chosen for the purification of lipase from fermented broth.

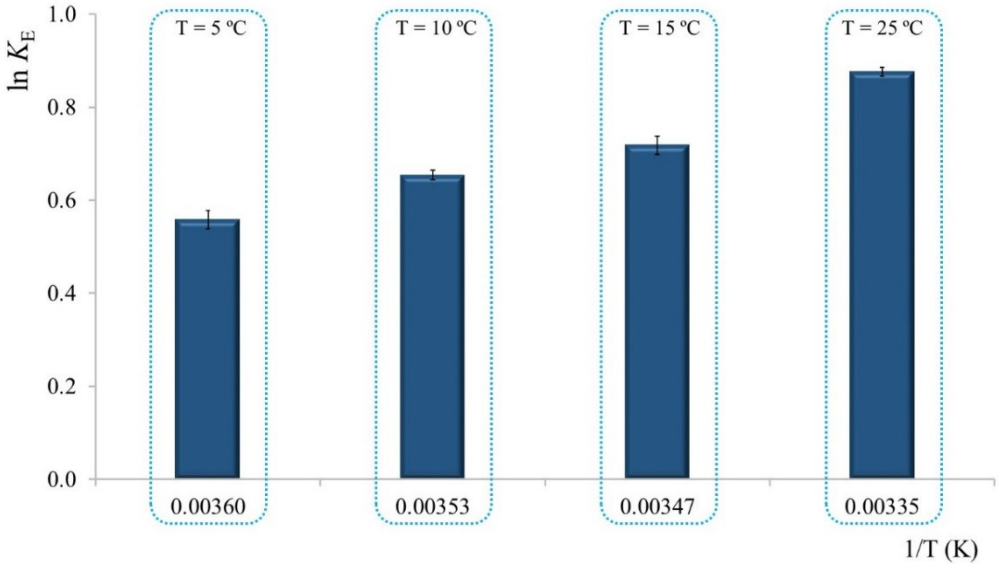


**Fig. 4:** Effect of temperature on partition coefficient ($K_E$) of lipase from *Burkholderia cepacia* for the ATPS based on THF + potassium phosphate buffer (pH 7).

*3.4. Purification lipase from Bacillus sp.ITP-001*

Before assessing the ability of this new ATPS in purifying extracellular lipase from *Bacillus* sp. ITP-001, the steps of production and pre-purification must be considered. The fermentation was 144 hours long, then was applied the salt $(NH_4)_2SO_4$ for the precipitation of lipase, followed by a dialysis step to remove low molecular weight compounds, including inorganic salts of the precipitation process, all this process is described in detail elsewhere [8, 50]. Table 3 reports the enzymatic activity (*EA* – $U.mL^{-1}$), total protein concentration (*C* – $mg.mL^{-1}$), specific activity (*SA* – $U.mg^{-1}$) and purification factor (*PF* - fold) in the fermented broth and dialyzed. The purification factor found on the dialysate was 12.7 ± 0.2 fold.

**Table 3:** Purification factor, enzymatic activity, specific activity, and protein concentration at the end of each step of the production and pre-purification of lipase produced by *Bacillus* sp. ITP-001.

| Steps | Process | *EA* (U.mL$^{-1}$) | *C* (mg.mL$^{-1}$) | *SA* (U.mg$^{-1}$) | *PF* (fold) |
|---|---|---|---|---|---|
| Production | Fermentation | 6,167.3 | 1.15 | 5,365.7 | – |
| Pre-purification | Dialyse | 6,135.4 | 0.09 | 68,171.1 | 12.7 ± 0.2 |
| Purification | ATPS | 36,210.4 | 0.06 | 557,846.9 | 103.9 ± 0.9 |

After the production and pre-purification step of extracellular lipase from *Bacillus* sp. ITP-001, the purification was assessed employing representative conditions of this new ATPS previously studied. The extraction systems were prepared by adding 20 wt% of THF + 20 wt% of potassium phosphate buffer (pH 7) + 60 wt% of dialysate solution containing the lipolytic enzyme produced. The systems were held at equilibrium for 18 hours at 25 °C. These optimized conditions were based on the partitioning of *BCL* to the bottom phase (rich in salt).

The proposed application of this ATPS revealed a good performance in the purification of the lipolytic lipase produced from *Bacillus* sp. ITP-001. The data suggest that the *PF* of the enzyme was increased from 12.7 to 103.9 ± 0.9 fold, comparing the steps of pre-purification (by use of dialysis) with the purification step using ATPS (Table 3). The increase of the purification factor achieved by the use of the ATPS is related to the selectivity of the phases constituting the system, resulting mainly from the removal of the contaminants which act as inhibitors [63]. The results show a lower selectivity of contaminating proteins to the bottom phase ($S$ = 1.69), compared with the enzyme ($S$ = 20.0). This is probably because the enzymes are almost completely recovered in the bottom phase ($R_{EB}$ = 95.2 ±1.1 %), due to their very low isoelectric point (p$I$ = 3.0) [8] and negatively charged at pH 7.0 [50, 52], which results in the increase of

its hydrophilic character, creating a higher affinity of the enzyme for the salt-rich phase. Furthermore, the migration of enzymes to bottom phase ($K_E$ = 0.12 ±0.02) and the contaminating proteins to the top phase ($K_P$ = 1.45 ±0.18) indicate an increase in the specific activity of the enzyme in the salt-rich phase (bottom phase), increasing the purification factor.

Previous studies by us, focused on the purification of enzymes, including lipase from *Bacillus* sp. ITP-001 using IL/salt ATPS, 25 wt% of [$C_8$mim]Cl and 30 wt% of phosphate buffer potassium (pH 7), showed lower purification values (*PF* = 51 ± 2 fold) than those obtained here [50]. However, the use of conventional ATPS (polyethylene glycol – PEG 8000 g.mol$^{-1}$ + phosphate buffer potassium) show higher values purification (*PF* = 201.53 fold) [8]. Although the system here reported shows a lower performance than the ATPS of PEG/salt, it is less expensive and the viscosity of the phases is reduced when compared with the PEG/salt system, enhancing the mass transfer, and simplifying the fluid flow problems when one considers the scaling up of the process.

To support our interpretation of the results concerning the purification capacity of the ATPS based in THF/potassium phosphate buffer, an electrophoresis analysis was performed using samples of the bottom phase (system 20 wt% of THF + 20 wt% of potassium phosphate buffer at pH 7 – considered in this work the best purification system) and crude fermentation broth. The three lanes shown in Fig. 5 correspond to the molecular mass standard (lane P), the crude fermentation broth (Lane 1) and bottom phase obtained from the THF-based ATPS (Lane 2).

The presence of multiple light bands in Lane 1 confirms the presence of some contaminant proteins. In Lane 2, it is possible to see the presence of the target enzyme with a molecular weight of around 54 kDa [8, 50] (here abbreviated as *Enz*) and the presence of few other protein bands (contaminants) which were not completely removed. The results from the electrophoresis are consistent with purification factor values reported.

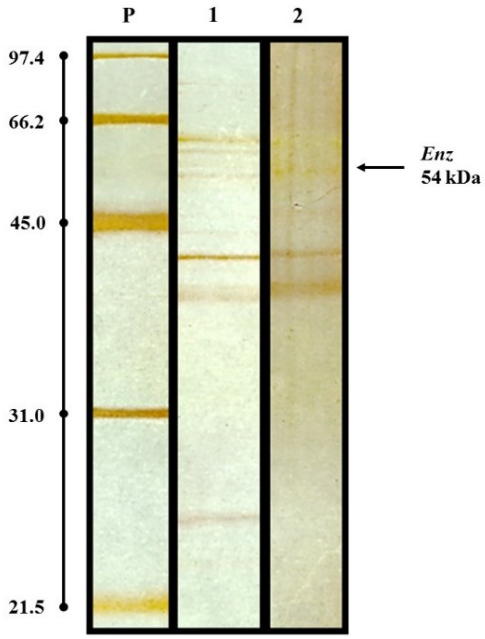


**Fig. 5:** SDS–PAGE analysis of purified lipase from *Bacillus* sp. ITP-001. The purity of partitioned lipase was assessed by 12 % acrylamide gel stained with silver nitrate solution. The molecular weights of the standard protein marker ranged between 21.5 – 97.4 kDa. Lane P: protein molecular markers; Lane 1: fermented broth; Lane 2: bottom phase obtained from the THF-based ATPS.

## 4. Conclusions

In this article, the binodal curve, tie-lines and critical point were obtained for the new THF−potassium phosphate buffer ATPS at 25 °C. The experimental binodal data was successfully correlated with the empirical nonlinear equations proposed by Merchuck or Hu. The ability of this new system to purify the lipase produced by the bacterium *Bacillus* sp. ITP-001 was demonstrated to be effective. The optimal condition for purification of lipase from a real matrix was optimized based on the partitioning of a commercial lipase from *Burkholderia cepacia*, used as model. The best extraction system was identified to be 20 wt% of THF and 20 wt% of potassium phosphate buffer (pH 7) at 25 ºC. The enzyme partitioning is spontaneous and governed by entropic effects. A *PF* of 103.9 ± 0.9 and $R_{EB}$ of 95.2 ± 1.1 % were achieved

for the lipase from *Bacillus* sp. ITP -001 using this recovery process from a fermentation broth. The THF/salt ATPS proved to be effective for the purification of solvent tolerant lipase and it was shown that the lipase's enzymatic activity was not affected by the organic solvent. Because of the ease of organic solvent recovery, lower viscosity and the cost-effectiveness of the process, the THF/salt ATPS could be potentially developed as a commercial recovery process for lipase derived from microbial sources.

**Acknowledgments**

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# Supporting Information

## Novel aqueous biphasic system based on tetrahydrofuran and potassium phosphate buffer for purification of lipase

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**Table A.1:** Data of enzyme recovery in the bottom phase ($R_{EB}$), partition coefficients ($K_E$) and ratio volumetric ($R_V$) of lipase from *Burkholderia cepacia*, for ATPS with different compositions of THF (wt%) and potassium phosphate buffer, pH 7 (wt%), at 25 ± (0.1) ºC.

| ATPS | | $R_V \pm \sigma$ | $K_E \pm \sigma$ | $R_{EB} \pm \sigma$ (%) |
|---|---|---|---|---|
| wt%, THF | wt%, salt | | | |
| 20 | 10 | 1.05 ± 0.2 | 0.73 ± 0.03 | 56.8 ± 1.0 |
| 20 | 15 | 1.05 ± 0.1 | 0.64 ± 0.05 | 59.7 ± 1.7 |
| 20 | 18 | 1.09 ± 0.2 | 0.60 ± 0.01 | 60.4 ± 0.4 |
| 20 | 20 | 1.25 ± 0.2 | 0.42 ± 0.01 | 65.8 ± 0.3 |
| 20 | 25 | 1.37 ± 0.1 | 1.18 ± 0.18 | 41.3 ± 1.9 |
| 10 | 20 | 0.14 ± 0.1 | 0.64 ± 0.02 | 91.8 ± 0.3 |
| 15 | 20 | 0.29 ± 0.1 | 0.53 ± 0.03 | 87.0 ± 0.5 |
| 20 | 20 | 1.25 ± 0.2 | 0.42 ± 0.01 | 65.8 ± 0.3 |

## ARTIGO II

# Aqueous two-phase systems based on cholinium salts and tetrahydrofuran and their use for lipase purification

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**ATPSTRACT**: Aqueous two-phase systems (ATPS) formed with cholinium-based ionic liquid – ILs (or salts) are a novel, low cost, and high efficient technique for the recovery of biomolecules. This study examines the formation of ATPS based on cholinium-based salts (cholinium chloride, cholinium bitartrate and cholinium dihydrogencitrate) and tetrahydrofuran (THF) for the purification of lipase from *Bacillus* sp. ITP-001, produced by submerged fermentation. The optimum conditions for this purification were determined to be 40 wt% of THF and 30 wt% of cholinium bitartrate at 25ºC. A purification factor of 130.1 ± 11.7 fold, a lipase yield of 90.0 ± 0.7% and a partition coefficient of enzyme for IL-rich phase ($K_E$ = 0.11 ± 0.01) and protein contaminants for THF-rich phase ($K_P$ = 1.16 ± 0.1) were achieved.

**KEYWORDS:** aqueous two-phase systems, lipase, cholinium-based ionic liquid, purification

## 1. Introduction

Lipases are glycerol ester hydrolases (EC 3.1.1.3), and those from microbial origin, occupy a place of prominence among biocatalysts in several sectors like in oleochemistry, organic synthesis, detergent formulation, nutrition, biosensors, bioremediation, among others [1-4]. Lipase preparations with a high degree of purity are used by the fine chemical industries, for example in the biocatalytic production of the pharmaceuticals and cosmetics [3, 5]. The main problem with the production of high purity enzymes is the purification process. In general it has a poor efficiency, causes loss of enzyme activity, and requires high consumption of energy and chemicals [6, 7]. To overcome these limitations, a significant effort has been made to develop novel techniques in order to reduce the costs related to the purification [6, 8].

Aqueous two-phase systems (ATPS) have been used for the separation and purification of a great number, of biological products as amino-acids [9, 10], proteins [11] and enzymes [8, 12-16] as their two phases having a rich water environment are favorable to the preservation of activity of biomolecules. Conventional ATPS are formed by two water-soluble polymer–polymer, polymer–salt or salt–salt combinations that phase separate above given concentrations [17]. Despite the well-known advantages offered by these systems, such as low interfacial tension, good biocompatibility, fast and high phase separation rates and low cost [8, 12], their performance is however significantly affected due to the small difference in polarity between the coexisting phases [18]. Currently, the number of systems capable of forming two aqueous phases is increasing and the alternatives include the use of alcohol/salt [19-21], acetonitrile/carbohydrates [22, 23], polymers(polyvinyl alcohol – PVA)/dextran [24] and other combinations including ionic liquids (ILs) [25, 26]. The numerous combinations of cations and anions that form ILs lead to a variety of physical properties, allowing the tailoring of their polarities, and for this reason have been regarded as important constituents of ATPS [27-29]. Although good results have been achieved using ATPS formed by ILs for the extraction of

amino acids, proteins, enzymes, pharmaceuticals and phenolic compounds [25, 30, 31], the use of these compounds may raise some issues concerning their water stability, price, and biodegradability [32-36]. Their toxicity has been shown to be at least equivalent to those of common organic solvents [37].

To overcome these issues, the search for safer and cheaper ILs for the formation of ATPS is still an imperative issue and in this context, the cholinium-based ionic liquids are a good option. This family is derived from quaternary ammonium salts described as important structures in living processes, used as precursors for the synthesis of vitamins (*e.g.* vitamin B complexes and thiamine) and enzymes that participate in the carbohydrate metabolism [38, 39]. Recent works have reported the synthesis of novel cholinium-based ILs with the cholinium cation combined with a variety of different anions [40-43]. Besides the exceptional properties shared with the ionic liquids, such as, non-flammability and negligible vapour pressure at ambient conditions, and high solvation ability, the cholinium ILs also have low toxicity, excellent biodegradability and can be produced at low cost since they can be obtained from cheap raw materials [42-46]. The interest in these compounds has increased in the past few years, with applications ranging from crosslinking agents for collagen based materials, solvents in the pre-treatment and dissolution of biomass, and use as co-substrates for microorganisms in the degradation of dyes [45-48]. Moreover, a number of works have described novel cholinium-based ILs in which protein structure and the enzyme function can be maintained or even increased [47, 49]. This fact, coupled with other advantages cited herein, motivated the application of these ILs to the formation of alternative ATPS, which served as a platform for the purification/separation of antibiotics [50, 51] and proteins [47].

Taking into account the ability of cholinium-based ILs to promote phase separation in ATPS, their low cost and the capacity to maintain the activity of the target compounds, they will be here explored combined with tetrahydrofuran. Tetrahydrofuran (THF) is an organic

solvent with excellent solvent power for numerous organic substances, and employed for the extraction of compounds from vegetables, including commercially important compounds such as carotenoids [52, 53]. The formation of aqueous two-phase systems using THF and a biological buffer, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), was first reported by Taha et al., [54]. Moreover, THF + potassium phosphate buffer based ATPS, was show to be effective for the purification of extracellular lipase from *Bacillus* sp. ITP-001 [55].

This work focuses in the design of ATPS based on tetrahydrofuran (THF) and cholinium-based ionic liquids (cholinium chloride, cholinium bitartrate and cholinium dihydrogencitrate). Aiming at exploring the applicability of those novel ATPS, the lipase from *Burkholderia cepacia* (commercially obtained) is here used as a model to evaluate the profile of the enzymatic partition and efficiency of extraction, namely considering cholinium-based ILs with different anions, overall system composition and temperature of equilibrium. Subsequently, representative conditions are employed with the objective of evaluating the possibility of applying these systems for the separation and purification of lipase from *Bacillus* sp.ITP-001 produced by submerged fermentation.

## 2. Materials and methods

### *2.1 Materials*

The organic solvent tetrahydrofuran (purity ≥99.9%), cholinium chloride (purity >98%); cholinium dihydrogencitrate (purity >98%); and cholinium bitartrate (purity >98%) were purchased from Sigma-Aldrich. Their chemical structures are shown in Fig. 1.

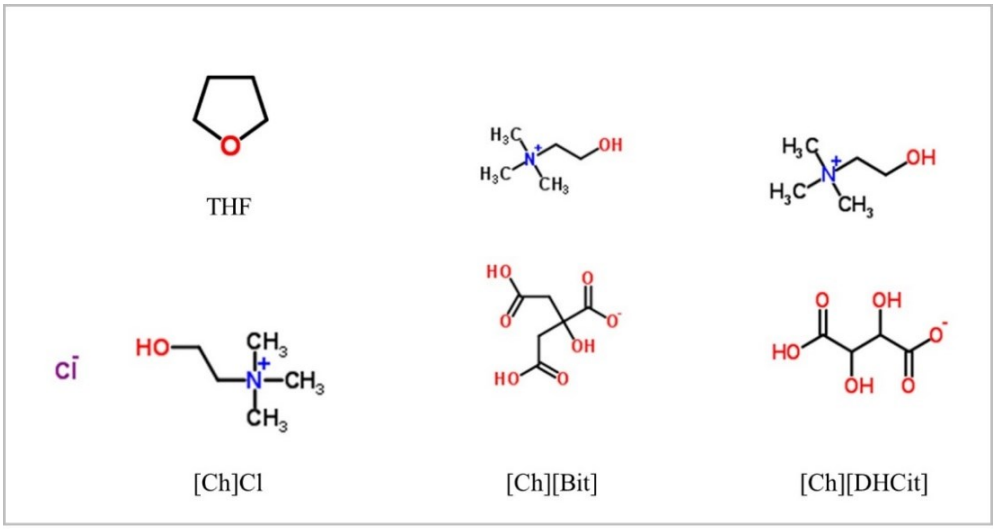


**Fig. 1:** Chemical structure and abbreviation name of the cholinium-based ILs and THF studied in this work.

The lipase from *Burkholderia cepacia– BCL* (≥ 30,000 U/g, pH 7.0, 50 °C - optimum pH and temperature) was also obtained from Sigma-Aldrich, and the lipase from *Bacillus* sp. ITP-001 was obtained by submerged fermentation, using $MgSO_4$ $7H_2O$ (purity ≥ 98%) obtained from Panreac, Triton X-100 purchased from Fisher Scientifc, and $NaNO_3$ (purity ≥ 99.5%), yeast extract, peptone, and starch purchased from Himedia. The ammonium sulphate (P.A.) was obtained from Synth (Brazil) and coconut oil was purchased at a local market. The protein bovine serum albumin (BSA, purity ≥ 97%) was obtained from Merck.

*2.2 Production of the lipase by Bacillus sp.ITP-001*

*2.2.1 Fermentation conditions*

The lipase was obtained by the fermentation of a *Bacillus* sp. ITP-001, isolated from an oil contaminated soil, stored at the Instituto de Tecnologia e Pesquisa - ITP (Aracaju - Sergipe, Brazil). The strain was cultivated in 500 mL erlenmeyer flasks containing 200 mL medium with the following composition (%, w/v): $KH_2PO_4$ (0.1), $MgSO_4 \cdot 7H_2O$ (0.05), $NaNO_3$ (0.3), yeast extract (0.6), peptone (0.13), and starch (2.0) as the carbon source. The fermentation

conditions were: initial pH 7; incubation temperature 37 ºC, and stirring speed 170 rpm. After 72 h of cultivation, coconut oil (4%, w/v) and Triton X-100 were added as inductors as described by Feitosa et al. [56].

*2.2.2 Pre-purification steps*

The pre-purification steps were performed according to the methodology proposed by Barbosa et al. [8]. The fermented broth was centrifuged at 3,000 rpm for 30 min, so that bottom phase was discharged (biomass) and the supernatant was used to determine the enzymatic activity and the total protein content. Protein contaminants in the cell-free fermented broth were precipitated using ammonium sulphate at 80% (w/v) saturation, the solution was prepared at room temperature and the broth was subsequently centrifuged at 3,000 rpm for 30 min, separating the aqueous solution and precipitate. The aqueous phase was dialyzed using MD 25 (cut-off: 10,000-12,000 Da) against ultra-pure water for 24 h at 4 ºC. The dialyzed solution containing the enzyme was then used to prepare the ATPS.

*2.3 Binodal curves and tie-lines*

The binodal curves were determined by the cloud-point titration method at 25 ± 1 ºC and at atmospheric pressure. In a test tube, a THF aqueous solution of known concentration was added, and then a cholinium-based ILs solution of known mass fraction was added dropwise until the mixture became turbid or cloudy; then, a known mass of water was added to make the mixture clear again. This procedure was repeated to obtain sufficient data for the construction of a liquid–liquid equilibrium binodal curve. The systems composition were determined by the weight quantification of all components added within an uncertainty of ± $10^{-5}$ g. The binodal curves data were correlated using the Merchuk equation [57].

$$[\mathrm{THF}] = A \times \exp\{(B \times [\mathrm{IL}]^{0.5}) - (C \times [\mathrm{IL}]^{3})\} \quad (1)$$

The determination of the tie-lines (TLs) was then accomplished by solving the following system of four equations (Eqs. (2)–(5)) for the four unknown values of $[THF]_T$, $[THF]_B$, $[IL]_T$ and $[IL]_B$,

$$[\mathrm{THF}] = A\exp\{(B \times [\mathrm{IL}]_T^{0.5}) - (C \times [\mathrm{IL}]_T^3)\} \quad (2)$$

$$[\mathrm{THF}] = A\exp\{(B \times [\mathrm{IL}]_B^{0.5}) - (C \times [\mathrm{IL}]_B^3)\} \quad (3)$$

$$[\mathrm{THF}]_T = ([\mathrm{THF}]_M/\alpha) - ((1-\alpha)/\alpha)[\mathrm{THF}]_B \quad (4)$$

$$[\mathrm{IL}]_T = ([\mathrm{IL}]_M/\alpha) - ((1-\alpha)/\alpha)[\mathrm{IL}]_B \quad (5)$$

where the subscripts *M, T* and *B* denote, respectively, the initial mixture, and the top and bottom phases. The value of $\alpha$ is the ratio between the mass of the top phase and the total weight of the mixture. The system solution results in the THF and cholinium-based ILs concentration in the top and bottom phases, and thus, TLs can be simply represented.

The tie line length (TLLs) were determined through the application of Eq. (6), which uses the concentrations of THF and ILs in the two phases.

$$\mathrm{TLL} = \sqrt{([\mathrm{THF}]_T - [\mathrm{THF}]_B)^2 + ([\mathrm{IL}]_T - [\mathrm{IL}]_B)^2} \quad (6)$$

The location of the critical point of the ternary systems was estimated by extrapolation from the TLs compositions applying the Eq. (7) [25].

$$[\mathrm{THF}] = f + \mathrm{g}[\mathrm{IL}] \quad (7)$$

where *f* and *g* are fitting parameters.

*2.4 Preparation of the ATPS*

The biphasic systems were prepared in graduated centrifuge tubes (15 mL) by weighing the appropriate amounts of THF (25–50 wt%) and cholinium-based ILs (15–35 wt%). All systems contained approximately 2 wt% of *BCL* (≈ 20 $mg.mL^{-1}$). For the lipase from *Bacillus* sp. ITP-

001, the THF and cholinium solutions were prepared with the dialysate solution (where the lipolytic lipase from *Bacillus* sp. ITP-001 is concentrated).

Each mixture was prepared gravimetrically within $\pm 10^{-5}$ g, vigorously stirred and left to equilibrate for at least 12 h (a time period established in previous optimizing experiments) and at 25 ± (0.1) ºC. After this treatment, the two phases became clear and transparent and the interface was well defined. The phases were carefully separated using a pipette for the top phase and a syringe with a long needle for the bottom phase. The volumes and weights were determined in graduated test tubes (the total mass of the extraction systems prepared is 5.0 g).

The partition coefficient was defined as the protein concentration ($K_P$) or enzyme activity ($K_E$) in the top phase, divided by the corresponding value in the bottom phase, as describe by Eqs. (8) and (9).

$$K_P = \frac{C_T}{C_B} \tag{8}$$

$$K_E = \frac{EA_T}{EA_B} \tag{9}$$

where $C_T$ and $C_B$ are, respectively, the total protein concentration (mg.mL$^{-1}$) in the top and bottom phases, and $EA_T$ and $EA_B$ are the enzyme activity (U.mL$^{-1}$) of the top and bottom phases, respectively.

In order to evaluate the purification process, the enzyme specific activity ($SA$, U.mg$^{-1}$ protein) was calculated using Eq. (10), the volume ratio between volumes of top and bottom phases ($R_v$), the contaminant protein recovered in the top phase ($R_{PT}$, %), the enzyme recovered in the bottom phase ($R_{EB}$, %),and the purification factor ($PF$ - fold) were calculated using Eqs. (11)–(13).

$$SA = \frac{EA}{C} \tag{10}$$

$$R_{\mathrm{PT}} = \frac{100}{1 + \left({}^{1}/_{R_{\mathrm{V}}K_{\mathrm{P}}}\right)} \tag{11}$$

$$R_{\mathrm{EB}} = \frac{100}{1 + R_{\mathrm{V}}K_{\mathrm{E}}} \tag{12}$$

$$PF = \frac{SA}{SA_i} \tag{13}$$

where C is the total protein concentration ($mg.mL^{-1}$). The purification factor (*PF*) was calculated by the ratio between the *SA* in the top or bottom phase and the initial specific activity (*SAi*).

*2.5 Enzyme assay*

Lipolytic activity was assayed using the modified oil emulsion method proposed by Soares *et al*. [58]. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of Arabic gum solution (7%, w/v). The reaction mixture containing 5 mL of the oil emulsion, 2 mL of 100 mM sodium phosphate buffer (pH 7) and enzyme extract (1 mL) was incubated in a thermostated batch reactor for 5 min at 37 °C. A blank titration was done on a sample where the enzyme was replaced with distilled water. The reaction was stopped by the addition of approximately 0.33 g of sample to a 2 mL of acetone–ethanol–water solution (1:1:1). The liberated fatty acids were titrated with 40 mM potassium hydroxide solution in presence of phenolphthalein as indicator. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min ($\mu mol.min^{-1}$) under the assay conditions (37 °C, pH 7, 120 rpm).

*2.6 Protein Assay*

Total protein concentration was determined by Bradford's method [59], using a SHIMADZU UV-1700, Pharma-Spec Spectrometer UV-Vis Spectrophotometer at 595 nm, and a calibration curve previously established for the standard protein bovine serum albumin (BSA).

*2.7 SDS-PAGE electrophoresis*

Electrophoresis was performed with the Mini-PROTEAN II System (BioRad, Brazil) using 12 % resolving gels and 5% stacking gels as described by Laemmli [60]. Proteins were visualized by staining with silver stain procedure. Protein markers used were trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine albumin (66.2 kDa), and phosphorylase (97.4 kDa) all purchased from BioRad (Brazil).

## 3. Results and discussion

*3.1 Binodal curves and tie-lines*

The ability of the various cholinium compounds to induce ATPS formation in presence of THF was evaluated. Aqueous solutions of each cholinium (from 20 wt% to 60 wt%) and of THF (from 80 wt% to pure THF) were initially prepared and used for the determination of the binodal curves at 25 ± 1 ºC and atmospheric pressure, through the cloud point titration method [18], and the results are shown in Fig. 2. The data of the binodal curves in mass fraction units, as well as the respective regression parameters (A, B and C) obtained by applying Eq. 1 [57], standard deviations (*std*) and correlation coefficients ($R^2$) are provided in Supporting Information (Table A.1).

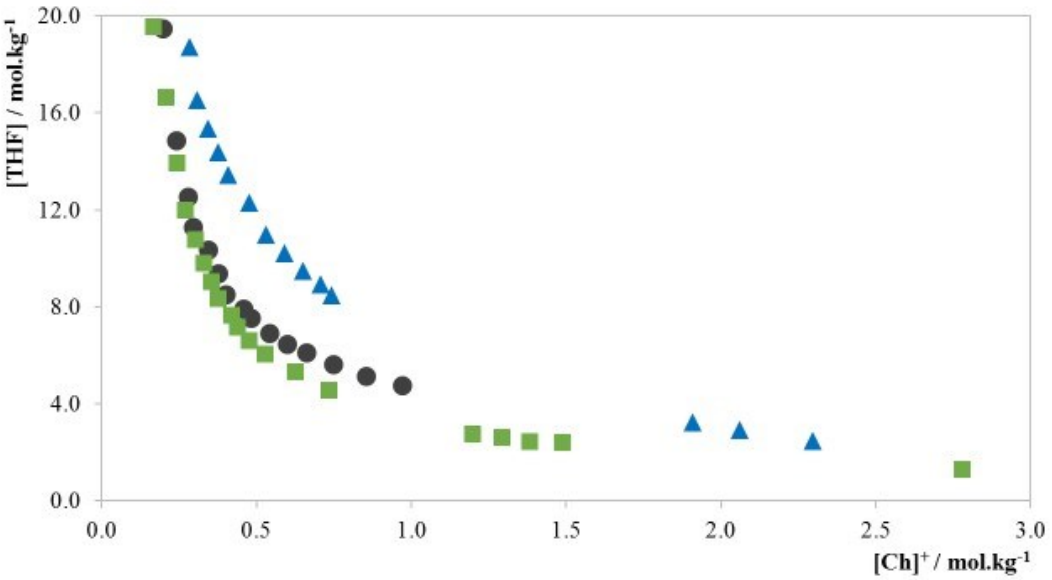


**Fig. 2:** Binodal curves for the ternary systems composed of THF + cholinium-based ILs + water, at 25 ± 1 ºC and atmospheric pressure. ● [Ch]Cl; ■ [Ch][Bit]; ▲ [Ch][DHCit].

The phase diagrams provide information about (*i*) the concentration of phase-forming components required to form two phases (total mixture compositions above the binodal curve fall into the biphasic regime, whereas mixture compositions below the solubility curve are homogeneous); (*ii*) the concentration of phase components in the top and bottom phases; and (iii) the ratio of phase volumes [25]. In addition, as shown in Fig. 3, the critical points for the studied systems were also estimated by extrapolation of the TLs compositions applying Eq. (3). In the *critical point*, where the two binodal nodes meet, the compositions of the two coexisting phases become equal, and the biphasic system ceases to exist [25]. The tie-line length (TLL) corresponds to the length of each tie-line and indicates the difference in composition of the two phases. The values of the TLLs are presented in Table A.2.

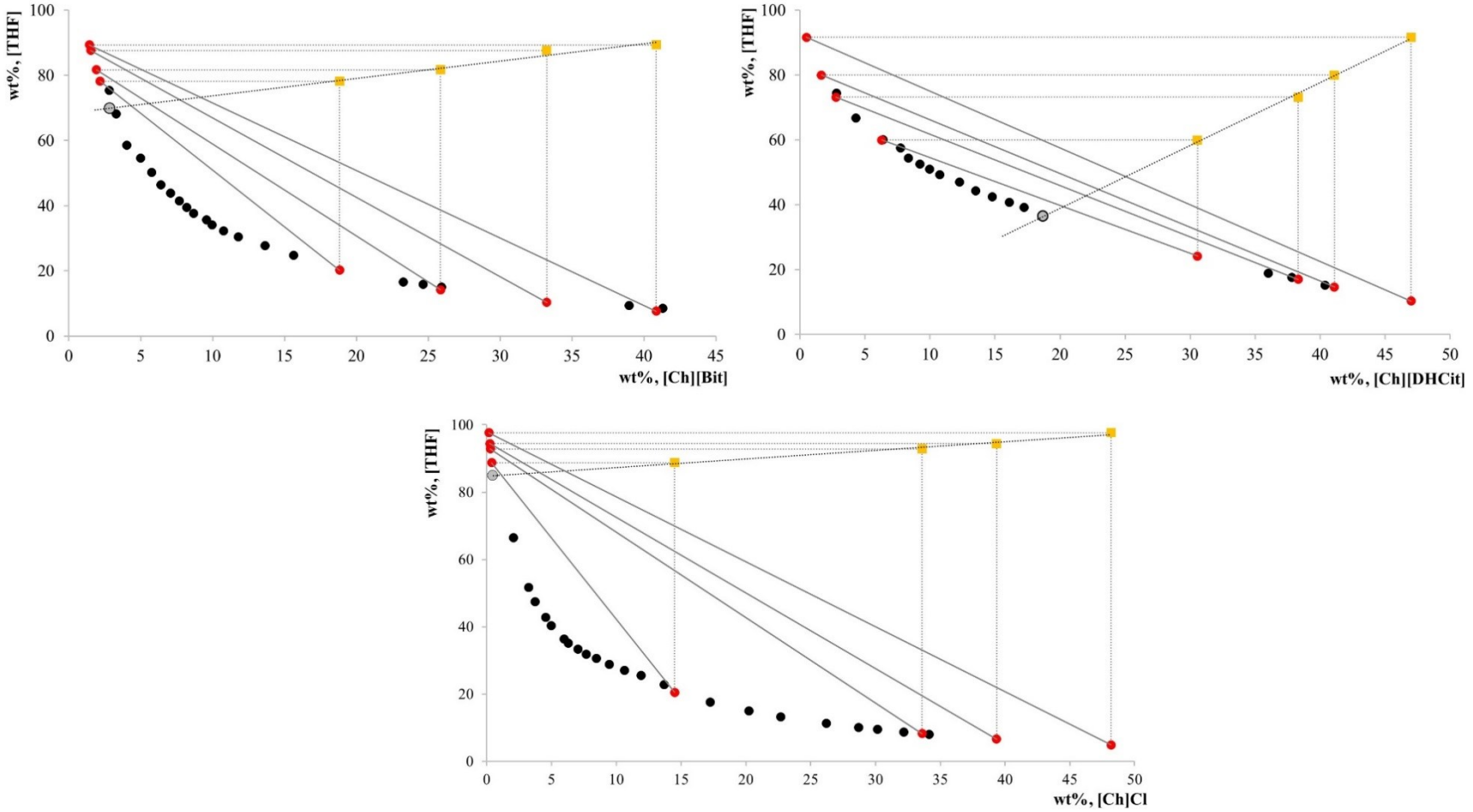


**Fig. 3:** Phase diagrams for ternary systems composed of THF + cholinium-based ILs + water, at 25 ± (0.1) ºC and atmospheric pressure. ●, experimental solubility data; ●, tie-line data; ▬, tie-lines; ■, auxiliary curve data; ●, critical point by Eq. (3).

Of the ATPS studied, [Ch][Bit] showed the highest ability to induce ATPS as shown in Fig. 2. The ability of the cholinium salts to promote the formation of ATPS with THF follows the trend: [Ch][Bit] > [Ch]Cl > [Ch][DHCit]. Considering that these ILs have the same cation but different anions, the phase-forming ability of these ILs must be determined by the nature of the anions. The ability to induce phase separation of these cholinium salts is determined by the capacity of the anion to form hydration complexes. Anions with higher charge densities have a strong hydration capacity than those with a lower charge density [61], leading to the exclusion of the more ‘‘hydrophobic’’ compounds (THF in this case) into a second liquid phase. The high polarity due to the charge of the carboxylate groups in both [Ch][Bit] and [Ch][DHCit], and the hydrogen bond acceptor ability of the chloride anion increases their affinity for water inducing a salting-out effect in THF leading to the formation of ATPS. Additionally, the ability of the [Ch][Bit] to promote the phase separation is affected by the pH of the system bottom phase (pH 3.8, Table A.3). In this system a portion of the bitartrate ions are completely deprotonated (presenting divalent charges), and therefore present a greater capacity to form two phases, as seen in Fig. 2 (the speciation curves for [Ch][Bit] is reported in Supporting Information, Fig A.1).

*3.2 Studies of partition of Burkholderia cepacia lipase*

In order to optimize the purification process, lipase from *Burkholderia cepacia* (*BCL*) was used. The initial mixture compositions were selected so that the liquid–liquid systems could be formed taking into account their phase diagrams. The volumetric ratio ($R_V$), pH, partition coefficients ($K_E$), and enzyme recovery in the bottom phase ($R_{EB}$) of *BCL* of the systems are presented in Table A.3.

It should be remarked that for the systems based on [Ch][Bit], [Ch][DHCit] and [Ch]Cl, the bottom phase is the IL-rich phase whereas the top phase corresponds to the THF-rich phase.

This first optimization step aimed at understanding the dependence of $K_E$ and $R_{EB}$ with the different cholinium-based ILs studied. The values of $K_E$ shown in Fig. 4 suggest that lipase preferentially migrates for the IL-rich phase ($K_E$ always < 0.41). This preference was also observed by Li et al.[47] using proteins of different molecular weights (lysozyme, papain, trypsin and BSA) in systems based on cholinium-based ILs and the lipase partitions into the IL-rich phase in the following order: [Ch][Bit] > [Ch][DHCit] > [Ch]Cl. This tendency seems to be dominated by the hydrophobicity of the anions since the highest values of $R_{EB}$ were attained for systems composed of the most hydrophobic ionic liquids, namely [Ch][Bit] and [Ch][DHCit], reflected by their octanol–water partition coefficients ($\log K_{ow}$ = -1.43 and -1.32, respectively, while for [Ch]Cl it is -3.70) [62]). The pH values of the bottom phases range between 3.5 and 4.5, and seem to have no significant influence on the preferential migration of the lipase for the IL-rich phase (Table A.3).

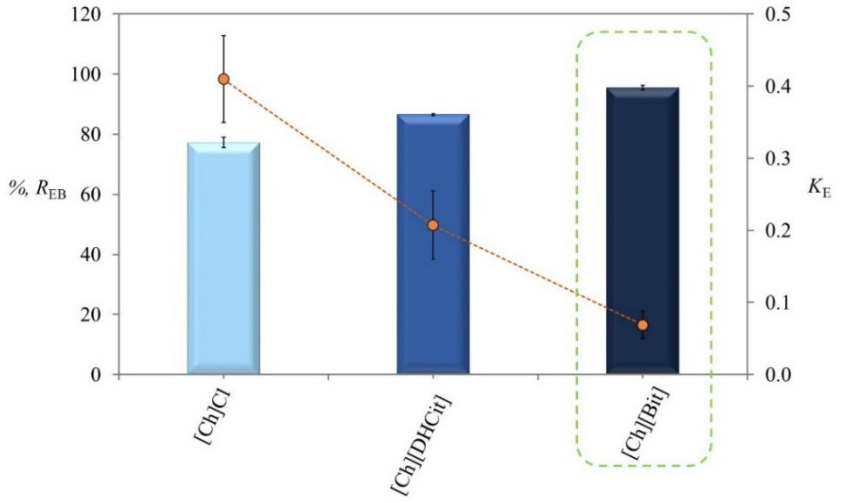


**Fig. 4:** Comparison of the enzyme recovery in the bottom data (%, $R_{EB}$ represented by the bars), and partition coefficient data ($K_E$ - represented by the symbols) for lipase from *Burkholderia cepcaia* using different cholinium-based ILs ATPS with THF. All ATPS are composed of 40 wt% THF and 25 wt% IL, at 25 ± (0.1) ºC, and atmospheric pressure.

Although we can say that the best $R_{EB}$ of lipase was achieved with [Ch][Bit], the concentrations of the phases, i.e. the tie line length may also be optimized to maximize the

extraction. In order to infer the effect of the composition of the ATPS on the partitioning of lipase, several experiments were carried out with varying concentrations of THF and [Ch][Bit] in the total mixture. In Fig. 5 are shown the effects caused by changing the concentration of THF (Fig. 5 (*i*)) and [Ch][Bit] (Fig. 5 (*ii*)) in the recovery and the partition coefficient of *BCL*, which is used as model for the further implementation with the real system. In addition, the effect of the THF solution on the lipolytic activity of lipase shows no deleterious effect when considering up to 18 hours of incubation (Figure A.2). The high tolerance of lipase from *Burkholderia* against organic solvent was previously reported [21, 63].

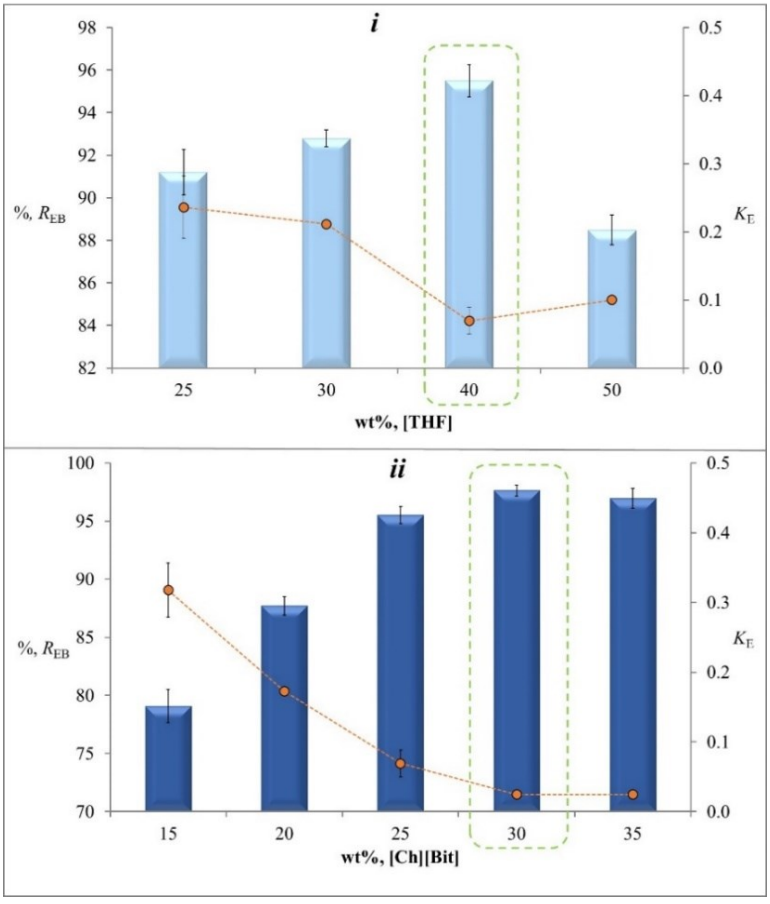


**Fig. 5:** Enzyme recovery in the bottom phase (%, $R_{EB}$ - represented by the bars) and partition coefficients ($K_E$ - represented by the symbols) of lipase from *Burkholderia cepacia*, for systems based in THF + [Ch][Bit] + water, at 25 ± (0.1) ºC and atmospheric pressure, as a function of concentration: (*i*), wt% THF + 25 wt% [Ch][Bit]; (*ii*) 40 wt% THF + wt% [Ch][Bit].

For the first analysis, the concentration of [Ch][Bit] was fixed in 25 wt% and the concentration of THF ranged from 25 to 50 wt%. An increase in the concentration of THF in the top phase (up to 40 wt%) enhances the migration of the enzyme to the bottom phase (rich in IL) as seen in Fig. 5 (*i*) when analyzing the partition coefficients and $R_{EB}$ for lipase in the IL-rich phase ($K_E$ = 0.24 ± 0.05 to 0.07 ± 0.02 and $R_{EB}$ = 91.2 ± 1.06 % to 95.5 ± 0.75 %, respectively for ATPS with 25 wt% and 40 wt% of THF). At concentrations above 40 wt% of THF, the migration of lipase appears to increase slightly for the top phase, this fact, coupled with the increase in the volumetric proportions between the phases ($R_V$ = 1.30), dramatically lowers the enzyme recovery of the system with 50 wt% of THF ($R_{EB}$ = 88.5 ± 0.7%). Clearly we can observe that 40% of THF is the concentration limit at which we have the best recovery of lipase in the bottom phase. The phenomena of intermolecular interactions between THF and water are key issues to understand these results. At low concentrations of THF the hydrogen bond between the water-THF has little effect on the water–water hydrogen bonded network. However, the addition of THF in water reduces the strength of hydrogen bonding, the tetrahedral structure of water breaks down, and a hydrogen bond is formed between water and THF [64]. In this case, the increase of the volumetric ratio between phases and the migration of enzymes for THF-rich phase is expected.

Following the study of optimization, the concentration of [THF] was fixed in 40 wt% and the concentration of [Ch][Bit] ranged from 15 to 35 wt% (Fig. 5 (*ii*)). The increase of the IL concentration leads to lower partition coefficients of lipase, that is, to a higher ability of lipase to migrate for the IL-rich phase. In accordance, the enzyme recovery in the bottom phase of lipase increased from 79.08 ± 1.4% to 97.61 ± 0.5%, respectively for ATPS with 15 wt% and 30 wt% of IL. Lipase was almost completely recovered in the IL-rich phase with the higher amount of IL (30 – 35 wt% of IL). This is a result of the salting-in effect of the IL over lipase which forces the biomolecule migration for the salt-rich phase. The lipases, due its hydrophilic

character tend to partition away from the solvent-rich phase. This preference is reported by several previous studies and corroborates our observations [8, 31, 65]. Data for the enzyme recovery ($R_{EB}$), partition coefficients of enzyme ($K_E$) and volumetric ratio ($R_V$) for ATPS with different compositions of THF and [Ch][Bit] in (wt%) are shown in Table A.4.

The influence of temperature on the lipase extraction was also studied using ATPS composed of THF at 40 wt% and [Ch][Bit] at 30 wt% (Fig. 6). The temperature of equilibrium was changed from 5 to 25 ºC. An increase in temperature slightly favors the migration of lipase for the IL-rich phase. The thermodynamic functions calculated for the transfer of lipase, namely the molar Gibbs energy ($\Delta G_m^o$) the molar enthalpy ($\Delta H_m^o$) and the molar entropy of transfer ($\Delta S_m^o$), Eqs. (10) - (12) were used.

$$\ln K_E = -\frac{\Delta H_m^O}{R} \times \frac{1}{T} + \frac{\Delta S_m^O}{R} \quad (10)$$

$$\Delta G_m^O = \Delta H_m^O - T\Delta S_m^O \quad (11)$$

$$\Delta G_m^O = -RT \ln(K_E) \quad (12)$$

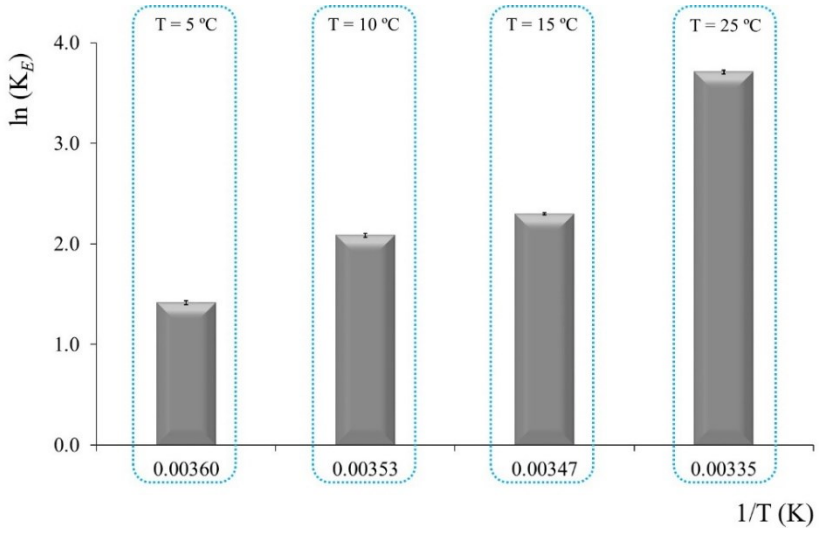


**Fig. 6:** Effect of temperature on partition coefficient ($K_E$) of lipase from *Burkholderia cepacia* for the ATPS based on THF and [Ch][Bit].

The calculated value for $\Delta G_m^o$ (-5.72 KJ/mol) is negative, reflecting therefore the spontaneous and preferential partitioning of lipase for the IL-rich phase ($K_E < 1$). The migration

process of lipase from the THF-rich phase to the IL-rich phase is endothermic ($\Delta H_m^o$ = 76.82 KJ/mol) and mainly governed by entropic forces ($\Delta S_m^o$= 287.7 J/mol.K), since $T \times \Delta S_m^o > \Delta H_m^o$.

In summary, the optimization tests using the lipase from *BCL* indicate that improved partition coefficients and recovery are obtained with ATPS composed of THF at 40 wt% and [Ch][Bit] at 30 wt%. Therefore, this cholinium-based IL was chosen, along with the THF, to conduct the purification of lipase from the fermented broth, which is described below.

### *3.3 Partition of lipase from Bacillus sp.ITP-001*

#### *3.3.1 Production and pre-purification of lipase*

The process, from production to the pre-purification step of extracellular lipase from *Bacillus* sp. ITP-001, considering the application of the salt $(NH_4)_2SO_4$ for the precipitation process of lipase, followed by a dialysis step to remove low molecular weight compounds, including inorganic salts of the precipitation process, was previously described by our group [8]. Table 1 reports the enzymatic activity (*EA* – $U.mL^{-1}$), total protein concentration (*C* – $mg.mL^{-1}$), specific activity (*SA* – $U.mL^{-1}$) and purification factor (*PF* – fold) in the fermented broth and dialyzed. The purification factor of the dialysate was found to be 12.7 ± 0.2 fold, confirming the values previously reported by our group at the stage of pre-purification by dialysis [8, 30, 55].

**Table 1:** Purification factor, enzymatic activity, specific activity, and protein concentration at the end of each step of the production and pre-purification of lipase produced by *Bacillus* sp. ITP-001.

| **Steps** | **Process** | **EA ($U.mL^{-1}$)** | ***C*** **($mg.mL^{-1}$)** | **SA ($U.mg^{-1}$)** | ***PF*** **(fold)** |
|---|---|---|---|---|---|
| Production | Fermentation | 6,167.3 | 1.15 | 5,365.7 | – |
| Pre-purification | Dialyse | 6,135.4 | 0.09 | 68,171.1 | 12.7 ± 0.2 |
| Purification | ATPS | 64,483.3 | 0.09 | 726,548.4 | 136.8 ± 0.5 |

*3.3.2 Purification of lipase using ATPS*

The extracellular lipase from *Bacillus* sp. ITP-001 was then purified pursuing the best conditions of composition and temperature of the systems, previously optimized. The extraction systems were prepared by adding 40 wt% of THF + 30 wt% of [Ch][Bit] + 30 wt% of dialysate solution containing the lipolytic enzyme produced.

The proposed application of this ATPS revealed a great performance in the purification of the lipolytic lipase produced from *Bacillus* sp. ITP-001. The data suggest that the *PF* of the enzyme was increased from 12.7 to 136.8 ± 0.5 fold, comparing the steps of pre-purification (by use of dialysis) with the proposed purification step (using ATPS). The increase of the purification factor achieved by the use of the ATPS is related with the large selectivity of the phases constituting the system, resulting mainly from the removal of the contaminants which act as inhibitors [31]. In this case, we can observe the increased recovery of the enzymes to the bottom phase, the IL-rich phase ($R_{EB}$ = 90.0 ± 0.7 %), in opposition to the contaminating proteins which migrate for the THF-rich phase ($R_{PT}$ = 54.5 ± 2.5 %) by following the partition coefficients of the enzyme ($K_E$ = 0.11 ± 0.01) and protein contaminants ($K_P$ = 1.16 ± 0.11). Previous studies from our group focused on the purification of lipases, including lipase from *Bacillus* sp. ITP-001 using IL/salt ATPS with 25 wt% of [$C_8$mim]Cl and 30 wt% of potassium phosphate buffer (pH 7). Showed lower purification factors than those here achieved (*PF* = 51 ± 2 fold) [30]. ATPS based in THF with the of potassium phosphate buffer (pH 7) were also tested and the results of purification (*PF* = 103.9 ± 0.9 fold) were again below to those described in this work [55]. All of these studies were reported for the lipase purification from *Bacillus* sp. ITP-001.

The highest purification factor of lipase from fermented broth was achieved in ATPS with a composition of 40 wt% THF, 30 wt% [Ch][Bit] at 25 ºC. The purity of the partitioned lipase was assessed with an SDS–PAGE [60]. The SDS–PAGE analysis is shown in Fig. 7. The

fermented broth contains multiple bands (Lane 1), which represent impurities present in the culture. In Lane 2, it is possible to see the presence of the target enzyme with a molecular weight of around 54 kDa (here abbreviated as *Enz*). The molecular weight of microbial lipase from *Bacillus* sp. ITP-001 was previously found to by 54 kDa [8, 30]. The results from the electrophoresis show the excellent purification ability of the THF/[Ch][Bit] based ATPS that made possible the separation of the enzyme from the contaminant compounds.

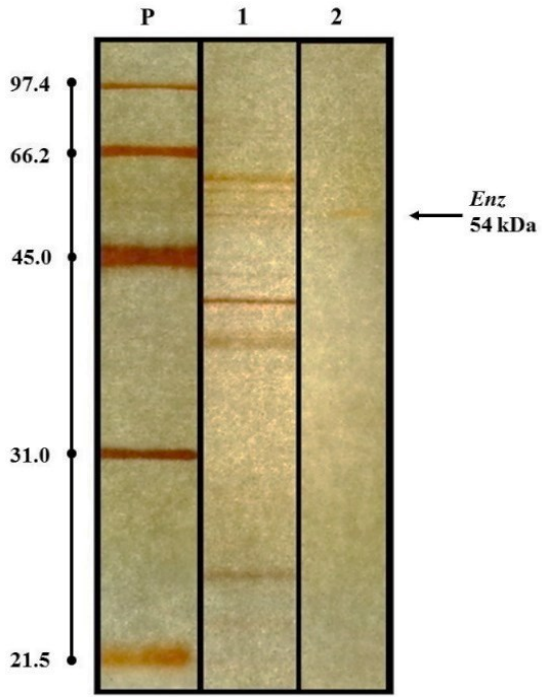


**Fig. 7:** SDS–PAGE analysis of purified lipase from *Bacillus* sp. ITP-001. The purity of partitioned lipase was assessed by 12 % acrylamide gel stained with silver nitrate solution. The molecular weights of the standard protein marker ranged between 21.5 – 97.4 kDa. Lane P: protein molecular markers; Lane 1: fermented broth; Lane 2: bottom phase obtained from the THF/[Ch][Bit]-based ATPS.

## 4. Conclusion

Cholinium-based ATPS were here successfully applied to the purification of lipase produced by the bacterium *Bacillus* sp. ITP-001, from a fermentation broth. For that purpose, novel ATPS

composed of tetrahydrofuran (THF) and three different cholinium-based ionic liquids were studied. The phase-forming ability of these ILs is determined by the nature of their anions.

The partition of lipase from *Burkholderia cepacia* (*BCL*) on these ATPS was studied and the operating conditions optimized, to be later applied to the purification using a real matrix. In the optimization study it was observed the preferential migration of lipase to the cholinium-rich phase, with the best partition being obtained for the [Ch][Bit]. The partition could be manipulated by the use of different tie line lengths to achieve the best extraction of the target biomolecule. The best recovery of enzyme was achieved using the ATPS composed of 40 wt% of THF and 30 wt% of [Ch][Bit] ($R_{EB}$ = 90 ± 0.7 %) at 25 ºC in the bottom phase ([Ch][Bit] - rich phase). After the optimization step, the best ATPS was applied to the purification of lipase, produced by the bacterium *Bacillus* sp. ITP-001 and a (*PF* = 136.8 ± 0.5) was achieved.

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# Supporting Information

## Aqueous tow-phase system based on cholinium salts and tetrahydrofuran and their use for lipase purification

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**Table A.1.** Parameters obtained through the Merchuk equation (Eq. 1) with the respective standard deviations (*std*) and correlation factors ($R^2$) along with the weight fraction data ($w$) for the systems composed of THF (1) + cholinium-based IL (2) + $H_2O$, at 25 ± 1 ºC.

| | **[Ch]Cl** | | **[Ch][Bit]** | | **[Ch][DCit]** | |
|---|---|---|---|---|---|---|
| **A** | 123.6 ± 5.4 | | 164.9 ± 6.2 | | 108.1 ± 1.3 | |
| **B** | -0.483 ± 0.020 | | -0.493 ± 0.016 | | -0.234 ± 0.005 | |
| **C** | $7.4\times10^{-14} \pm 4.3\times10^{-6}$ | | $1.4\times10^{-13} \pm 5.3\times10^{-6}$ | | $7.1\times10^{-6} \pm 6.0\times10^{-7}$ | |
| **R²** | 0.9949 | | 0.9969 | | 0.9992 | |
| | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ |
| | 66.49 | 2.05 | 75.28 | 2.82 | 77.79 | 1.74 |
| | 58.41 | 2.67 | 68.13 | 3.33 | 74.36 | 2.84 |
| | 51.72 | 3.23 | 58.51 | 4.05 | 66.62 | 4.33 |
| | 47.45 | 3.75 | 54.53 | 5.01 | 60.00 | 6.41 |
| | 44.89 | 3.96 | 50.09 | 5.77 | 57.46 | 7.75 |
| | 42.71 | 4.57 | 46.38 | 6.41 | 54.38 | 8.35 |
| | 40.32 | 4.98 | 43.73 | 7.10 | 52.53 | 9.24 |
| | 38.03 | 5.29 | 41.39 | 7.69 | 50.89 | 9.99 |
| | 36.33 | 5.99 | 39.41 | 8.22 | 49.25 | 10.76 |
| | 35.18 | 6.31 | 37.58 | 8.69 | 47.00 | 12.30 |
| | 33.29 | 7.03 | 35.53 | 9.60 | 44.18 | 13.54 |
| | 31.77 | 7.69 | 34.00 | 9.99 | 42.39 | 14.82 |
| | 30.62 | 8.45 | 32.25 | 10.79 | 40.65 | 16.12 |
| | 28.87 | 9.46 | 30.40 | 11.82 | 39.12 | 17.25 |
| | 27.06 | 10.65 | 27.67 | 13.66 | 33.87 | 22.35 |
| | 25.57 | 11.93 | 24.76 | 15.67 | 18.87 | 36.05 |
| | 22.75 | 13.69 | 16.53 | 23.29 | 17.47 | 37.83 |
| | 17.54 | 17.25 | 15.84 | 24.65 | 15.12 | 40.40 |
| | 14.93 | 20.26 | 14.99 | 25.93 | | |
| | 13.24 | 22.68 | 9.30 | 38.97 | | |
| | 11.23 | 26.22 | 8.43 | 41.31 | | |
| | 10.08 | 28.69 | | | | |
| | 9.47 | 30.16 | | | | |
| | 8.71 | 32.19 | | | | |
| | 8.05 | 34.16 | | | | |

**Table A.2.** Experimental data of TLs, TLLs and critical point values of the coexisting phases for the THF + cholinium-based IL systems at 25 ± (0.1) ºC.

| IL | Weight fraction/(wt%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $[THF]_M$ | $[IL]_M$ | $[THF]_T$ | $[IL]_T$ | $[THF]_B$ | $[IL]_B$ | TLL | $[Y]_{Critical}$ | $[X]_{Critical}$ |
| [Ch][Bit] | 30.16 | 15.98 | 78.20 | 2.19 | 18.84 | 20.20 | 60.34 | 69.86 | 2.85 |
| | 31.73 | 19.64 | 81.70 | 1.93 | 14.20 | 25.85 | 71.62 | | |
| | 30.35 | 25.02 | 87.66 | 1.54 | 10.28 | 33.24 | 83.62 | | |
| | 29.85 | 30.09 | 89.34 | 1.44 | 7.76 | 40.85 | 90.70 | | |
| [Ch][DHCit] | 32.56 | 24.86 | 59.95 | 6.29 | 24.13 | 30.57 | 43.27 | 36.44 | 18.71 |
| | 30.67 | 29.65 | 73.20 | 2.76 | 16.96 | 38.31 | 66.53 | | |
| | 25.12 | 34.78 | 80.00 | 1.65 | 14.66 | 41.09 | 76.32 | | |
| | 50.00 | 24.31 | 91.63 | 0.50 | 10.33 | 47.00 | 93.67 | | |
| [Ch]Cl | 30.18 | 12.51 | 88.74 | 0.39 | 14.51 | 20.52 | 69.66 | 84.84 | 0.48 |
| | 30.20 | 24.95 | 92.85 | 0.28 | 8.23 | 33.61 | 90.95 | | |
| | 50.08 | 19.99 | 94.42 | 0.24 | 6.61 | 39.35 | 96.12 | | |
| | 40.02 | 29.98 | 97.60 | 0.17 | 4.86 | 48.19 | 104.43 | | |

**Table A.3:** Enzyme recovery in the bottom phase (%, $R_{EB}$), partition coefficients ($K_E$), volumetric ratio ($R_V$) and pH of the bottom phase for the ATPS composed of 40 wt% of THF + 25 wt% of IL, for partition of the lipase from *Burkholderia cepacia* at 25 ± (0.1) ºC.

| ATPS | THF + IL | $R_V \pm \sigma$ | pH ± σ | $K_E \pm \sigma$ | $R_{EB} \pm \sigma$ (%) |
|---|---|---|---|---|---|
| | [Ch][Bit] | 0.79 ± 0.03 | 3.8 ± 0.2 | 0.07 ± 0.02 | 94.2 ± 1.0 |
| | [Ch][DHCit] | 0.67 ± 0.02 | 4.1 ± 0.1 | 0.21 ± 0.05 | 86.5 ± 0.3 |
| | [Ch]Cl | 0.72 ± 0.01 | 4.4 ± 0.1 | 0.41 ± 0.06 | 76.5 ± 1.3 |

**Table A.4:** Enzyme recovery in the bottom phase (%, $R_{EB}$), partition coefficients ($K_E$), volumetric ratio ($R_V$) for ATPS with different compositions of THF (wt%) and [Ch][Bit] (wt%), for lipase from *Burkholderia cepacia*, at 25 ± (0.1) ºC.

| ATPS | | $R_V \pm \sigma$ | $K_E \pm \sigma$ | $EE \pm \sigma$ (%) |
|---|---|---|---|---|
| wt%, THF | wt%, [Ch][Bit] | | | |
| 25 | 25 | 0.36 ± 0.02 | 0.24 ± 0.05 | 91.2 ± 1.1 |
| 30 | 25 | 0.37 ± 0.02 | 0.21 ± 0.01 | 92.8 ± 0.4 |
| 40 | 25 | 0.80 ± 0.03 | 0.07 ± 0.02 | 95.5 ± 0.7 |
| 50 | 25 | 1.30 ± 0.01 | 0.10 ± 0.01 | 88.5 ± 0.7 |
| 40 | 15 | 0.75 ± 0.10 | 0.32 ± 0.04 | 79.1 ± 1.4 |
| 40 | 20 | 0.85 ± 0.08 | 0.17 ± 0.01 | 87.7 ± 0.8 |
| 40 | 25 | 0.80 ± 0.03 | 0.07 ± 0.02 | 95.5 ± 0.7 |
| 40 | 30 | 1.00 ± 0.02 | 0.02 ± 0.01 | 97.6 ± 0.5 |
| 40 | 35 | 1.09 ± 0.34 | 0.02 ± 0.02 | 97.0 ± 0.9 |

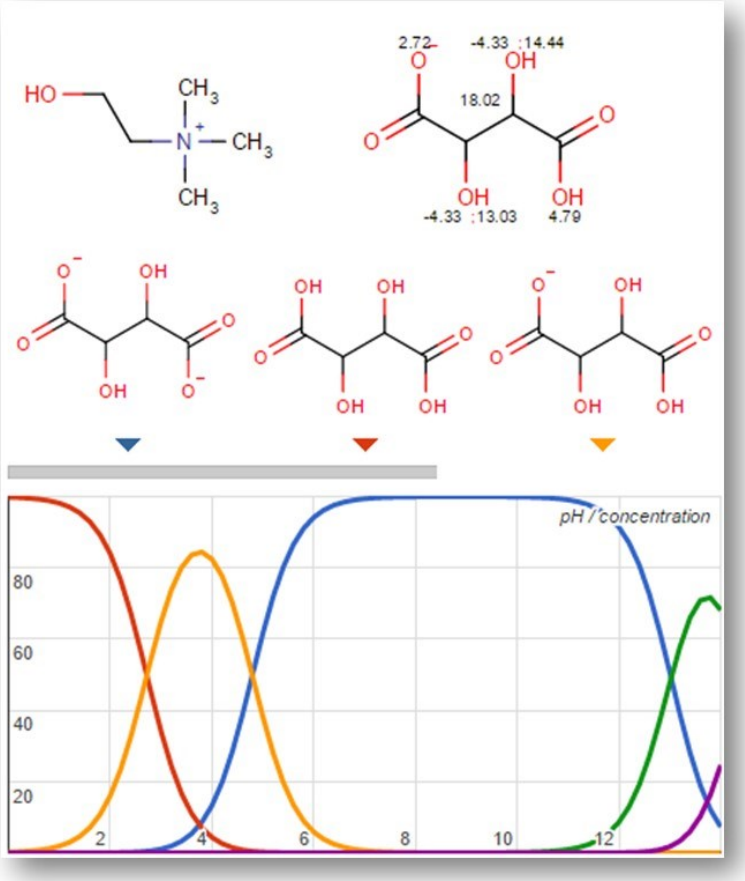


**Fig. A.1:** Speciation curve of [Ch][Bit] as a function of pH. This content was adapted from the Chemspider chemical database [1].

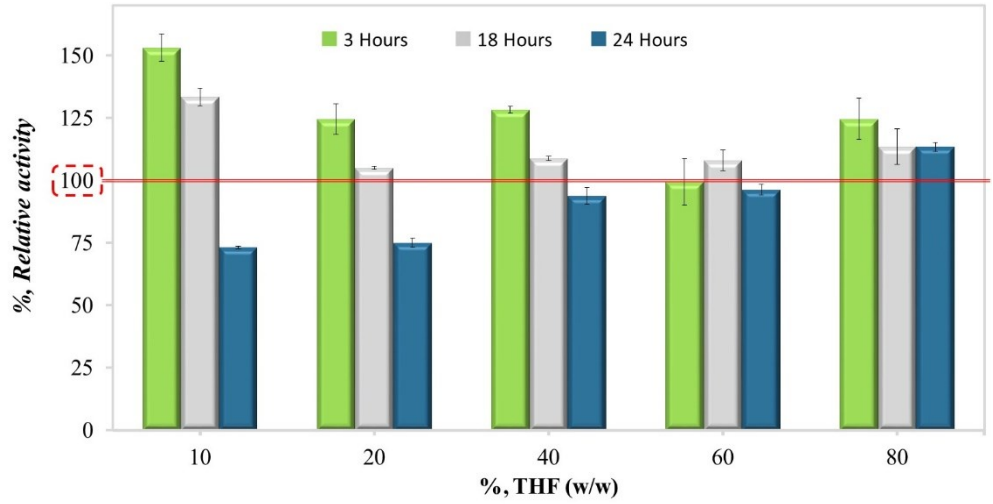


**Figure. A.1:** Effect of concentration of THF on the stability of lipase from *Burkholderia cepacia.* The crude lipase feedstock was incubated at room temperature up to 24 h. The relative activity was measured using a lipase assay. The lipase activity of phosphate buffer (pH 7.0) was used as the control. The THF concentrations were expressed as (wt%) [2].

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## ARTIGO III

# Effect of ionic liquids as adjuvants on PEG-based ABS formation and the extraction of two probe dyes

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**Abstract**

Aqueous biphasic systems (ABS) are relevant for the development of environmentally friendly and "biocompatible" separation processes. However, the common polyethylene glycol (PEG) polymers present a limited range of applicability, due to the low polarity of the PEG-rich phase. To overcome this limitation, a new approach was recently proposed based on the use of ionic liquids (ILs) as adjuvants in ABS, enlarging the polarity range of these systems. This work addresses the use of imidazolium-based ILs as ionic adjuvant compounds in the formation of ABS, namely potassium salts + water + PEG (1500, 4000, 6000 and 8000). To explore the differences induced by the presence of the IL as adjuvant the partition behavior of two dyes, Chloranilic Acid and Rhodamine 6G, is analyzed and correlated with the phase behavior and the IL distribution on the ABS under study.

*Keywords:* Aqueous Biphasic Systems, Ionic Liquids, Adjuvant, Partition, Dyes

## 1. Introduction

The application of aqueous biphasic systems (ABS) for liquid-liquid extraction processes was originally proposed by Albertsson in 1958 [1]. These systems form two aqueous phases that coexist in equilibrium due to the dissolution, at appropriate concentrations, of pairs of solutes in water [2]. ABS composed of polymers (namely polymer-polymer, or polymer-salt), were recognized as "biocompatible" systems to cells, organelles and biologically active substances, properties that make them well known as good systems to be applied in the recovery and purification of biomolecules [3]. Despite the well-known advantages offered by these systems, such as low interfacial tension, good biocompatibility, fast and high phase separation rates and low cost [4, 5], their performance is however significantly affected by the limited range of polarities of the coexisting phases, that can be an important issue to take into account when the goal is to apply these systems in the extraction of biomolecules.

Since 2003 [6], alternative ABS constituted by ionic liquids (ILs) have been proposed to replace ABS based on polymeric matrices. Contrarily to the common polymer-based ABS, they do not suffer from high viscosity [7, 8], formation of opaque aqueous solutions, and display a much broader range of polarities [2, 9] since ILs cover the whole hydrophilicity/hydrophobicity range [10, 11]. One of the main advantages of the application of ILs in ABS is the possibility of manipulating their physicochemical properties [12] by a proper combination/manipulation of the cation, anion and alkyl chains of the ILs [13]. Due to their advantages, these systems have been extensively studied [14-16] and applied in the extraction of wide variety of compounds such as amino-acids [17-21], drugs [22], phenolic compounds [21], alkaloids [23-27], antibiotics [24, 28] and anti-inflammatory compounds [29], proteins [30-33], enzymes[30, 34, 35] and natural colorants [36]. When dealing with ILs very miscible in water at room temperature, large concentrations of salts are necessary to promote the ABS formation, making the extraction process more expensive and less sustainable. The use of small quantities of ILs

as adjuvants [20] appears as an alternative to overcome this difficulty. Pereira et al. [20] have demonstrated that the incorporation of 5 wt% of an IL in a polymer + salt ABS is capable of modifying the polarities of both phases leading to more advantageous separation processes, with improved extraction parameters (*i.e.* higher partition coefficients and extraction efficiencies).

This work focuses in the design of several quaternary systems, based in four polymers (PEG 1500, PEG 4000, PEG 6000 and PEG 8000), three potassium salts ($K_3PO4$, $K_2HPO_4$ and the potassium phosphate buffer $K_2HPO_4/KH_2PO_4$) and four imidazolium-based ILs as adjuvants ([$C_2$mim]Cl, [$C_4$mim]Cl, [$C_6$mim]Cl and [$C_8$mim]Cl) at 5 wt%. Thus, various parameters were investigated in terms of their effect on the characteristics of the ABS, namely the ILs cation alkyl chain length, the polymers molecular weights, different potassium-based salts and finally, different pH values associated with the salt type applied. To evaluate this effect upon the separation potential, the partition of two dyes, Rhodamine 6G (R6G) and Chloranillic Acid (CA), was investigated, and the results discussed based on the partition of each IL in the various ABS.

## 2. Experimental section

### 2.1. Materials

The present study was carried out using different polyethylene glycol polymers (average molecular weight of 1500, 4000, 6000 and 8000 $g.mol^{-1}$), abbreviated as PEG 1500, PEG 4000, PEG 6000 and PEG 8000, respectively. These polymers were supplied by Sigma-Aldrich and were used as received. The inorganic salts used in the formation of the phase diagrams were the potassium phosphate tribasic ($K_3PO_4$), the potassium phosphate dibasic ($K_2HPO_4$) and the potassium phosphate buffer composed of $K_2HPO_4/KH_2PO_4$ at pH 7 [37]. These salts were

purchased from Sigma-Aldrich®, with purities higher than 98 wt%. The ILs studied were 1-ethyl-3-methylimidazolium chloride ([$C_2$mim]Cl), 1-butyl-3-methylimidazolium chloride ([$C_4$mim]Cl), 1-hexyl-3-methylimidazoliumchloride ([$C_6$mim]Cl) and 1-methyl-3-octylimidazolium chloride ([$C_8$mim]Cl). All ILs were purchased from Iolitec (Ionic Liquid Technologies, Germany). The Chloranilic Acid [CA, purity > 99 wt%] was purchased from Merck and the Rhodamine 6G (R6G, content ≈ 95 wt%) was supplied by Sigma-Aldrich®. The chemical structures of the ILs and dyes are presented in Fig. 1 (A and B) along with the abbreviations used.

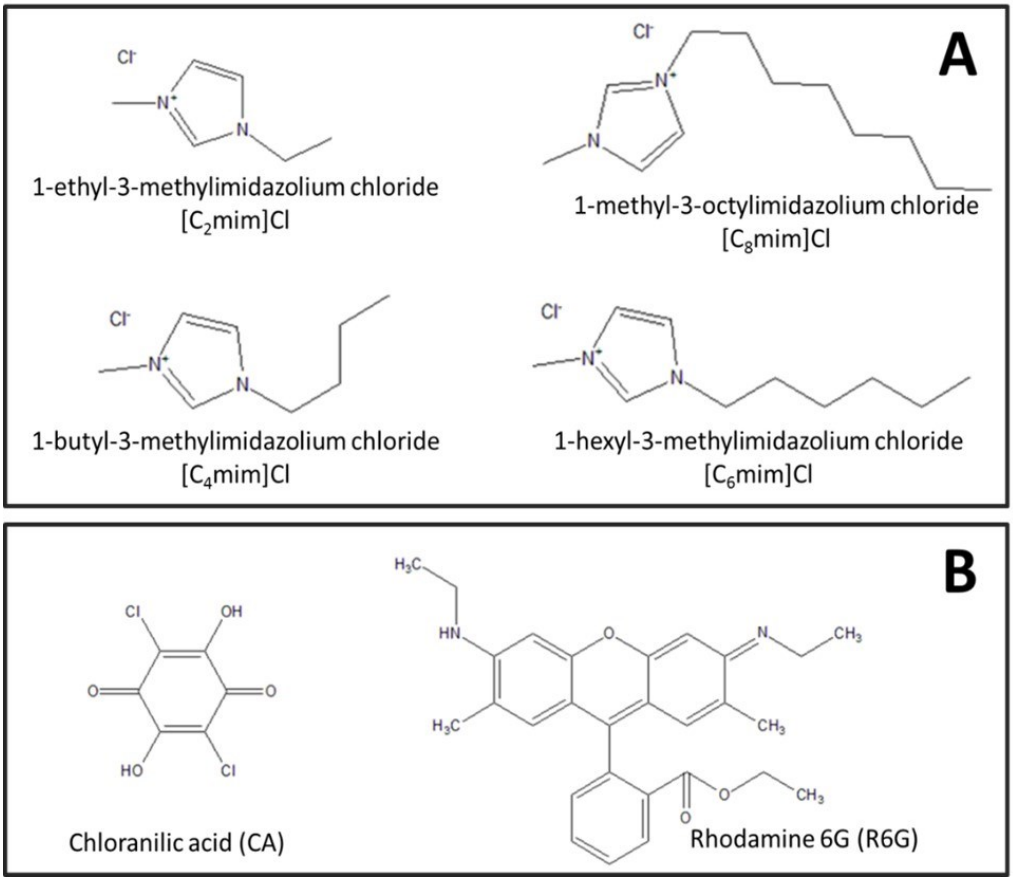


**Fig. 1:** Chemical structure, full name and abbreviation of the ILs (A) and dyes (B)

### 2.2. Phase Diagrams

The quaternary phase diagrams were determined at 298 (± 1) K and at atmospheric pressure, by the cloud point titration method [20]. The quaternary systems were prepared considering

stock solutions of each salt at 25 wt% plus 5 wt% of each IL, PEG at 40 wt% plus 5 wt% of each IL and finally, water solutions with 5 wt% of each IL studied. The systems composition was determined by the weight quantification of all components added within an uncertainty of ± $10^{-4}$ g. The binodal curves data were correlated using the Merchuk equation [38], described as follows:

$$Y = \mathrm{A} \times \exp[(\mathrm{B}X^{0.5}) - (\mathrm{C}X^{3})] \quad (1)$$

where Y and X are respectively, the PEG and inorganic salt weight percentages.

### 2.3. Dyes partition

A mixture point into the biphasic region was selected, composed of PEG at 15 wt%, salt at 15 wt% and IL at 5 wt%, being used to evaluate the partition of both dyes, the Chloranilic Acid (CA) and the Rhodamine 6G (R6G), by the combination of four imidazolium-based ILs ([$C_2$mim]Cl, [$C_4$mim]Cl, [$C_6$mim]Cl and [$C_8$mim]Cl), two polymers PEG 1500 and PEG 8000, and by the application of the potassium phosphate buffer.

In the preparation of each extraction system, circa 0.30 mg of each dye was introduced into the glass tubes already containing a total mass of the quaternary system of 5 g. The ABS was then allowed to equilibrate at 298 (± 1) K and atmospheric pressure conditions during 12 h to reach equilibrium. The top and bottom phases were then carefully separated, and the partition coefficients of each dye and the IL were evaluated. Thus, the ILs (211 nm) and the dyes (332 nm for CA and 527 nm for R6G) were quantified in both phases, through UV-Vis spectroscopy using a SHIMADZU UV-1700 Pharma-Spec spectrometer. The possible interferences from the phase promoters (salt, IL and polymer) were taken into account and found to be of no significance at the dilution levels used. Moreover, at least three samples of each extraction system were prepared, being the IL and dyes precisely quantified in both aqueous phases. Thus,

the partition coefficients of both ILs ($K_{IL}$) and dyes ($K_{dye}$) were determined, in accordance with Eq. 2 and 3:

$$K_{IL} = \frac{Abs_{IL_T}}{Abs_{IL_B}} \times df \quad (2)$$

$$K_{dye} = \frac{Abs_{dye\,T}}{Abs_{dye\,B}} \times df \quad (3)$$

where $Abs_{IL_T}$ and $Abs_{IL_B}$ are the absorbance data of IL in the top (PEG-rich) and bottom (salt-rich) phases, $Abs_{dye_T}$ and $Abs_{dye_B}$ represent the dye absorbance data in the top and bottom phases, respectively, and *df* represents the dilution factor.

## 3. Results and Discussion

One of the major drawbacks in the application of conventional polymer-salt based ABS is their limited polarity window between both aqueous phases. In this context, the number of studies describing new ABS and their application as extraction techniques is increasing. Initially, this work studies the phase behavior of different quaternary ABS formed by different combinations of PEG + potassium salts + water + imidazolium-based ILs, being ILs here applied as adjuvants. In this work several parameters were investigated, namely the salt, their pH, the PEG molecular weight and the IL alkyl chain length.

The mass fraction solubility data of all systems are presented in Supporting Information (Tables A.1 to A.6). The set of solubility curves obtained is discussed in this work, having into account two criteria: **(a)** The effect of the various salts in the ABS formation of PEG 1500-based systems with and without ILs, **(b)** The effect of the PEG molecular weight in the formation of ABS, using the potassium buffer solution ($K_2HPO_4/KH_2PO_4$) at pH 7 and ILs as adjuvants. All phase diagrams are presented in molality units to avoid discrepancies in the phase diagrams behavior, which could be a result of the differences between the PEG, salt and IL

molecular weights. All binodal curves were determined at 298 (± 1) K and atmospheric pressure. The data was correlated using Eq. 1 with the regression parameters reported in Tables A.7 and A.8. Furthermore, the partition of two different dyes (here utilized as probe molecules) is discussed based on the information collected about the phase diagrams, since our main objective is to evaluate the differences in the extraction capacity of these ABS induced by the addition of ILs as adjuvants.

### *3.1. Analysis of the Phase Diagrams*

#### *3.1.1. Effect of Salts*

The effect of using ILs as additives upon the formation of ABS composed by PEG 1500, potassium phosphate salts [phosphate buffer $K_2HPO_4/KH_2PO_4$ (pH 7), $K_2HPO_4$ or $K_3PO_4$] was analyzed in this study (Fig. 2 and Figs. A.1 and A.2, Supporting Information). The results depicted in Fig. 2 show that the presence of 5 wt% of [$C_n$mim]Cl-based ILs produce a small effect in the ABS formation. In general, it seems that ILs with smaller chains, namely the [$C_2$mim]Cl, tend to increase the two phase region when compared with the remaining ILs, thus following the tendency: [$C_2$mim]Cl > [$C_4$mim]Cl > [$C_6$mim]Cl. This behavior is independent of the salt used ($K_2HPO_4/KH_2PO_4$, $K_2HPO_4$ and $K_3PO_4$ are depicted in Figs. 2, A.1 and A.2) and it is in close agreement with the increase in the ILs hydrophobic nature, from [$C_2$mim]Cl to [$C_6$mim]Cl. The analysis of the phase diagrams should start by the less complex ABS, namely the PEG + salt + water (Fig. A.3, Supporting Information). In this Figure, the PEG 1500 + salt + water systems were depicted and the results suggest that the ability of the various potassium phosphate salts to form ABS follows the trend: $K_3PO_4$> $K_2HPO_4$> $K_2HPO_4/KH_2PO_4$, describing the well-known Hofmeister series [39] and the "salting-out" ability of these salts. When IL is added, the changes in the binodal curves follow the same trend observed for the PEG + IL + water systems [2], indicating that the ILs are preferentially interacting with the polymer-rich

phase, when the quaternary systems are considered. Because the interaction of the IL as adjuvant is important to understand the characteristics of these new systems, the partition coefficient of each IL ($K_{IL}$) tested was determined considering the PEG 1500 and PEG 8000 systems, both with the buffer solution (Fig. 3).

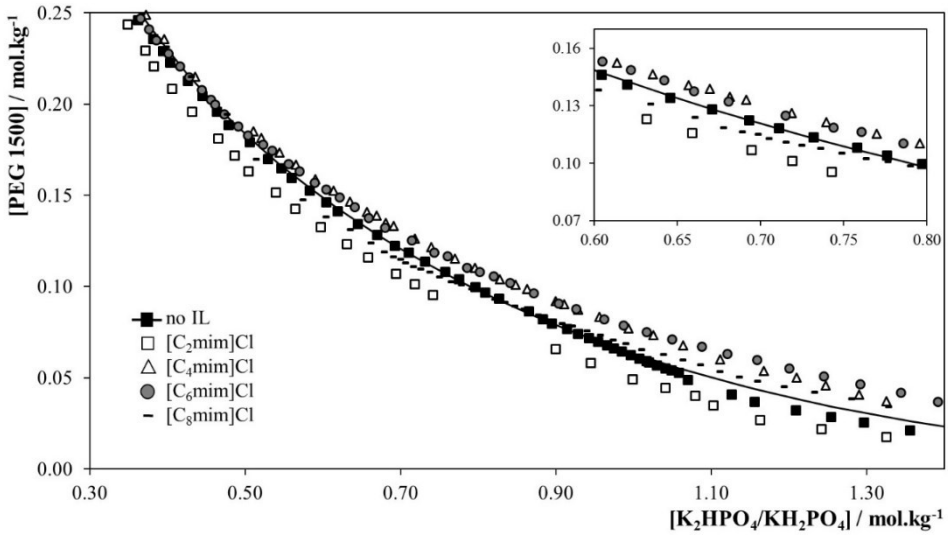


**Fig. 2:** Binodal curves for the quaternary systems composed of PEG 1500 + $K_2HPO_4/KH_2PO_4$ (pH 7) + 5 wt% of ILs (when present) + water, at 298 (± 1) K.

This salt was used, since it allows the pH to be kept constant during the partition experiments avoiding changes in the charge of the molecules [29, 40]. The $K_{IL}$ data were calculated according to Eq. 2, taking into account the PEG-rich phase as reference. Here, it seems that the various ILs have different affinities for the PEG-rich phase, which can be explained by their interactions with each one of the phases. Moreover, when the results obtained for the $K_{IL}$ were compared considering both PEG 1500 and PEG 8000, in general, it becomes clear that the IL migration for the top phase is favored for polymers with lower molecular weight. Meanwhile, it is observed that the migration of the ILs species increases with the IL' hydrophobicity. The exception observed for the [$C_8$mim]Cl, seems to be related with the possible micelle formation by the IL self-aggregation, which is promoted by the longer alkyl chains of this IL, helped by the presence of the salt, which is acting as a "salting-out" agent. As recently discussed, the

phase formation is always the result of a delicate balance between entropic effects (described by the decrease in the solubility and the consequent "salting-out" effects promoted by the presence of ILs) and the tendency of ILs to auto-aggregate when in aqueous media [41]. Their self-aggregation only happens for ILs containing longer alkyl chains higher than 6 carbons (not included) and above their corresponding critical micelle concentration (CMC) [42]. Furthermore, it is well-known that the presence of "salting-out" inducing salts is also responsible for the decrease of the CMC, facilitating more the aggregation of ILs, due to the decreased reduction of the repulsion between the cation core groups [41].

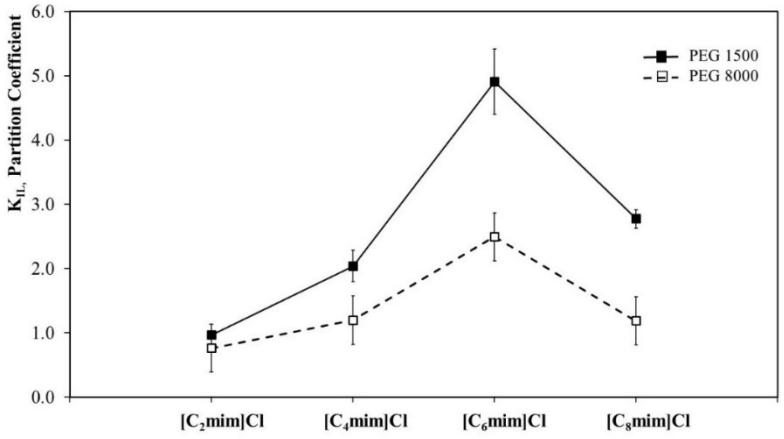


**Fig. 3:** ILs distribution between the two phases in terms of their partition coefficient ($K_{IL}$) and their respective standard deviations, for systems based in PEG (1500 and 8000) + 5 wt% of [$C_n$mim]Cl + water + $K_2HPO_4$/$KH_2PO_4$ (pH 7).

### *3.1.2. Effect of Polymer*

In this work, four PEGs (PEG 1500, PEG 4000, PEG 6000 and PEG 8000) were used to test the formation of quaternary ABS using the same ILs as adjuvants. In this case, the polymer also offers a certain level of tunability by the variation of the polymeric chain length and the average molecular weight. This effect was analyzed by studying ABS formed by the co-dissolution of the potassium phosphate buffer ($K_2HPO_4$/$KH_2PO_4$ at pH 7) and 5 wt% of imidazolium-based ILs. Fig. 4 presents the binodal curves for PEG (1500, 4000, 6000 and 8000) +

$K_2HPO_4/KH_2PO_4$ (pH 7) + water + IL. The influence of the PEG molecular weight (*i.e.* alkyl chain length) on the phase diagrams is clear with the two phase region increasing in the order: PEG 1500 < PEG 4000 < PEG 6000 < PEG 8000. This effect is well described in literature [43-45]. PEGs with a higher molecular weight are more hydrophobic, facilitating the ABS formation, since these polymers present a lower affinity for the water molecules, being more easily "salted-out".

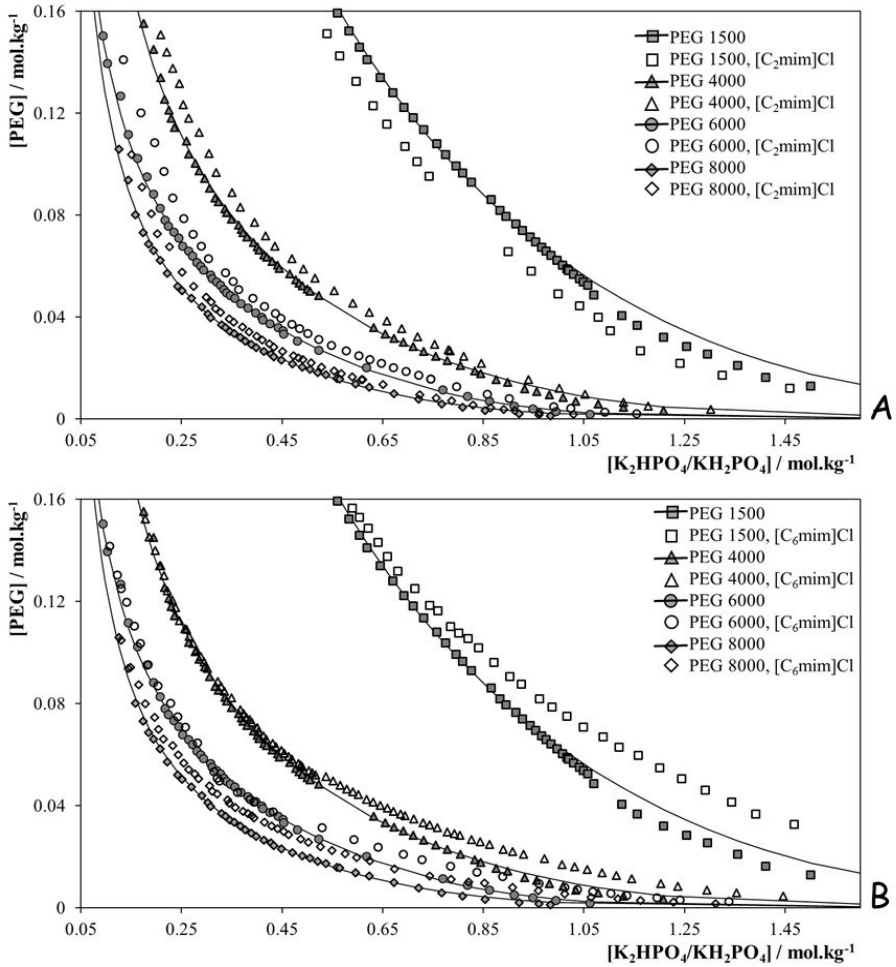


**Fig. 4:** Binodal curves for the quaternary systems, at 298 (± 1) K, composed of PEG (1500, 4000, 6000, and 8000) + $K_2HPO_4/KH_2PO_4$ (pH 7) + water + 5 wt% of $[C_2mim]Cl$ (A), or 5 wt% of $[C_6mim]Cl$ (B).

Fig. 4 also shows the impact of the $[C_nmim]Cl$ series on the phase separation. The binodal curves are depicted in Figs. 4A and 4B for $[C_2mim]Cl$ and $[C_6mim]Cl$ (respectively) for a better analysis of their effects in the ABS formation. The remaining systems are depicted in Figs. 5A

and 5B for [$C_4$mim]Cl and [$C_8$mim]Cl, respectively, their behaviors being similar to those reported in Fig. 4. The binodal curves of Figs. 4A and 4B indicate that the IL effect upon the phase separation is stronger for the IL with shorter alkyl chains, *i.e.* [$C_2$mim]Cl. In this case, the binodal data of the systems with and without IL are more deviated from each other, and the biphasic region of the system with IL is, in general, larger for the ternary system, being the only exception observed for PEG 1500, the more hydrophilic polymer, with [$C_2$mim]Cl. This synergistic effect upon ABS formation of the more hydrophilic ILs was previously observed for other low molecular weight PEG with various ILs [20].

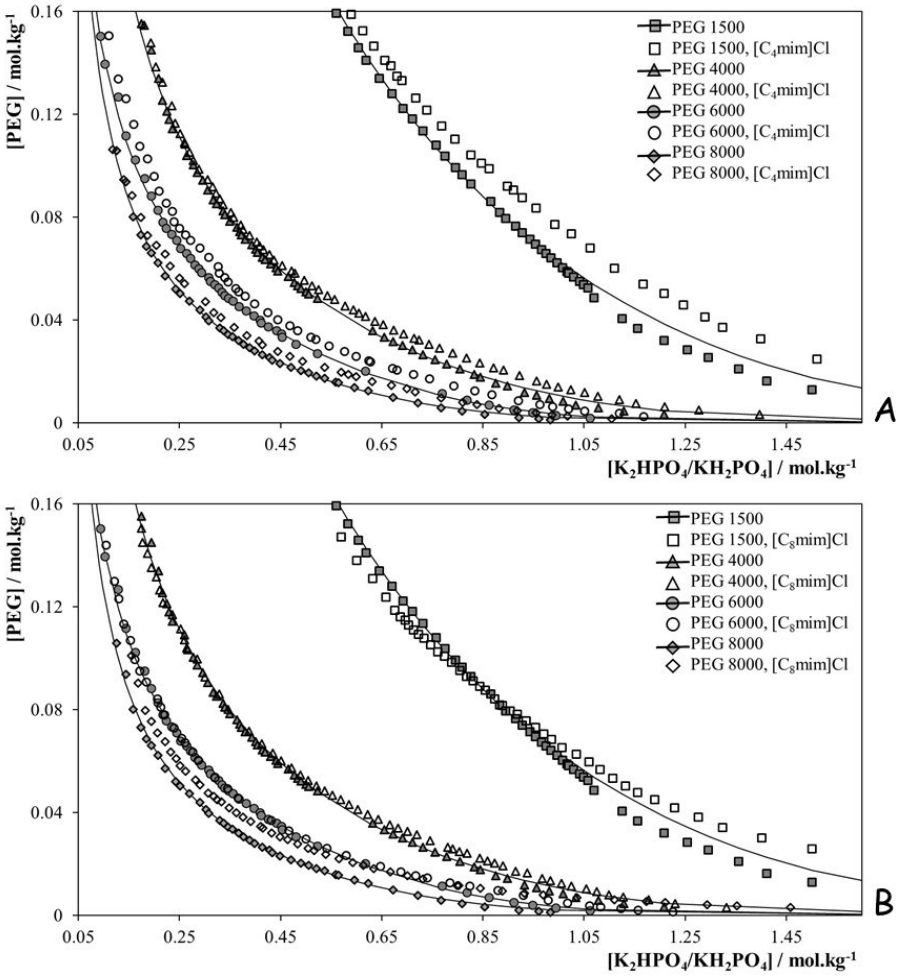


**Fig. 5:** Binodal curves for the quaternary systems, at 298 (± 1) K, composed of PEG (1500, 4000, 6000, and 8000) + $K_2HPO_4/KH_2PO_4$ (pH 7) + water + 5wt% of [$C_4$mim]Cl (A), or 5wt% of [$C_8$mim]Cl (B).

### *3.2. Dyes partition*

It is previously shown that the presence of the IL does not have a significant impact upon the phase separation. To further understand the nature of these systems, and the impact of the use of the IL as adjuvant, partition studies of two probe dyes in ABS systems with and without ILs were carried out. Rhodamine 6G (R6G) and the Chloranilic Acid (CA) (their structures being presented in Fig. 1B) were adopted in the partition tests due to their different natures since at pH 7, the CA is negatively charged and R6G is mainly in its neutral form (the speciation curves for these molecules are reported in Supporting Information, Figs. A.4 and A.5). The results of the partition experiments are depicted in Fig. 6 (***i)*** and (***ii***). These were done for the following ABS: PEG 1500 and PEG 8000 + $KH_2PO_4/K_2HPO_4$ + $[C_nmim]Cl$. In addition, the mass fraction of the systems, and the partition coefficients of ILs, CA and R6G, are shown respectively in Tables A.9, A.10 and A.11 (in Supporting Information). The results of Fig. 6 show that both dyes have more affinity for the more hydrophobic phase ($K_{dyes} > 1$), the PEG-rich phase, which is in agreement with their octanol-water partition coefficients ($\log K_{ow} > 1$), suggesting the hydrophobic interactions as the controlling forces of the dyes partition. However, based on the $K_{dyes}$ data, it seems that more than just the hydrophobic interactions control the partition, which is modulated by the ILs presence. The impact of the alkyl chain of the IL cation upon the partition coefficients is opposite when both dyes are analyzed. While the anionic CA becomes more concentrated, the neutral R6G follows the opposite trend and becomes less concentrated in the PEG-rich phase, as the additives change $[C_2mim]Cl$ to $[C_6mim]Cl$. These results may be explained by additional interactions between the anionic CA and ILs, in particular the electrostatic interactions. The more concentrated the IL in the PEG-rich phase (according to Fig. 3 this increases with the alkyl chain length), the larger the partition of CA towards this phase.

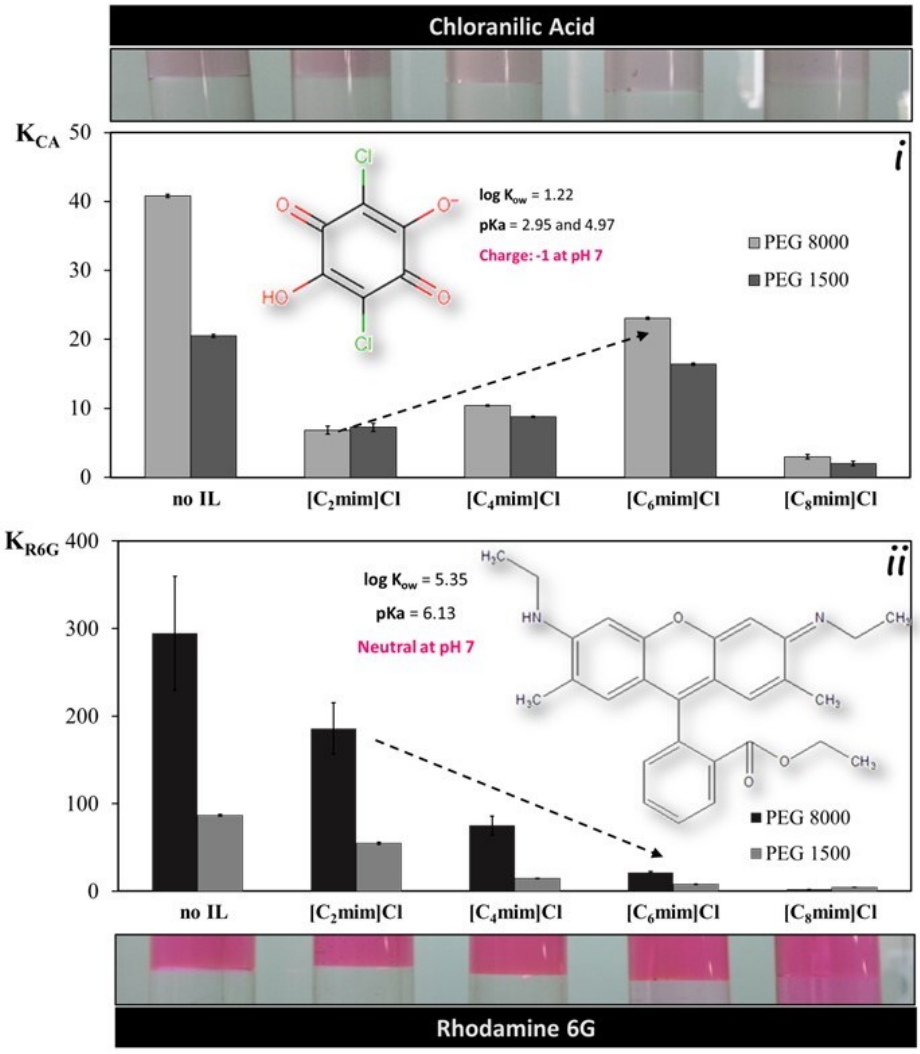


**Fig. 6:** Partition coefficient results for the ***(i)*** CA ($K_{CA}$) and ***(ii)*** R6G ($K_{R6G}$), by applying the systems based in PEG (1500 and 8000) + water + $K_2HPO_4/KH_2PO_4$ (pH 7) without and with 5 wt% of [$C_n$mim]Cl, at 298 (± 1) K. The visual aspect of the extraction systems for CA and R6G is depicted for systems based in PEG 8000.

The decrease observed for [$C_8$mim]Cl further supports this behavior ($K_{[C_8mim]Cl}$ is the lowest). Associated with the lowest amount of [$C_8$mim]Cl system in the PEG-rich phase (Fig. 3), the CA partition coefficient is also influenced by the IL self-aggregation [46], since this phenomenon is promoting the alteration of the interactions acting in the partition of the dye when different ILs with shorter and longer alkyl chains are compared. A decrease in the IL concentration in the PEG-rich phase has a direct and proportional impact in the CA migration. The neutral R6G presents a different behavior. Since it is not charged, the presence of the IL in

the PEG-rich phase becomes deleterious to the partition of the compound towards this phase, due to the enhanced polar and Coulombic interactions that would be present on this phase due to the IL. As the concentration of the IL in the PEG-rich phase increases with the alkyl chain length then the partition becomes less favorable towards this phase. Meanwhile, when the whole picture is observed, it seems that, despite the clear affinity of both dyes for the PEG-rich phase, when the ILs are not present, the partition coefficients of both dyes are higher. This can be easily explained by the increased hydrophobic nature of the PEG-rich phase [47]. It is noticeable the huge impact that a small quantity of IL has on the dyes partition.

## 4. Conclusions

The effect of ILs used as adjuvants in small concentrations (5 wt%), in the ABS composed of PEG with some potassium salts is here investigated. It is shown that, while in most cases the effect is small, it seems that the presence of ILs with smaller chains tend to increase the two phase region, being this behavior independent of the salt associated. In this specific work, all ILs have higher interaction with the most hydrophobic phase, the polymer-rich phase, which is shown by the partition coefficients of the ILs investigated. This migration of the adjuvants seems to be favored when polymers with lower molecular weights are presented. The results obtained from a more general analysis suggest that synergistic effects are playing a key role upon ABS formation.

The performance of the ILs as additives was further investigated through the study of the partition coefficients of Rhodamine 6G and the Chloranilic Acid, being established that while the presence of a small concentration of additive may have a minor effect upon the ABS formation it has a major impact in the PEG-rich phase characteristics, and thus on the partition coefficients of the dyes studied. Although both dyes have a higher affinity for the PEG-rich phase, their partition coefficients react differently to the presence of the IL depending on the

charge of the dye, and thus on the additional interactions that the IL induces in the PEG-rich phase.

**Acknowledgments**

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# Supporting Information

## Effect of ionic liquids as adjuvants on PEG-based ABS formation and the extraction of two probe dyes

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**Fluid Phase Equilibria** 375 **(2014)** 30–36

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**Table A.1:** Binodal weight fraction data for the systems composed of PEG 1500 (1) + $[K_2HPO_4]$ (2) + 5 wt% of ILs (when present) + $H_2O$ at 298 (± 1) K. The uncertainty associated with the weight quantification of all components added is within ± $10^{-4}$g.

| **no IL** | | **[C$_2$mim]Cl** | | **[C$_4$mim]Cl** | | **[C$_6$mim]Cl** | | **[C$_8$mim]Cl** | |
|---|---|---|---|---|---|---|---|---|---|
| **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** |
| 68.33 | 0.44 | 44.23 | 2.33 | 69.28 | 0.57 | 66.69 | 0.16 | 68.06 | 0.36 |
| 60.02 | 0.79 | 41.31 | 2.62 | 63.70 | 0.77 | 47.40 | 1.28 | 62.93 | 0.75 |
| 53.41 | 1.02 | 38.84 | 2.91 | 55.31 | 1.26 | 44.57 | 1.73 | 60.37 | 0.86 |
| 50.00 | 1.31 | 36.73 | 3.19 | 42.80 | 2.05 | 39.52 | 2.04 | 56.35 | 1.09 |
| 47.79 | 1.34 | 34.84 | 3.41 | 41.33 | 2.26 | 37.72 | 2.38 | 52.25 | 1.31 |
| 46.78 | 1.68 | 33.56 | 3.67 | 39.51 | 2.41 | 36.29 | 2.72 | 49.23 | 1.50 |
| 43.77 | 1.95 | 32.40 | 3.89 | 38.42 | 2.68 | 35.02 | 2.86 | 45.63 | 1.68 |
| 42.08 | 2.16 | 31.39 | 4.07 | 37.37 | 2.81 | 33.74 | 3.20 | 44.15 | 1.88 |
| 40.15 | 2.29 | 30.32 | 4.26 | 36.20 | 3.03 | 32.97 | 3.28 | 42.61 | 2.03 |
| 38.69 | 2.47 | 28.24 | 4.68 | 34.93 | 3.18 | 32.42 | 3.41 | 41.51 | 2.22 |
| 37.72 | 2.68 | 27.47 | 4.81 | 33.25 | 3.38 | 31.57 | 3.50 | 39.97 | 2.40 |
| 36.44 | 2.84 | 25.02 | 5.52 | 32.94 | 3.46 | 31.06 | 3.61 | 38.80 | 2.49 |
| 35.34 | 3.00 | 23.70 | 5.84 | 32.46 | 3.55 | 30.27 | 3.68 | 37.57 | 2.65 |
| 34.30 | 3.17 | 22.45 | 6.22 | 31.91 | 3.65 | 29.85 | 3.80 | 36.41 | 2.76 |
| 33.37 | 3.33 | 21.14 | 6.63 | 31.63 | 3.77 | 29.23 | 4.02 | 35.85 | 2.92 |
| 32.30 | 3.45 | 20.19 | 6.96 | 31.22 | 3.85 | 28.10 | 4.22 | 35.04 | 3.08 |
| 31.77 | 3.62 | 19.18 | 7.33 | 30.43 | 4.11 | 27.25 | 4.51 | 33.84 | 3.14 |
| 30.97 | 3.73 | 18.25 | 7.69 | 29.62 | 4.20 | 26.53 | 4.66 | 32.65 | 3.23 |
| 30.34 | 3.89 | 16.08 | 8.53 | 29.08 | 4.33 | 26.05 | 4.81 | 31.87 | 3.36 |
| 29.72 | 4.01 | 15.55 | 8.81 | 28.62 | 4.39 | 25.41 | 4.96 | 31.14 | 3.68 |
| 29.53 | 4.06 | 14.20 | 9.37 | 28.40 | 4.51 | 24.90 | 5.14 | 30.45 | 3.77 |
| 28.84 | 4.25 | 13.12 | 9.84 | 28.01 | 4.56 | 24.48 | 5.28 | 30.11 | 3.87 |
| 28.24 | 4.38 | 11.91 | 10.39 | 27.68 | 4.72 | 24.01 | 5.48 | 29.72 | 4.00 |
| 27.61 | 4.51 | 10.59 | 10.99 | 27.12 | 4.75 | 23.42 | 5.61 | 29.20 | 4.08 |
| 27.09 | 4.61 | 9.66 | 11.43 | 26.87 | 4.88 | 22.97 | 5.76 | 28.77 | 4.19 |
| 26.42 | 4.82 | 8.64 | 11.97 | 26.52 | 4.94 | 22.51 | 5.88 | 28.25 | 4.29 |
| 26.10 | 4.87 | 7.57 | 12.53 | 26.13 | 5.02 | 22.11 | 6.05 | 27.81 | 4.35 |
| 25.17 | 5.10 | 6.45 | 13.13 | 25.93 | 5.10 | 21.54 | 6.24 | 27.43 | 4.48 |
| 24.77 | 5.20 | 5.81 | 13.59 | 25.49 | 5.20 | 20.91 | 6.56 | 27.01 | 4.56 |
| 24.18 | 5.38 | 4.51 | 14.29 | 25.04 | 5.36 | 20.51 | 6.63 | 26.69 | 4.66 |
| 23.57 | 5.59 | 3.77 | 14.87 | 24.28 | 5.59 | 19.88 | 6.89 | 26.25 | 4.73 |
| 23.24 | 5.67 | 3.08 | 15.77 | 23.80 | 5.71 | 19.52 | 7.00 | 25.92 | 4.84 |
| 22.92 | 5.76 | 2.33 | 16.92 | 23.38 | 5.87 | 19.25 | 7.11 | 25.62 | 4.94 |
| 22.60 | 5.84 | 1.54 | 18.07 | 23.02 | 5.98 | 18.88 | 7.29 | 25.29 | 5.00 |
| 22.04 | 6.07 | 0.77 | 19.82 | 22.80 | 6.09 | 18.59 | 7.43 | 25.00 | 5.10 |
| 21.73 | 6.13 | | | 22.55 | 6.17 | 18.30 | 7.53 | 24.75 | 5.18 |
| 21.45 | 6.20 | | | 22.19 | 6.23 | 17.93 | 7.66 | 24.44 | 5.29 |
| 20.98 | 6.38 | | | 21.95 | 6.41 | 17.62 | 7.82 | 24.04 | 5.34 |
| 20.54 | 6.51 | | | 21.73 | 6.43 | 17.12 | 8.05 | 23.82 | 5.43 |
| 20.32 | 6.60 | | | 21.56 | 6.49 | 16.70 | 8.26 | 23.34 | 5.60 |
| 19.91 | 6.76 | | | 21.21 | 6.59 | 16.30 | 8.43 | 22.98 | 5.66 |
| 19.49 | 6.88 | | | 21.03 | 6.72 | 15.99 | 8.60 | 22.77 | 5.74 |
| 19.11 | 7.03 | | | 20.60 | 6.83 | 15.69 | 8.74 | 22.55 | 5.82 |
| 18.78 | 7.14 | | | 20.25 | 6.98 | 15.49 | 8.82 | 22.35 | 5.91 |
| 18.45 | 7.27 | | | 19.99 | 7.06 | 15.21 | 8.95 | 21.91 | 6.04 |
| 18.08 | 7.41 | | | 19.63 | 7.19 | 14.81 | 9.15 | 21.61 | 6.08 |
| 17.95 | 7.47 | | | 19.27 | 7.39 | 14.51 | 9.27 | 21.33 | 6.23 |
| 17.77 | 7.53 | | | 19.04 | 7.44 | 14.06 | 9.49 | 21.08 | 6.27 |
| 17.46 | 7.64 | | | 18.52 | 7.63 | 13.69 | 9.69 | 20.75 | 6.46 |
| 17.33 | 7.70 | | | 17.76 | 7.97 | 13.43 | 9.80 | 20.40 | 6.56 |
| 17.02 | 7.81 | | | 17.37 | 8.13 | 13.17 | 9.98 | 20.14 | 6.60 |

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|---|---|
| 16.73 | 7.93 |
| 16.45 | 7.91 |
| 16.18 | 8.01 |
| 15.79 | 8.17 |
| 15.68 | 8.22 |
| 15.40 | 8.32 |
| 15.10 | 8.46 |
| 14.77 | 8.58 |
| 14.41 | 8.76 |
| 14.30 | 8.80 |
| 14.11 | 8.87 |
| 13.81 | 9.00 |
| 13.56 | 9.12 |
| 13.31 | 9.23 |
| 13.13 | 9.29 |
| 12.80 | 9.45 |
| 12.56 | 9.54 |
| 12.34 | 9.63 |
| 11.97 | 9.78 |
| 11.56 | 10.24 |
| 11.40 | 10.35 |
| 11.02 | 10.51 |
| 10.82 | 10.62 |
| 10.38 | 10.80 |
| 10.21 | 10.91 |
| 9.70 | 11.10 |
| 9.47 | 11.24 |
| 9.24 | 11.38 |
| 8.99 | 11.53 |
| 8.41 | 11.73 |
| 8.09 | 11.88 |
| 7.80 | 12.02 |
| 7.58 | 12.19 |
| 7.34 | 12.36 |
| 6.65 | 12.60 |
| 6.39 | 12.77 |
| 6.11 | 12.94 |
| 5.90 | 13.09 |
| 5.28 | 13.33 |
| 4.91 | 13.52 |
| 4.66 | 13.70 |
| 4.31 | 13.90 |
| 4.01 | 14.19 |
| 3.64 | 14.38 |
| 3.25 | 14.61 |
| 3.18 | 14.77 |
| 2.98 | 14.98 |
| 2.52 | 15.23 |
| 2.25 | 15.74 |
| 1.28 | 17.00 |
| 1.76 | 16.24 |
| 0.70 | 18.28 |
| 0.45 | 24.50 |

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|---|---|---|---|
| 16.92 | 8.36 | 12.71 | 10.27 |
| 16.45 | 8.52 | 11.86 | 10.74 |
| 15.72 | 8.88 | 11.08 | 11.21 |
| 15.40 | 9.06 | 9.93 | 11.86 |
| 14.81 | 9.30 | 9.29 | 12.22 |
| 14.23 | 9.66 | 8.86 | 12.53 |
| 13.82 | 9.80 | 8.17 | 12.92 |
| 13.51 | 10.00 | 7.70 | 13.40 |
| 12.83 | 10.33 | 7.24 | 13.60 |
| 12.33 | 10.60 | 6.85 | 13.91 |
| 11.63 | 10.94 | 6.39 | 14.27 |
| 11.13 | 11.26 | 5.96 | 14.61 |
| 10.87 | 11.42 | 5.47 | 14.96 |
| 10.17 | 11.89 | 5.00 | 15.37 |
| 9.31 | 12.31 | 4.53 | 15.85 |
| 8.18 | 13.03 | 4.07 | 16.25 |
| 7.55 | 13.35 | 3.53 | 16.92 |
| 6.70 | 13.84 | 3.04 | 17.81 |
| 6.33 | 14.17 | 2.43 | 18.85 |
| 5.57 | 14.72 | 1.73 | 20.71 |
| 4.68 | 15.24 | 0.87 | 22.66 |
| 4.27 | 15.62 | | |
| 3.51 | 16.36 | | |
| 2.69 | 17.50 | | |
| 2.16 | 23.01 | | |
| 1.39 | 24.36 | | |

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|---|---|
| 19.97 | 6.65 |
| 19.80 | 6.72 |
| 19.63 | 6.78 |
| 19.48 | 6.86 |
| 19.28 | 6.90 |
| 19.13 | 6.96 |
| 18.91 | 7.05 |
| 18.78 | 7.10 |
| 18.64 | 7.13 |
| 18.49 | 7.18 |
| 18.25 | 7.32 |
| 18.08 | 7.36 |
| 17.92 | 7.41 |
| 17.81 | 7.47 |
| 17.71 | 7.53 |
| 17.58 | 7.57 |
| 17.43 | 7.63 |
| 17.33 | 7.66 |
| 17.11 | 7.77 |
| 17.01 | 7.80 |
| 16.83 | 7.89 |
| 16.73 | 7.91 |
| 16.61 | 7.95 |
| 16.48 | 8.00 |
| 16.18 | 8.16 |
| 16.06 | 8.21 |
| 15.95 | 8.24 |
| 14.75 | 8.87 |
| 14.47 | 9.00 |
| 14.20 | 9.14 |
| 14.06 | 9.19 |
| 13.87 | 9.31 |
| 13.57 | 9.44 |
| 13.34 | 9.56 |
| 13.24 | 9.62 |
| 13.10 | 9.69 |
| 12.92 | 9.79 |
| 12.25 | 10.13 |
| 11.96 | 10.28 |
| 11.56 | 10.47 |
| 11.38 | 10.59 |
| 11.00 | 10.79 |
| 10.66 | 10.98 |
| 10.29 | 11.16 |
| 10.07 | 11.31 |
| 9.68 | 11.49 |
| 9.24 | 11.72 |
| 9.05 | 11.85 |
| 8.67 | 12.07 |
| 8.42 | 12.23 |
| 7.89 | 12.49 |
| 7.62 | 12.69 |
| 7.40 | 12.83 |
| 7.07 | 12.95 |
| 6.88 | 13.10 |
| 6.58 | 13.26 |
| 6.37 | 13.41 |
| 6.21 | 13.57 |
| 5.77 | 13.80 |
| 5.06 | 14.31 |

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|---|---|
| 4.86 | 14.58 |
| 4.16 | 14.97 |
| 3.78 | 15.25 |
| 3.63 | 15.43 |
| 3.30 | 15.67 |
| 3.17 | 16.01 |
| 2.66 | 16.51 |
| 2.21 | 16.88 |
| 1.83 | 17.57 |
| 1.36 | 18.81 |
| 0.94 | 22.35 |
| 0.43 | 24.79 |

**Table A.2:** Binodal weight fraction data for the systems composed of PEG 1500 (1) + [$K_3PO_4$] (2) + 5 wt% of ILs (when present) + $H_2O$ at 298 (± 1) K. The uncertainty associated with the weight quantification of all components added is within ± $10^{-4}$g.

| no IL | | [$C_2$mim]Cl | | [$C_4$mim]Cl | | [$C_6$mim]Cl | | [$C_8$mim]Cl | |
|---|---|---|---|---|---|---|---|---|---|
| 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ |
| 68.08 | 0.70 | 45.70 | 1.92 | 67.88 | 0.44 | 68.57 | 0.36 | 69.65 | 0.36 |
| 57.62 | 1.12 | 41.60 | 2.38 | 63.58 | 0.84 | 61.70 | 0.67 | 63.12 | 0.67 |
| 51.08 | 1.44 | 40.32 | 2.42 | 58.21 | 1.01 | 58.44 | 1.03 | 56.99 | 1.01 |
| 47.62 | 1.77 | 38.77 | 2.66 | 54.05 | 1.22 | 53.62 | 1.28 | 49.77 | 1.17 |
| 42.42 | 2.21 | 37.23 | 2.96 | 40.75 | 2.10 | 49.53 | 1.57 | 47.86 | 1.37 |
| 41.25 | 2.49 | 35.04 | 3.19 | 40.02 | 2.31 | 45.59 | 1.82 | 45.88 | 1.54 |
| 39.11 | 2.68 | 33.94 | 3.30 | 39.09 | 2.39 | 42.62 | 2.07 | 43.77 | 1.72 |
| 37.63 | 2.84 | 32.59 | 3.55 | 30.36 | 4.38 | 40.26 | 2.22 | 41.35 | 1.88 |
| 36.77 | 3.12 | 31.62 | 3.66 | 29.32 | 4.65 | 38.72 | 2.49 | 40.40 | 2.07 |
| 35.20 | 3.24 | 30.55 | 3.88 | 28.63 | 4.77 | 37.02 | 2.65 | 38.80 | 2.59 |
| 34.49 | 3.52 | 29.51 | 4.08 | 28.37 | 4.80 | 35.84 | 2.78 | 37.83 | 2.75 |
| 32.98 | 3.69 | 28.51 | 4.31 | 27.86 | 4.96 | 34.97 | 2.94 | 36.90 | 2.88 |
| 31.63 | 4.04 | 27.29 | 4.45 | 27.45 | 5.09 | 33.71 | 3.06 | 36.11 | 3.02 |
| 30.11 | 4.45 | 26.50 | 4.61 | 26.85 | 5.33 | 33.43 | 3.23 | 35.12 | 3.16 |
| 28.53 | 4.58 | 25.74 | 4.77 | 26.36 | 5.35 | 32.86 | 3.44 | 33.91 | 3.42 |
| 28.10 | 4.76 | 23.91 | 5.29 | 25.79 | 5.64 | 31.86 | 3.58 | 32.85 | 3.73 |
| 27.53 | 4.93 | 22.56 | 5.62 | 25.25 | 5.69 | 31.08 | 3.72 | 31.53 | 3.94 |
| 26.91 | 5.07 | 21.01 | 6.13 | 24.82 | 5.84 | 30.52 | 3.91 | 30.53 | 3.99 |
| 26.24 | 5.16 | 20.03 | 6.33 | 24.45 | 5.95 | 29.93 | 4.02 | 30.10 | 4.12 |
| 25.76 | 5.31 | 18.89 | 6.72 | 24.05 | 6.07 | 29.16 | 4.10 | 29.48 | 4.23 |
| 25.17 | 5.41 | 17.89 | 7.06 | 23.50 | 6.19 | 28.74 | 4.24 | 29.07 | 4.34 |
| 24.59 | 5.64 | 16.88 | 7.39 | 23.25 | 6.23 | 28.41 | 4.33 | 28.54 | 4.43 |
| 24.18 | 5.79 | 15.78 | 7.78 | 22.87 | 6.33 | 28.02 | 4.45 | 28.15 | 4.54 |
| 23.78 | 5.89 | 15.03 | 8.06 | 22.56 | 6.44 | 27.63 | 4.60 | 27.65 | 4.61 |
| 23.10 | 6.11 | 14.35 | 8.29 | 22.27 | 6.53 | 27.16 | 4.61 | 27.34 | 4.73 |
| 22.79 | 6.11 | 13.40 | 8.68 | 21.88 | 6.68 | 26.70 | 4.74 | 26.66 | 4.93 |
| 22.59 | 6.23 | 12.51 | 9.00 | 21.78 | 6.70 | 26.31 | 4.89 | 26.20 | 4.98 |
| 22.22 | 6.32 | 11.41 | 9.43 | 21.49 | 6.77 | 25.77 | 4.98 | 25.81 | 5.10 |
| 21.95 | 6.37 | 10.42 | 9.84 | 21.27 | 6.86 | 25.43 | 5.10 | 25.34 | 5.31 |
| 21.76 | 6.49 | 10.74 | 9.69 | 21.04 | 6.93 | 24.95 | 5.17 | 24.75 | 5.34 |
| 21.36 | 6.55 | 8.62 | 10.57 | 20.77 | 7.03 | 24.66 | 5.26 | 24.46 | 5.45 |
| 21.06 | 6.61 | 7.02 | 11.35 | 20.67 | 7.07 | 24.34 | 5.36 | 24.09 | 5.49 |
| 20.65 | 6.78 | 5.44 | 12.06 | 20.44 | 7.14 | 23.76 | 5.68 | 23.69 | 5.66 |
| 20.36 | 6.87 | 4.40 | 12.74 | 20.07 | 7.33 | 22.85 | 5.92 | 23.46 | 5.72 |
| 20.16 | 6.93 | 3.48 | 13.46 | 19.82 | 7.42 | 22.31 | 6.10 | 23.22 | 5.79 |
| 19.97 | 6.99 | 2.93 | 13.79 | 19.56 | 7.49 | 21.61 | 6.29 | 22.95 | 5.87 |
| 19.29 | 7.23 | 2.48 | 14.15 | 19.22 | 7.62 | 20.95 | 6.53 | 22.72 | 5.94 |
| 18.40 | 7.55 | 2.15 | 14.53 | 18.89 | 7.77 | 20.50 | 6.66 | 22.47 | 6.02 |
| 17.60 | 7.85 | 2.08 | 14.99 | 18.72 | 7.82 | 20.11 | 6.82 | 22.27 | 6.08 |
| 16.66 | 8.20 | 1.65 | 18.34 | 18.50 | 7.90 | 19.61 | 6.96 | 21.89 | 6.21 |
| 15.70 | 8.57 | 1.10 | 19.62 | 18.16 | 8.06 | 19.31 | 7.13 | 21.51 | 6.43 |
| 15.10 | 8.83 | | | 18.01 | 8.11 | 18.91 | 7.28 | 21.10 | 6.47 |
| 14.19 | 9.19 | | | 17.79 | 8.18 | 18.54 | 7.38 | 20.88 | 6.53 |
| 13.26 | 9.57 | | | 17.49 | 8.31 | 18.27 | 7.53 | 20.49 | 6.67 |
| 12.26 | 9.96 | | | 17.35 | 8.39 | 18.02 | 7.60 | 20.18 | 6.80 |
| 11.28 | 10.35 | | | 17.11 | 8.47 | 17.67 | 7.74 | 20.01 | 6.85 |
| 10.40 | 10.74 | | | 16.84 | 8.60 | 17.51 | 7.79 | 19.85 | 6.91 |
| 9.34 | 11.15 | | | 16.60 | 8.70 | 17.32 | 7.90 | 19.69 | 6.95 |
| 8.35 | 11.58 | | | 16.26 | 8.87 | 16.99 | 8.02 | 19.54 | 7.00 |

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| 7.32 | 12.13 | 16.06 | 8.93 | 16.59 | 8.23 | 19.38 | 7.04 |
| 6.27 | 12.67 | 15.83 | 9.03 | 16.47 | 8.39 | 19.10 | 7.17 |
| 5.23 | 13.18 | 15.60 | 9.13 | 16.29 | 8.48 | 18.92 | 7.22 |
| 4.77 | 13.52 | 15.44 | 9.20 | 16.02 | 8.58 | 18.65 | 7.36 |
| 3.82 | 14.04 | 15.17 | 9.33 | 15.86 | 8.64 | 18.49 | 7.40 |
| 3.05 | 14.75 | 15.02 | 9.40 | 15.65 | 8.77 | 18.34 | 7.45 |
| 1.16 | 17.86 | 14.67 | 9.59 | 15.54 | 8.80 | 18.20 | 7.51 |
| 0.98 | 18.40 | 14.46 | 9.69 | 15.39 | 8.89 | 18.04 | 7.56 |
| 0.85 | 18.90 | 14.26 | 9.77 | 15.06 | 9.07 | 17.82 | 7.67 |
| 0.71 | 24.49 | 14.07 | 9.85 | 14.64 | 9.24 | 17.68 | 7.70 |
| | | 13.85 | 9.95 | 14.32 | 9.44 | 17.47 | 7.80 |
| | | 13.48 | 10.24 | 14.06 | 9.58 | 17.35 | 7.84 |
| | | 13.27 | 10.35 | 13.63 | 9.79 | 17.13 | 7.96 |
| | | 13.06 | 10.45 | 13.39 | 9.90 | 16.99 | 8.00 |
| | | 12.97 | 10.46 | 13.16 | 10.02 | 16.88 | 8.05 |
| | | 12.80 | 10.55 | 13.03 | 10.08 | 16.60 | 8.18 |
| | | 12.66 | 10.61 | 12.92 | 10.15 | 16.49 | 8.22 |
| | | 12.55 | 10.65 | 12.67 | 10.28 | 16.29 | 8.32 |
| | | 12.32 | 10.77 | 12.56 | 10.34 | 16.18 | 8.35 |
| | | 12.16 | 10.84 | 12.41 | 10.43 | 16.00 | 8.44 |
| | | 11.98 | 10.93 | 12.31 | 10.48 | 15.52 | 8.84 |
| | | 11.78 | 11.05 | 12.20 | 10.54 | 15.40 | 8.89 |
| | | 11.55 | 11.16 | 11.99 | 10.65 | 15.29 | 8.92 |
| | | 11.35 | 11.24 | 11.88 | 10.71 | 15.23 | 8.96 |
| | | 11.11 | 11.37 | 11.78 | 10.76 | 15.07 | 9.03 |
| | | 10.95 | 11.45 | 11.63 | 10.86 | 14.96 | 9.06 |
| | | 10.80 | 11.53 | 11.45 | 10.95 | 14.80 | 9.13 |
| | | 10.59 | 11.65 | 11.30 | 11.06 | 14.73 | 9.17 |
| | | 10.47 | 11.70 | 11.21 | 11.10 | 14.48 | 9.29 |
| | | 10.18 | 11.85 | 11.08 | 11.16 | 14.38 | 9.32 |
| | | 10.08 | 11.89 | 10.94 | 11.27 | 14.26 | 9.39 |
| | | 9.76 | 12.07 | 10.85 | 11.30 | 14.12 | 9.46 |
| | | 9.45 | 12.23 | 10.74 | 11.37 | 14.05 | 9.50 |
| | | 9.17 | 12.40 | 10.64 | 11.43 | 13.91 | 9.56 |
| | | 8.86 | 12.56 | 10.56 | 11.47 | 13.82 | 9.58 |
| | | 8.69 | 12.66 | 10.40 | 11.59 | 13.71 | 9.64 |
| | | 8.18 | 12.93 | 10.26 | 11.64 | 13.58 | 9.69 |
| | | 7.51 | 13.31 | 10.16 | 11.71 | 13.51 | 9.72 |
| | | 6.79 | 13.76 | 9.99 | 11.81 | 13.24 | 9.84 |
| | | 6.08 | 14.20 | 9.87 | 11.91 | 13.13 | 9.91 |
| | | 5.66 | 14.50 | 9.65 | 12.04 | 12.97 | 10.02 |
| | | 4.94 | 15.24 | 9.50 | 12.12 | 12.89 | 10.04 |
| | | 3.98 | 15.75 | 9.33 | 12.24 | 12.77 | 10.10 |
| | | 3.52 | 16.18 | 9.24 | 12.29 | 12.69 | 10.12 |
| | | 3.03 | 16.63 | 9.13 | 12.37 | 12.54 | 10.21 |
| | | 2.62 | 17.24 | 9.06 | 12.41 | 12.44 | 10.26 |
| | | 2.10 | 17.67 | 9.00 | 12.44 | 12.33 | 10.31 |
| | | 1.60 | 18.86 | 8.92 | 12.49 | 12.25 | 10.34 |
| | | 1.10 | 22.82 | 8.84 | 12.54 | 12.11 | 10.43 |
| | | 0.68 | 24.61 | 8.74 | 12.61 | 12.02 | 10.48 |
| | | | | 8.68 | 12.63 | 11.95 | 10.49 |
| | | | | 8.60 | 12.70 | 11.84 | 10.56 |
| | | | | 8.51 | 12.74 | 11.78 | 10.57 |
| | | | | 8.39 | 12.84 | 11.66 | 10.66 |
| | | | | 8.28 | 12.91 | 11.50 | 10.72 |
| | | | | 8.19 | 13.00 | 11.40 | 10.78 |
| | | | | 8.13 | 13.01 | 11.27 | 10.86 |
| | | | | 8.05 | 13.04 | 11.16 | 10.89 |
| | | | | 7.97 | 13.08 | 11.07 | 10.93 |
| | | | | 6.57 | 14.32 | 10.89 | 11.02 |

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| 6.41 | 14.30 | 10.75 | 11.08 |
| 6.12 | 14.53 | 10.61 | 11.16 |
| 5.70 | 14.83 | 10.42 | 11.25 |
| 5.53 | 14.99 | 10.30 | 11.34 |
| 5.37 | 15.12 | 10.24 | 11.35 |
| 5.25 | 15.22 | 10.17 | 11.38 |
| 5.17 | 15.25 | 10.02 | 11.46 |
| 5.05 | 15.35 | 9.93 | 11.52 |
| 4.92 | 15.47 | 9.86 | 11.57 |
| 4.26 | 16.59 | 9.80 | 11.60 |
| 3.63 | 17.77 | 9.69 | 11.64 |
| 3.51 | 17.85 | 9.57 | 11.71 |
| 3.14 | 18.55 | 9.44 | 11.77 |
| 3.09 | 18.58 | 9.37 | 11.83 |
| 3.00 | 18.63 | 9.30 | 11.85 |
| 2.94 | 18.68 | 9.25 | 11.89 |
| 2.85 | 18.75 | 9.19 | 11.92 |
| 2.78 | 18.82 | 9.13 | 11.95 |
| 2.64 | 18.94 | 9.07 | 11.98 |
| 2.54 | 19.05 | 9.01 | 12.01 |
| 2.42 | 19.15 | 8.95 | 12.05 |
| 2.36 | 19.18 | 8.89 | 12.08 |
| 2.26 | 19.24 | 8.83 | 12.10 |
| 2.21 | 19.29 | 8.80 | 12.11 |
| 2.17 | 19.33 | 8.68 | 12.18 |
| 2.12 | 19.36 | 8.65 | 12.19 |
| 2.03 | 19.45 | 8.60 | 12.22 |
| 1.98 | 19.50 | 8.54 | 12.26 |
| 1.90 | 19.66 | 8.46 | 12.29 |
| 1.77 | 19.76 | 8.41 | 12.32 |
| 1.61 | 20.03 | 8.36 | 12.35 |
| 1.47 | 20.19 | 8.31 | 12.38 |
| 1.38 | 20.34 | 8.28 | 12.39 |
| 1.30 | 20.49 | 8.22 | 12.43 |
| | | 8.18 | 12.45 |
| | | 8.13 | 12.47 |
| | | 8.09 | 12.50 |
| | | 8.04 | 12.52 |
| | | 7.98 | 12.57 |
| | | 7.92 | 12.60 |
| | | 7.88 | 12.62 |
| | | 7.83 | 12.65 |
| | | 7.75 | 12.62 |
| | | 7.71 | 12.64 |
| | | 7.66 | 12.67 |
| | | 7.62 | 12.69 |
| | | 7.57 | 12.72 |
| | | 7.55 | 12.72 |
| | | 7.49 | 12.77 |
| | | 7.45 | 12.79 |
| | | 7.41 | 12.82 |
| | | 7.37 | 12.83 |
| | | 7.30 | 12.89 |
| | | 7.28 | 12.89 |
| | | 7.21 | 12.93 |
| | | 7.18 | 12.94 |
| | | 7.10 | 13.01 |
| | | 7.06 | 13.02 |
| | | 7.00 | 13.06 |
| | | 6.96 | 13.08 |

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|---|---|
| 6.95 | 13.09 |
| 6.68 | 13.10 |
| 6.18 | 13.36 |
| 5.80 | 13.62 |
| 5.26 | 13.94 |
| 4.83 | 14.25 |
| 4.50 | 14.52 |
| 4.05 | 14.90 |
| 3.62 | 15.35 |
| 2.87 | 15.89 |
| 2.60 | 16.07 |
| 2.39 | 16.32 |
| 2.16 | 16.73 |
| 1.82 | 17.40 |
| 1.24 | 18.36 |
| 0.81 | 24.58 |

**Table A.3:** Binodal weight fraction data for the systems composed of PEG 1500 (1) + [$K_2HPO_4$/$KH_2PO_4$] (2) + 5 wt% of ILs (when present) + $H_2O$ at 298 (± 1) K. The uncertainty associated with the weight quantification of all components added is within ± $10^{-4}$g.

| no IL | | [$C_2$mim]Cl | | [$C_4$mim]Cl | | [$C_6$mim]Cl | | [$C_8$mim]Cl | |
|---|---|---|---|---|---|---|---|---|---|
| 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ |
| 45.50 | 1.85 | 43.03 | 2.44 | 59.52 | 1.26 | 67.11 | 0.73 | 44.74 | 2.23 |
| 43.16 | 2.21 | 40.46 | 2.74 | 55.79 | 1.51 | 61.69 | 1.03 | 43.37 | 2.35 |
| 41.64 | 2.53 | 38.75 | 2.96 | 53.13 | 1.70 | 58.96 | 1.33 | 42.44 | 2.46 |
| 39.57 | 2.82 | 37.18 | 3.20 | 50.14 | 1.78 | 54.36 | 1.49 | 41.62 | 2.62 |
| 37.82 | 2.93 | 35.62 | 3.41 | 48.53 | 2.01 | 50.90 | 1.75 | 40.62 | 2.76 |
| 36.57 | 3.17 | 34.38 | 3.66 | 46.15 | 2.15 | 47.39 | 1.94 | 39.48 | 2.89 |
| 34.94 | 3.54 | 32.96 | 3.93 | 45.46 | 2.18 | 45.15 | 2.12 | 38.56 | 3.00 |
| 33.85 | 3.82 | 31.39 | 4.17 | 44.11 | 2.35 | 43.54 | 2.36 | 37.46 | 3.22 |
| 31.95 | 4.17 | 30.36 | 4.33 | 42.99 | 2.52 | 41.47 | 2.50 | 36.30 | 3.41 |
| 31.04 | 4.35 | 28.85 | 4.71 | 41.82 | 2.67 | 40.56 | 2.67 | 35.64 | 3.50 |
| 30.26 | 4.49 | 28.04 | 4.83 | 40.79 | 2.79 | 39.22 | 2.85 | 35.01 | 3.60 |
| 29.44 | 4.66 | 26.73 | 5.15 | 39.42 | 2.93 | 38.42 | 3.02 | 34.03 | 3.81 |
| 28.68 | 4.82 | 25.58 | 5.46 | 38.38 | 3.11 | 37.10 | 3.18 | 33.00 | 4.02 |
| 27.88 | 5.03 | 24.83 | 5.62 | 37.92 | 3.14 | 35.68 | 3.45 | 32.07 | 4.20 |
| 26.93 | 5.34 | 23.80 | 5.94 | 36.62 | 3.42 | 34.86 | 3.57 | 30.99 | 4.43 |
| 26.10 | 5.60 | 22.66 | 6.29 | 35.80 | 3.54 | 34.23 | 3.70 | 30.59 | 4.52 |
| 25.53 | 5.77 | 21.33 | 6.75 | 34.98 | 3.65 | 33.66 | 3.82 | 30.20 | 4.61 |
| 25.00 | 5.90 | 20.46 | 7.03 | 34.21 | 3.78 | 33.02 | 3.94 | 29.92 | 4.68 |
| 24.16 | 6.21 | 19.64 | 7.27 | 33.75 | 3.90 | 32.36 | 4.07 | 29.23 | 4.84 |
| 23.42 | 6.47 | 18.48 | 7.74 | 32.98 | 4.04 | 31.84 | 4.17 | 28.33 | 5.06 |
| 22.66 | 6.73 | 17.58 | 8.07 | 31.99 | 4.26 | 31.28 | 4.28 | 27.21 | 5.36 |
| 22.00 | 6.92 | 16.55 | 8.50 | 30.62 | 4.57 | 30.79 | 4.40 | 25.74 | 5.79 |
| 21.14 | 7.29 | 15.55 | 8.93 | 29.52 | 4.84 | 30.29 | 4.51 | 24.37 | 6.22 |
| 20.26 | 7.60 | 14.76 | 9.28 | 28.48 | 5.10 | 29.56 | 4.73 | 22.54 | 6.85 |
| 19.77 | 7.83 | 13.80 | 9.74 | 27.20 | 5.47 | 29.06 | 4.84 | 20.26 | 7.36 |
| 19.27 | 8.00 | 13.13 | 10.04 | 26.10 | 5.78 | 28.61 | 4.94 | 18.07 | 8.14 |
| 18.58 | 8.31 | 12.48 | 10.34 | 24.38 | 6.34 | 28.38 | 5.05 | 17.13 | 8.53 |
| 17.94 | 8.58 | 8.94 | 12.26 | 21.73 | 7.34 | 27.91 | 5.15 | 16.40 | 8.94 |
| 17.43 | 8.78 | 7.96 | 12.80 | 21.40 | 7.48 | 27.36 | 5.32 | 15.64 | 9.28 |
| 16.72 | 9.11 | 6.83 | 13.43 | 20.64 | 7.79 | 27.01 | 5.37 | 15.08 | 9.50 |
| 16.08 | 9.43 | 6.22 | 13.92 | 20.01 | 8.07 | 26.51 | 5.53 | 14.82 | 9.64 |
| 15.47 | 9.72 | 5.61 | 14.35 | 19.23 | 8.39 | 26.03 | 5.66 | 14.68 | 9.76 |
| 15.05 | 9.94 | 4.91 | 14.62 | 18.60 | 8.69 | 25.44 | 5.87 | 14.46 | 9.85 |
| 14.52 | 10.20 | 3.82 | 15.29 | 18.01 | 8.97 | 24.83 | 6.07 | 14.24 | 9.97 |
| 13.91 | 10.53 | 3.13 | 16.16 | 17.44 | 9.25 | 24.32 | 6.24 | 14.07 | 10.09 |
| 13.45 | 10.75 | 2.49 | 17.07 | 17.23 | 9.41 | 23.72 | 6.46 | 13.90 | 10.23 |
| 12.94 | 11.01 | 1.73 | 18.47 | 16.81 | 9.56 | 23.25 | 6.62 | 13.61 | 10.39 |
| 12.62 | 11.17 | | | 16.64 | 9.69 | 23.01 | 6.69 | 13.30 | 10.58 |
| 12.22 | 11.38 | | | 15.92 | 10.03 | 22.55 | 6.85 | 13.10 | 10.74 |
| 11.42 | 11.85 | | | 15.41 | 10.29 | 21.94 | 7.09 | 12.84 | 10.90 |
| 10.92 | 12.06 | | | 14.74 | 10.67 | 21.47 | 7.26 | 12.47 | 11.11 |
| 10.63 | 12.21 | | | 14.19 | 10.99 | 21.01 | 7.52 | 12.20 | 11.27 |
| 10.26 | 12.44 | | | 13.50 | 11.38 | 20.72 | 7.67 | 12.02 | 11.43 |
| 9.96 | 12.60 | | | 13.14 | 11.63 | 20.01 | 7.95 | 11.77 | 11.59 |
| 9.66 | 12.77 | | | 12.89 | 11.81 | 19.63 | 8.14 | 11.58 | 11.72 |
| 9.40 | 12.90 | | | 12.11 | 12.25 | 19.01 | 8.39 | 11.19 | 11.95 |
| 9.16 | 13.04 | | | 11.93 | 12.38 | 18.64 | 8.58 | 10.88 | 12.13 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 8.98 | 13.15 | 11.58 | 12.59 | 18.22 | 8.81 | 10.63 | 12.31 |
| 8.75 | 13.26 | 11.12 | 12.92 | 17.66 | 9.06 | 10.48 | 12.48 |
| 8.51 | 13.40 | 10.36 | 13.36 | 17.08 | 9.29 | 10.16 | 12.69 |
| 8.28 | 13.53 | 9.92 | 13.73 | 16.50 | 9.56 | 9.85 | 12.90 |
| 8.09 | 13.64 | 9.23 | 14.16 | 15.76 | 9.99 | 9.54 | 13.09 |
| 8.01 | 13.67 | 8.24 | 14.71 | 15.07 | 10.35 | 9.28 | 13.28 |
| 7.82 | 13.79 | 7.47 | 15.34 | 14.84 | 10.56 | 8.88 | 13.52 |
| 7.60 | 13.92 | 6.99 | 15.80 | 14.17 | 10.87 | 8.57 | 13.84 |
| 7.44 | 14.01 | 6.40 | 16.22 | 13.88 | 11.07 | 8.19 | 14.13 |
| 7.27 | 14.11 | 5.78 | 16.68 | 13.63 | 11.30 | 7.82 | 14.39 |
| 6.76 | 14.25 | 5.25 | 17.05 | 13.23 | 11.55 | 7.38 | 14.67 |
| 5.71 | 14.88 | 4.65 | 17.85 | 12.58 | 11.92 | 6.98 | 14.95 |
| 5.20 | 15.21 | 3.55 | 18.99 | 11.94 | 12.30 | 6.67 | 15.22 |
| 4.57 | 15.80 | | | 11.58 | 12.57 | 6.30 | 15.59 |
| 4.05 | 16.30 | | | 10.91 | 12.99 | 5.87 | 16.03 |
| 3.64 | 16.75 | | | 10.53 | 13.29 | 5.39 | 16.54 |
| 3.00 | 17.39 | | | 10.08 | 13.64 | 4.84 | 17.05 |
| 2.36 | 17.97 | | | 9.58 | 14.01 | 4.28 | 17.87 |
| 1.86 | 18.90 | | | 9.09 | 14.45 | 3.70 | 18.90 |
| 1.20 | 20.88 | | | 8.61 | 14.82 | | |
| | | | | 8.18 | 15.25 | | |
| | | | | 7.57 | 15.71 | | |
| | | | | 7.03 | 16.19 | | |
| | | | | 6.44 | 16.71 | | |
| | | | | 5.83 | 17.26 | | |
| | | | | 5.19 | 17.76 | | |
| | | | | 4.65 | 18.56 | | |

**Table A.4:** Binodal weight fraction data for the systems composed of PEG 4000 (1) + [$K_2HPO_4$/$KH_2PO_4$] (2) + 5 wt% of ILs (when present) + $H_2O$ at 298 (± 1) K. The uncertainty associated with the weight quantification of all components added is within ± $10^{-4}$g.

| no IL | | [$C_2$mim]Cl | | [$C_4$mim]Cl | | [$C_6$mim]Cl | | [$C_8$mim]Cl | |
|---|---|---|---|---|---|---|---|---|---|
| 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ | 100 $w_1$ | 100 $w_2$ |
| 45.41 | 1.76 | 44.85 | 2.32 | 45.30 | 1.93 | 45.92 | 1.86 | 39.37 | 2.47 |
| 43.40 | 1.93 | 41.68 | 2.71 | 44.21 | 2.04 | 43.59 | 2.03 | 37.58 | 2.63 |
| 41.93 | 2.08 | 40.08 | 3.02 | 43.20 | 2.12 | 42.55 | 2.15 | 36.73 | 2.68 |
| 40.48 | 2.37 | 37.63 | 3.13 | 42.04 | 2.29 | 41.34 | 2.41 | 36.05 | 2.81 |
| 38.32 | 2.64 | 36.54 | 3.31 | 39.93 | 2.55 | 39.27 | 2.54 | 35.09 | 2.93 |
| 36.72 | 2.93 | 35.50 | 3.48 | 38.21 | 2.72 | 37.87 | 2.68 | 34.50 | 3.07 |
| 34.88 | 3.14 | 34.50 | 3.67 | 37.15 | 2.91 | 36.75 | 2.81 | 33.60 | 3.17 |
| 33.43 | 3.25 | 33.05 | 3.78 | 35.65 | 3.05 | 35.91 | 2.98 | 32.74 | 3.26 |
| 32.67 | 3.37 | 32.06 | 3.94 | 34.64 | 3.25 | 34.91 | 3.10 | 31.87 | 3.53 |
| 32.07 | 3.43 | 31.05 | 4.23 | 33.03 | 3.52 | 34.23 | 3.23 | 30.83 | 3.76 |
| 31.40 | 3.53 | 29.42 | 4.51 | 31.78 | 3.61 | 33.20 | 3.33 | 29.99 | 3.88 |
| 30.40 | 3.87 | 27.92 | 4.72 | 31.04 | 3.76 | 32.53 | 3.46 | 29.26 | 3.97 |
| 29.39 | 3.93 | 26.21 | 5.16 | 30.27 | 3.89 | 32.00 | 3.56 | 28.53 | 4.23 |
| 28.63 | 4.13 | 24.94 | 5.39 | 29.62 | 4.01 | 31.04 | 3.68 | 27.05 | 4.46 |
| 28.04 | 4.23 | 23.56 | 5.78 | 28.97 | 4.13 | 30.41 | 3.83 | 25.63 | 4.85 |
| 27.40 | 4.39 | 22.34 | 6.07 | 28.31 | 4.27 | 29.83 | 3.95 | 24.27 | 5.10 |
| 26.62 | 4.53 | 21.34 | 6.33 | 27.46 | 4.53 | 29.30 | 4.06 | 22.28 | 5.68 |
| 25.78 | 4.69 | 20.10 | 6.78 | 26.85 | 4.67 | 28.78 | 4.16 | 21.07 | 6.02 |
| 25.45 | 4.78 | 19.06 | 7.10 | 26.33 | 4.76 | 28.27 | 4.29 | 20.15 | 6.32 |
| 24.83 | 4.96 | 18.12 | 7.40 | 25.84 | 4.87 | 27.79 | 4.38 | 19.41 | 6.55 |
| 24.45 | 5.01 | 16.74 | 7.90 | 25.34 | 4.96 | 27.29 | 4.47 | 18.66 | 6.81 |
| 23.90 | 5.15 | 15.40 | 8.40 | 24.66 | 5.18 | 26.18 | 4.76 | 17.92 | 7.05 |
| 23.32 | 5.34 | 14.31 | 8.82 | 24.22 | 5.25 | 25.76 | 4.85 | 17.37 | 7.25 |
| 22.93 | 5.41 | 13.31 | 9.26 | 23.55 | 5.45 | 25.34 | 4.94 | 16.75 | 7.48 |
| 22.61 | 5.47 | 12.41 | 9.64 | 23.13 | 5.51 | 24.77 | 5.14 | 16.22 | 7.69 |
| 22.20 | 5.56 | 11.42 | 10.05 | 22.57 | 5.69 | 23.70 | 5.39 | 15.76 | 7.89 |
| 21.70 | 5.74 | 10.49 | 10.46 | 21.98 | 5.88 | 23.33 | 5.48 | 15.25 | 8.10 |
| 21.27 | 5.85 | 9.77 | 10.80 | 21.60 | 5.96 | 23.00 | 5.55 | 14.72 | 8.32 |
| 20.95 | 5.89 | 9.65 | 10.84 | 21.22 | 6.06 | 22.78 | 5.64 | 14.17 | 8.55 |
| 20.50 | 6.03 | 8.96 | 11.18 | 20.73 | 6.22 | 22.50 | 5.70 | 13.60 | 8.80 |
| 20.27 | 6.10 | 8.05 | 11.61 | 20.23 | 6.35 | 22.03 | 5.86 | 12.99 | 9.07 |
| 19.85 | 6.25 | 5.78 | 12.73 | 19.65 | 6.57 | 21.63 | 5.88 | 12.37 | 9.36 |
| 19.42 | 6.40 | 4.59 | 13.45 | 19.12 | 6.82 | 21.23 | 6.02 | 11.95 | 9.58 |
| 19.11 | 6.45 | 3.76 | 14.04 | 18.87 | 6.91 | 20.93 | 6.09 | 11.58 | 9.80 |
| 18.53 | 6.74 | 2.51 | 14.90 | 18.17 | 7.15 | 20.50 | 6.27 | 11.13 | 10.04 |
| 17.90 | 6.89 | 1.96 | 15.46 | 17.59 | 7.36 | 20.17 | 6.37 | 10.42 | 10.36 |
| 17.58 | 6.98 | 1.44 | 16.81 | 17.17 | 7.53 | 19.78 | 6.51 | 9.61 | 10.80 |
| 17.29 | 7.06 | 0.91 | 22.23 | 16.54 | 7.76 | 19.52 | 6.58 | 9.35 | 10.93 |
| 16.94 | 7.20 | | | 16.11 | 7.95 | 19.15 | 6.70 | 9.05 | 11.09 |
| 16.68 | 7.27 | | | 15.41 | 8.22 | 18.87 | 6.79 | 8.78 | 11.27 |
| 16.25 | 7.50 | | | 14.94 | 8.43 | 18.44 | 6.97 | 8.16 | 11.58 |
| 12.55 | 8.93 | | | 14.56 | 8.58 | 18.29 | 7.02 | 7.84 | 11.76 |
| 11.75 | 9.22 | | | 14.19 | 8.75 | 18.10 | 7.06 | 7.49 | 11.94 |
| 11.23 | 9.45 | | | 13.65 | 8.98 | 17.70 | 7.24 | 7.14 | 12.13 |
| 10.73 | 9.68 | | | 13.25 | 9.20 | 17.43 | 7.34 | 6.45 | 12.42 |
| 10.18 | 9.92 | | | 12.63 | 9.46 | 17.20 | 7.41 | 6.21 | 12.70 |
| 9.57 | 10.20 | | | 12.16 | 9.71 | 17.05 | 7.70 | 5.43 | 13.05 |
| 8.95 | 10.49 | | | 11.64 | 9.97 | 16.59 | 7.88 | 5.13 | 13.24 |
| 8.39 | 10.78 | | | 11.44 | 10.10 | 16.12 | 8.06 | 4.72 | 13.49 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 7.71 | 11.10 | 10.89 | 10.34 | 15.70 | 8.24 | 4.33 | 13.72 |
| 6.97 | 11.44 | 10.40 | 10.61 | 15.39 | 8.39 | 3.93 | 14.06 |
| 6.63 | 11.59 | 9.78 | 10.92 | 15.10 | 8.54 | 3.53 | 14.30 |
| 5.81 | 11.96 | 9.15 | 11.25 | 14.55 | 8.75 | 3.07 | 14.57 |
| 5.44 | 12.23 | 8.50 | 11.58 | 14.22 | 8.92 | 2.80 | 14.89 |
| 4.55 | 12.63 | 7.90 | 11.90 | 13.86 | 9.10 | 2.34 | 15.45 |
| 4.11 | 12.93 | 7.61 | 12.14 | 13.49 | 9.28 | 1.77 | 16.03 |
| 3.68 | 13.21 | 6.88 | 12.52 | 13.14 | 9.44 | 1.24 | 17.12 |
| 3.23 | 13.53 | 6.11 | 12.92 | 12.80 | 9.62 | 0.69 | 20.06 |
| 2.73 | 13.83 | 5.67 | 13.15 | 12.61 | 9.73 | | |
| 2.28 | 14.34 | 5.31 | 13.42 | 12.20 | 9.94 | | |
| 1.80 | 14.91 | 4.90 | 13.71 | 11.73 | 10.18 | | |
| 1.28 | 15.79 | 4.48 | 14.02 | 11.52 | 10.34 | | |
| 0.90 | 21.17 | 3.96 | 14.32 | 11.14 | 10.53 | | |
| | | 3.49 | 14.80 | 10.65 | 10.78 | | |
| | | 2.92 | 15.17 | 10.42 | 10.95 | | |
| | | 2.48 | 15.80 | 10.19 | 11.08 | | |
| | | 1.93 | 16.53 | 9.63 | 11.37 | | |
| | | 1.30 | 17.82 | 9.36 | 11.58 | | |
| | | 0.65 | 20.21 | 9.04 | 11.80 | | |
| | | | | 8.40 | 12.13 | | |
| | | | | 8.12 | 12.36 | | |
| | | | | 7.73 | 12.62 | | |
| | | | | 7.15 | 13.10 | | |
| | | | | 6.37 | 13.51 | | |
| | | | | 6.00 | 13.79 | | |
| | | | | 5.62 | 14.09 | | |
| | | | | 5.18 | 14.43 | | |
| | | | | 4.78 | 14.74 | | |
| | | | | 4.37 | 15.08 | | |
| | | | | 3.68 | 15.73 | | |
| | | | | 3.22 | 16.10 | | |
| | | | | 2.70 | 16.72 | | |
| | | | | 2.28 | 17.36 | | |
| | | | | 1.76 | 18.33 | | |
| | | | | 1.19 | 19.99 | | |

**Table A.5:** Binodal weight fraction data for the systems composed of PEG 6000 (1) + [$K_2HPO_4$/$KH_2PO_4$] (2) + 5 wt% of ILs (when present) + $H_2O$ at 298 (± 1) K. The uncertainty associated with the weight quantification of all components added is within ± $10^{-4}$g.

| **no IL** | | **[$C_2$mim]Cl** | | **[$C_4$mim]Cl** | | **[$C_6$mim]Cl** | | **[$C_8$mim]Cl** | |
|---|---|---|---|---|---|---|---|---|---|
| **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** |
| 51.08 | 1.03 | 45.81 | 2.06 | 47.43 | 1.70 | 45.91 | 1.66 | 46.30 | 1.61 |
| 47.40 | 1.45 | 41.87 | 2.58 | 44.50 | 1.98 | 43.87 | 1.88 | 43.77 | 1.87 |
| 45.54 | 1.57 | 39.39 | 2.98 | 43.02 | 2.21 | 42.82 | 1.99 | 42.44 | 1.99 |
| 43.18 | 1.96 | 36.78 | 3.24 | 39.20 | 2.61 | 41.76 | 2.15 | 40.43 | 2.15 |
| 40.07 | 2.20 | 34.20 | 3.50 | 38.10 | 2.82 | 39.80 | 2.39 | 39.02 | 2.34 |
| 37.99 | 2.47 | 32.03 | 3.81 | 36.47 | 3.02 | 38.27 | 2.56 | 37.36 | 2.47 |
| 36.27 | 2.75 | 30.26 | 4.07 | 35.05 | 3.17 | 36.30 | 2.79 | 36.27 | 2.61 |
| 34.59 | 2.93 | 28.86 | 4.31 | 33.84 | 3.36 | 34.23 | 3.07 | 35.23 | 2.80 |
| 33.11 | 3.13 | 27.31 | 4.51 | 33.00 | 3.55 | 32.40 | 3.43 | 33.52 | 3.12 |
| 31.88 | 3.27 | 25.53 | 4.99 | 31.91 | 3.60 | 30.93 | 3.64 | 32.66 | 3.22 |
| 31.22 | 3.37 | 24.37 | 5.21 | 30.44 | 3.92 | 29.72 | 3.86 | 31.81 | 3.32 |
| 30.51 | 3.52 | 23.28 | 5.36 | 29.80 | 4.07 | 27.88 | 4.21 | 30.39 | 3.60 |
| 29.84 | 3.66 | 22.05 | 5.75 | 28.93 | 4.33 | 25.85 | 4.70 | 29.14 | 3.81 |
| 28.91 | 3.78 | 20.86 | 6.07 | 27.78 | 4.57 | 25.26 | 4.80 | 28.63 | 3.91 |
| 28.32 | 3.94 | 19.81 | 6.37 | 26.62 | 4.84 | 23.13 | 5.32 | 27.32 | 4.21 |
| 27.72 | 4.06 | 19.07 | 6.51 | 25.85 | 4.86 | 22.17 | 5.57 | 24.53 | 4.82 |
| 26.89 | 4.18 | 18.17 | 6.81 | 25.23 | 5.03 | 21.20 | 5.83 | 21.99 | 5.45 |
| 26.38 | 4.29 | 17.42 | 7.01 | 24.71 | 5.11 | 20.56 | 6.01 | 20.52 | 5.84 |
| 25.91 | 4.36 | 16.69 | 7.23 | 23.80 | 5.32 | 19.13 | 6.43 | 17.74 | 6.63 |
| 25.25 | 4.56 | 15.67 | 7.52 | 23.38 | 5.40 | 18.80 | 6.53 | 15.70 | 7.28 |
| 24.77 | 4.62 | 14.70 | 7.85 | 22.75 | 5.59 | 18.32 | 6.71 | 14.03 | 7.84 |
| 24.38 | 4.74 | 13.74 | 8.24 | 21.62 | 5.83 | 15.77 | 7.61 | 13.21 | 8.33 |
| 23.91 | 4.85 | 12.88 | 8.56 | 20.38 | 6.19 | 13.75 | 8.43 | 12.08 | 8.81 |
| 23.27 | 4.94 | 12.15 | 8.85 | 19.30 | 6.47 | 12.35 | 9.05 | 11.04 | 9.28 |
| 22.89 | 5.01 | 11.45 | 9.14 | 18.37 | 6.78 | 11.06 | 9.69 | 9.83 | 9.84 |
| 22.48 | 5.12 | 10.67 | 9.44 | 17.66 | 6.99 | 10.07 | 10.17 | 8.63 | 10.41 |
| 22.07 | 5.24 | 9.92 | 9.76 | 16.54 | 7.34 | 8.78 | 10.86 | 7.72 | 10.87 |
| 21.32 | 5.41 | 9.21 | 10.08 | 16.21 | 7.48 | 7.63 | 11.52 | 6.75 | 11.36 |
| 20.63 | 5.60 | 8.45 | 10.38 | 15.15 | 7.82 | 6.69 | 12.13 | 5.42 | 12.02 |
| 19.92 | 5.82 | 8.43 | 10.39 | 14.15 | 8.22 | 5.28 | 12.99 | 4.36 | 12.62 |
| 19.26 | 5.98 | 6.98 | 11.02 | 13.32 | 8.53 | 4.44 | 13.58 | 3.30 | 13.20 |
| 18.85 | 6.02 | 5.38 | 11.76 | 12.53 | 8.84 | 4.03 | 13.88 | 2.67 | 13.64 |
| 18.25 | 6.21 | 4.41 | 12.28 | 11.14 | 9.44 | 3.55 | 14.23 | 2.05 | 14.14 |
| 17.64 | 6.42 | 2.63 | 13.33 | 10.88 | 9.56 | 3.19 | 14.63 | 1.77 | 14.40 |
| 17.14 | 6.56 | 2.25 | 13.72 | 10.06 | 9.97 | 2.73 | 14.99 | 1.40 | 14.86 |
| 16.60 | 6.58 | 1.50 | 14.49 | 9.17 | 10.38 | 2.15 | 15.66 | 1.08 | 15.24 |
| 15.46 | 6.95 | 1.10 | 15.21 | 7.87 | 10.96 | 1.67 | 16.16 | 0.75 | 15.99 |
| 13.85 | 7.51 | | | 6.84 | 11.47 | 1.38 | 17.21 | 0.41 | 20.25 |
| 10.67 | 8.75 | | | 6.10 | 11.88 | 0.89 | 20.02 | | |
| 6.29 | 10.67 | | | 4.81 | 12.50 | | | | |
| 4.96 | 11.28 | | | 3.93 | 13.00 | | | | |
| 3.90 | 11.81 | | | 3.45 | 13.30 | | | | |
| 2.82 | 12.39 | | | 2.98 | 13.67 | | | | |
| 2.26 | 12.84 | | | 2.53 | 14.04 | | | | |
| 1.62 | 13.37 | | | 2.07 | 14.82 | | | | |

**Table A.6:** Binodal weight fraction data for the systems composed of PEG 8000 (1) + [$K_2HPO_4$/$KH_2PO_4$] (2) + 5 wt% of ILs (when present) + $H_2O$ at 298 (± 1) K. The uncertainty associated with the weight quantification of all components added is within ± $10^{-4}$g.

| **no IL** | | **[C₂mim]Cl** | | **[C₄mim]Cl** | | **[C₆mim]Cl** | | **[C₈mim]Cl** | |
|---|---|---|---|---|---|---|---|---|---|
| **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** | **100 $w_1$** | **100 $w_2$** |
| 45.89 | 1.91 | 45.35 | 2.29 | 45.97 | 1.80 | 45.59 | 1.98 | 44.71 | 2.35 |
| 42.88 | 2.18 | 42.10 | 2.59 | 43.11 | 2.13 | 42.98 | 2.26 | 41.97 | 2.55 |
| 39.09 | 2.40 | 39.22 | 2.87 | 41.45 | 2.37 | 41.14 | 2.50 | 38.93 | 2.75 |
| 36.89 | 2.63 | 36.74 | 3.05 | 39.00 | 2.63 | 39.00 | 2.69 | 37.62 | 2.94 |
| 35.42 | 2.79 | 35.06 | 3.39 | 36.87 | 2.82 | 37.40 | 2.96 | 36.23 | 3.11 |
| 34.59 | 2.93 | 31.50 | 3.75 | 35.62 | 3.07 | 35.84 | 3.07 | 35.07 | 3.33 |
| 33.22 | 3.11 | 29.42 | 4.08 | 34.44 | 3.32 | 34.59 | 3.22 | 33.71 | 3.48 |
| 31.35 | 3.33 | 27.63 | 4.43 | 32.76 | 3.46 | 33.66 | 3.48 | 32.80 | 3.65 |
| 29.37 | 3.63 | 26.86 | 4.58 | 31.01 | 3.74 | 32.41 | 3.64 | 31.75 | 3.76 |
| 28.74 | 3.75 | 25.70 | 4.69 | 30.22 | 3.88 | 31.25 | 3.80 | 30.95 | 3.91 |
| 27.47 | 4.02 | 25.09 | 4.84 | 27.40 | 4.46 | 30.23 | 3.93 | 29.62 | 4.20 |
| 26.01 | 4.31 | 23.92 | 5.09 | 25.57 | 4.70 | 29.60 | 4.11 | 28.89 | 4.32 |
| 24.74 | 4.48 | 23.33 | 5.20 | 24.64 | 4.89 | 28.81 | 4.22 | 27.53 | 4.55 |
| 24.10 | 4.56 | 22.37 | 5.44 | 22.92 | 5.26 | 27.63 | 4.53 | 27.06 | 4.69 |
| 22.84 | 4.87 | 21.41 | 5.63 | 21.41 | 5.61 | 26.74 | 4.61 | 26.48 | 4.79 |
| 22.24 | 4.97 | 20.57 | 5.83 | 20.25 | 5.94 | 25.28 | 5.03 | 25.10 | 5.13 |
| 21.62 | 5.10 | 19.82 | 5.99 | 19.53 | 6.03 | 24.67 | 5.08 | 24.20 | 5.32 |
| 21.15 | 5.21 | 19.10 | 6.18 | 18.21 | 6.45 | 23.91 | 5.25 | 23.39 | 5.51 |
| 20.43 | 5.39 | 18.43 | 6.30 | 17.11 | 6.77 | 23.17 | 5.47 | 22.60 | 5.72 |
| 19.67 | 5.52 | 17.49 | 6.54 | 16.05 | 7.12 | 22.82 | 5.59 | 21.70 | 5.99 |
| 18.94 | 5.69 | 16.54 | 6.84 | 14.74 | 7.69 | 22.44 | 5.71 | 21.33 | 6.04 |
| 18.35 | 5.82 | 16.03 | 6.97 | 13.57 | 8.12 | 21.95 | 5.77 | 20.40 | 6.28 |
| 17.53 | 6.03 | 15.71 | 7.08 | 13.06 | 8.31 | 21.10 | 6.05 | 19.69 | 6.51 |
| 16.93 | 6.23 | 14.93 | 7.30 | 12.53 | 8.51 | 20.39 | 6.24 | 19.05 | 6.71 |
| 16.33 | 6.31 | 14.02 | 7.58 | 11.46 | 8.92 | 19.81 | 6.41 | 18.21 | 6.98 |
| 15.64 | 6.50 | 13.09 | 7.91 | 10.46 | 9.41 | 19.25 | 6.57 | 17.28 | 7.26 |
| 14.76 | 6.81 | 12.40 | 8.10 | 9.56 | 9.80 | 18.61 | 6.70 | 16.07 | 7.54 |
| 13.99 | 7.07 | 11.68 | 8.38 | 8.72 | 10.00 | 17.01 | 7.22 | 15.12 | 7.84 |
| 13.43 | 7.27 | 11.04 | 8.62 | 7.22 | 10.64 | 16.10 | 7.55 | 13.36 | 8.43 |
| 12.77 | 7.47 | 11.03 | 8.65 | 6.06 | 11.16 | 15.24 | 7.85 | 12.62 | 8.69 |
| 12.12 | 7.69 | 9.70 | 9.17 | 5.34 | 11.52 | 14.43 | 8.19 | 11.80 | 8.99 |
| 11.31 | 7.98 | 7.09 | 10.10 | 4.37 | 12.04 | 13.67 | 8.49 | 11.16 | 9.23 |
| 11.18 | 8.05 | 6.17 | 10.51 | 3.71 | 12.46 | 12.84 | 8.84 | 10.73 | 9.40 |
| 9.94 | 8.46 | 5.38 | 10.88 | 3.04 | 12.91 | 12.39 | 9.13 | 10.43 | 9.52 |
| 9.11 | 8.82 | 4.21 | 11.41 | 2.45 | 13.28 | 11.48 | 9.53 | 9.56 | 9.88 |
| 7.39 | 9.45 | 3.34 | 11.83 | 2.03 | 13.64 | 10.02 | 10.23 | 8.01 | 10.57 |
| 5.85 | 10.06 | 2.96 | 12.14 | 1.30 | 14.63 | 8.79 | 10.88 | 7.40 | 10.86 |
| 4.51 | 10.64 | 2.58 | 12.41 | 0.92 | 19.96 | 7.22 | 11.68 | 6.61 | 11.80 |
| 3.47 | 11.16 | 1.99 | 12.99 | | | 6.04 | 12.35 | 5.10 | 12.60 |
| 2.49 | 11.70 | 1.41 | 13.77 | | | 5.04 | 12.95 | 3.39 | 13.59 |
| 1.64 | 12.50 | 1.03 | 22.19 | | | 4.16 | 13.57 | 3.06 | 13.84 |
| 1.21 | 12.97 | | | | | 3.24 | 14.18 | 2.38 | 14.26 |
| 0.87 | 13.24 | | | | | 2.70 | 14.77 | 2.00 | 14.71 |
| 0.83 | 21.20 | | | | | 2.21 | 15.28 | 1.40 | 15.39 |
| | | | | | | 1.79 | 16.01 | 0.97 | 15.94 |

**Table A.7:** Correlation parameters used in Eq.1 plus the respective standard deviation data used to describe the binodal curves for systems based in the PEG 1500 with different inorganic salts and imidazolium-based ILs, at 298 (± 1) K.

| Salt | Ionic Liquid | $A \pm \sigma$ | $B \pm \sigma$ | $C \pm \sigma$ | $r^2$ |
|---|---|---|---|---|---|
| $K_3PO_4$ | no IL | 108.5 ± 1.3 | -0.604 ± 0.007 | $3.0 \times 10^{-4} \pm 1.8 \times 10^{-5}$ | 0.9990 |
| | $[C_2mim]Cl$ | 108.9 ± 2.2 | -0.629 ± 0.011 | $4.0 \times 10^{-4} \pm 2.0 \times 10^{-5}$ | 0.9995 |
| | $[C_4mim]Cl$ | 100.2 ± 1.0 | -0.564 ± 0.056 | $2.0 \times 10^{-4} \pm 1.0 \times 10^{-5}$ | 0.9983 |
| | $[C_6mim]Cl$ | 101.4 ± 0.6 | -0.602 ± 0.003 | $2.0 \times 10^{-4} \pm 5.0 \times 10^{-6}$ | 0.9992 |
| | $[C_8mim]Cl$ | 96.04 ± 0.7 | -0.566 ± 0.004 | $2.0 \times 10^{-6} \pm 6.7 \times 10^{-6}$ | 0.9987 |
| $K_2HPO_4$ | no IL | 92.8 ± 1.3 | -0.532 ± 0.007 | $4.0 \times 10^{-4} \pm 1.1 \times 10^{-5}$ | 0.9991 |
| | $[C_2mim]Cl$ | 110.8 ± 2.5 | -0.613 ± 0.010 | $3.0 \times 10^{-4} \pm 1.3 \times 10^{-5}$ | 0.9996 |
| | $[C_4mim]Cl$ | 100.2 ± 1.0 | -0.564 ± 0.006 | $2.0 \times 10^{-4} \pm 1.1 \times 10^{-5}$ | 0.9983 |
| | $[C_6mim]Cl$ | 101.4 ± 0.6 | -0.602 ± 0.004 | $2.0 \times 10^{-4} \pm 5.0 \times 10^{-6}$ | 0.9992 |
| | $[C_8mim]Cl$ | 96.0 ± 0.7 | -0.566 ± 0.004 | $2.0 \times 10^{-4} \pm 6.7 \times 10^{-6}$ | 0.9987 |
| $K_2HPO_4$/ $KH_2PO_4$ | no IL | 92.4 ± 1.0 | -0.514 ± 0.006 | $2.0 \times 10^{-4} \pm 5.7 \times 10^{-6}$ | 0.9996 |
| | $[C_2mim]Cl$ | 110.3 ± 1.5 | -0.605 ± 0.007 | $2.0 \times 10^{-4} \pm 8.2 \times 10^{-6}$ | 0.9998 |
| | $[C_4mim]Cl$ | 113.4 ± 1.5 | -0.603 ± 0.008 | $8.4 \times 10^{-5} \pm 9.1 \times 10^{-6}$ | 0.9990 |
| | $[C_6mim]Cl$ | 113.3 ± 1.0 | -0.613 ± 0.005 | $6.1 \times 10^{-5} \pm 7.0 \times 10^{-6}$ | 0.9990 |
| | $[C_8mim]Cl$ | 110.0 ± 0.8 | -0.599 ± 0.004 | $1.0 \times 10^{-4} \pm 4.1 \times 10^{-6}$ | 0.9998 |

**Table A.8:** Correlation parameters used in Eq. 1 plus the respective standard deviation data used to describe the binodal curves for systems based in the [$K_2HPO_4$/$KH_2PO_4$] buffer, considering different PEGs and ILs, at 298 (± 1) K.

| Polymer | Ionic Liquid | $A \pm \sigma$ | $B \pm \sigma$ | $C \pm \sigma$ | $r^2$ |
|---|---|---|---|---|---|
| PEG 1500 | no IL | 92.4 ± 1.0 | -0.514 ± 0.006 | $2.0x10^{-4} \pm 5.7x10^{-6}$ | 0.9996 |
| | [$C_2$mim]Cl | 110.3 ± 1.5 | -0.605 ± 0.007 | $2.0x10^{-4} \pm 8.2x10^{-6}$ | 0.9998 |
| | [$C_4$mim]Cl | 113.4 ± 1.5 | -0.603 ± 0.008 | $8.4x10^{-5} \pm 9.1x10^{-6}$ | 0.9990 |
| | [$C_6$mim]Cl | 113.3 ± 1.0 | -0.613 ± 0.005 | $6.1x10^{-5} \pm 7.0x10^{-6}$ | 0.9990 |
| | [$C_8$mim]Cl | 110.0 ± 0.8 | -0.599 ± 0.004 | $1.0x10^{-4} \pm 4.1x10^{-6}$ | 0.9998 |
| PEG 4000 | no IL | 103.2 ± 1.3 | -0.615 ± 0.007 | $4.0 x10^{-4} \pm 1.3x10^{-5}$ | 0.9995 |
| | [$C_2$mim]Cl | 121.8 ± 3.0 | -0.653 ± 0.013 | $3.0x10^{-4} \pm 1.9x10^{-5}$ | 0.9994 |
| | [$C_4$mim]Cl | 110.1 ± 1.1 | -0.639 ± 0.005 | $3.0x10^{-4} \pm 8.0x10^{-6}$ | 0.9997 |
| | [$C_6$mim]Cl | 111.0 ± 0.9 | -0.653 ± 0.004 | $2.0x10^{-4} \pm 6.1x10^{-6}$ | 0.9997 |
| | [$C_8$mim]Cl | 109.0 ± 0.7 | -0.642 ± 0.017 | $3.0x10^{-6} \pm 2.2x10^{-5}$ | 0.9982 |
| PEG 6000 | no IL | 96.6 ± 1.2 | -0.596 ± 0.008 | $7.0x10^{-4} \pm 3.1x10^{-5}$ | 0.9994 |
| | [$C_2$mim]Cl | 124.1 ± 3.4 | -0.678 ± 0.015 | $5.0x10^{-4} \pm 2.8x10^{-5}$ | 0.9992 |
| | [$C_4$mim]Cl | 107.3 ± 1.3 | -0.618 ± 0.007 | $5.0x10^{-4} \pm 1.4x10^{-5}$ | 0.9997 |
| | [$C_6$mim]Cl | 103.9 ± 1.0 | -0.625 ± 0.006 | $4.0x10^{-4} \pm 9.6x10^{-6}$ | 0.9999 |
| | [$C_8$mim]Cl | 100.8 ± 2.3 | -0.618 ± 0.014 | $5.0x10^{-4} \pm 2.3x10^{-5}$ | 0.9997 |
| PEG 8000 | no IL | 124.3 ± 2.9 | -0.732 ± 0.013 | $7.0x10^{-4} \pm 3.7x10^{-5}$ | 0.9993 |
| | [$C_2$mim]Cl | 138.8 ± 3.2 | -0.740 ± 0.013 | $6.0x10^{-4} \pm 2.7 x10^{-5}$ | 0.9995 |
| | [$C_4$mim]Cl | 111.7 ± 2.3 | -0.648 ± 0.012 | $5.0x10^{-4} \pm 2.5 x10^{-5}$ | 0.9995 |
| | [$C_6$mim]Cl | 118.1 ± 1.2 | -0.671 ± 0.005 | $3.0x10^{-4} \pm 1.1 x10^{-5}$ | 0.9998 |
| | [$C_8$mim]Cl | 114.3 ± 3.7 | -0.636 ± 0.017 | $5.0x10^{-4} \pm 3.4 x10^{-5}$ | 0.9988 |

**Table A.9:** Partition coefficients of each IL ($K_{IL}$) with the respective standard deviations and mass fraction compositions of the quaternary systems at 298 (± 1) K.

| Quaternary System | | Mass Fraction Composition ± $\sigma$ / (wt%) | | | $K_{IL} \pm \sigma$ |
|---|---|---|---|---|---|
| | | PEG | Salt | IL | |
| PEG 1500 | [$C_2$mim]Cl | 15.05 ± 0.11 | 14.98 ± 0.06 | 5.04 ± 0.12 | 0.97 ± 014 |
| | [$C_4$mim]Cl | 15.05 ± 0.21 | 14.97 ± 0.05 | 5.09 ± 0.08 | 2.04 ± 0.04 |
| | [$C_6$mim]Cl | 15.10 ± 0.06 | 15.05 ± 0.06 | 5.06 ± 0.09 | 4.91 ± 0.51 |
| | [$C_8$mim]Cl | 15.04 ± 0.14 | 15.08 ± 0.02 | 5.09 ± 0.08 | 2.77 ± 0.14 |
| PEG 8000 | [$C_2$mim]Cl | 15.03 ± 0.05 | 15.12 ± 0.05 | 5.10 ± 0.06 | 0.76 ± 0.04 |
| | [$C_4$mim]Cl | 15.01 ± 0.03 | 15.11 ± 0.03 | 5.14 ± 0.31 | 1.208 ± 0.001 |
| | [$C_6$mim]Cl | 15.06 ± 0.05 | 15.07 ± 0.09 | 5.09 ± 0.09 | 2.49 ± 0.07 |
| | [$C_8$mim]Cl | 15.03 ± 0.09 | 15.05 ± 0.06 | 5.05 ± 0.03 | 1.19 ± 0.07 |

**Table A.10:** Partition coefficients of each Chloranilic Acid ($K_{CA}$) with the respective standard deviations and mass fraction compositions of the quaternary systems at 298 (± 1) K.

| Quaternary System | | Mass Fraction Composition ± $\sigma$ / (wt%) | | | $K_{CA} \pm \sigma$ |
|---|---|---|---|---|---|
| | | PEG | Salt | IL | |
| PEG 1500 | no IL | 15.02 ± 0.04 | 15.03 ± 0.01 | | 20.62 ± 0.25 |
| | [$C_2$mim]Cl | 15.05 ± 0.03 | 15.03 ± 0.26 | 5.05 ± 0.17 | 7.60 ± 0.60 |
| | [$C_4$mim]Cl | 15.08 ± 0.31 | 15.05 ± 0.22 | 5.10 ± 0.11 | 8.77 ± 0.11 |
| | [$C_6$mim]Cl | 15.05 ± 0.05 | 15.08 ± 0.25 | 5.09 ± 0.11 | 16.33 ± 0.16 |
| | [$C_8$mim]Cl | 15.05 ± 0.10 | 15.05 ± 0.16 | 5.13 ± 0.04 | 4.55 ± 0.33 |
| PEG 8000 | no IL | 15.00 ± 0.08 | 15.11 ± 0.06 | | 40.65 ± 2.55 |
| | [$C_2$mim]Cl | 15.00 ±0.06 | 14.98 ± 0.06 | 5.09 ± 0.09 | 6.84 ± 0.03 |
| | [$C_4$mim]Cl | 14.98 ± 0.10 | 14.99 ± 0.05 | 5.14 ± 0.25 | 10.42 ± 0.11 |
| | [$C_6$mim]Cl | 15.05 ± 0.10 | 14.99 ± 0.10 | 5.09 ± 0.09 | 23.05 ± 0.23 |
| | [$C_8$mim]Cl | 14.99 ± 0.08 | 15.00 ± 0.07 | 5.05 ± 0.03 | 3.02 ± 0.03 |

**Table A.11:** Partition coefficients of Rhodamine 6G ($K_{R6G}$) with the respective standard deviations and the mass fraction compositions of the quaternary systems at 298 (± 1) K.

| Quaternary System | | Mass Fraction Composition ± $\sigma$ / (wt%) | | | $K_{R6G} \pm \sigma$ |
|---|---|---|---|---|---|
| | | PEG | Salt | IL | |
| PEG 1500 | no IL | 14.92 ± 0.05 | 15.12 ± 0.21 | | 86.53 ± 0.93 |
| | [$C_2$mim]Cl | 15.16 ± 0.38 | 14.89 ± 0.15 | 4.97 ± 0.05 | 54.78 ± 1.22 |
| | [$C_4$mim]Cl | 15.22 ± 0.29 | 14.77 ± 0.15 | 5.12 ± 0.08 | 14.73 ± 0.15 |
| | [$C_6$mim]Cl | 15.06 ± 0.06 | 15.03 ± 0.21 | 5.01 ± 0.10 | 7.81 ± 0.14 |
| | [$C_8$mim]Cl | 15.00 ± 0.14 | 15.16 ± 0.02 | 5.05 ± 0.08 | 4.53 ± 0.05 |
| PEG 8000 | no IL | 14.97 ± 0.08 | 15.20 ± 0.06 | | 294.59 ± 65.17 |
| | [$C_2$mim]Cl | 15.10 ± 0.11 | 15.20 ± 0.05 | 5.07 ± 0.06 | 184.57 ± 20.77 |
| | [$C_4$mim]Cl | 15.02 ± 0.22 | 15.23 ± 0.09 | 5.14 ± 0.05 | 78.24 ± 10.76 |
| | [$C_6$mim]Cl | 14.96 ± 0.14 | 15.19 ± 0.10 | 5.05 ± 0.04 | 21.00 ± 0.94 |
| | [$C_8$mim]Cl | 14.99 ± 0.09 | 15.13 ± 0.16 | 5.13 ± 0.09 | 2.07 ± 0.06 |

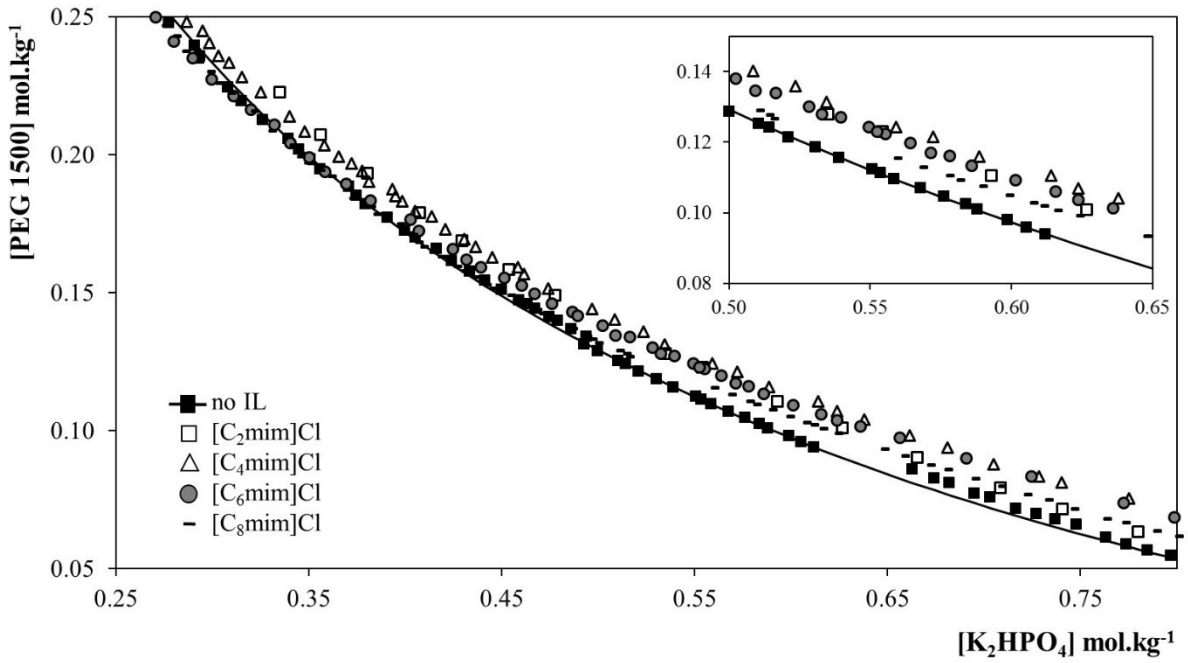


**Fig. A.1:** Binodal curves representation for the quaternary systems composed of [PEG 1500] + $[K_2HPO_4]$ + 5 wt% of ILs (when present) + water, at 298 (± 1) K.

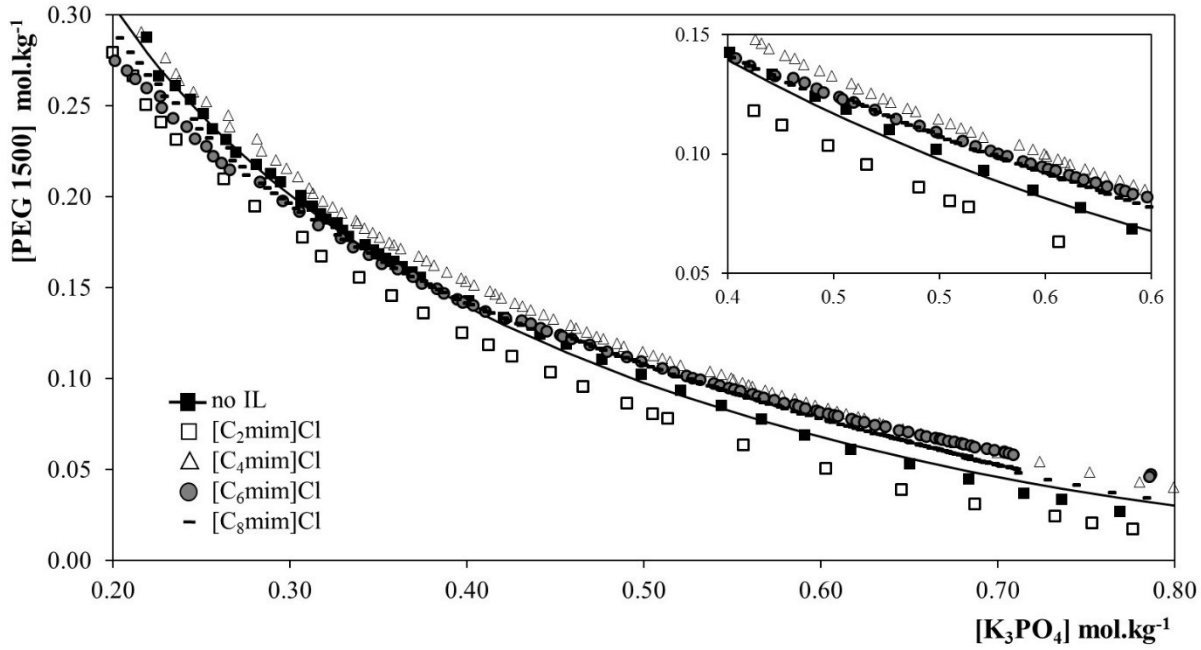


**Fig. A.2:** Binodal curves representation for the quaternary systems composed of [PEG 1500] + $K_3PO_4$ + 5 wt% of ILs (when present) + water, at 298 (± 1) K.

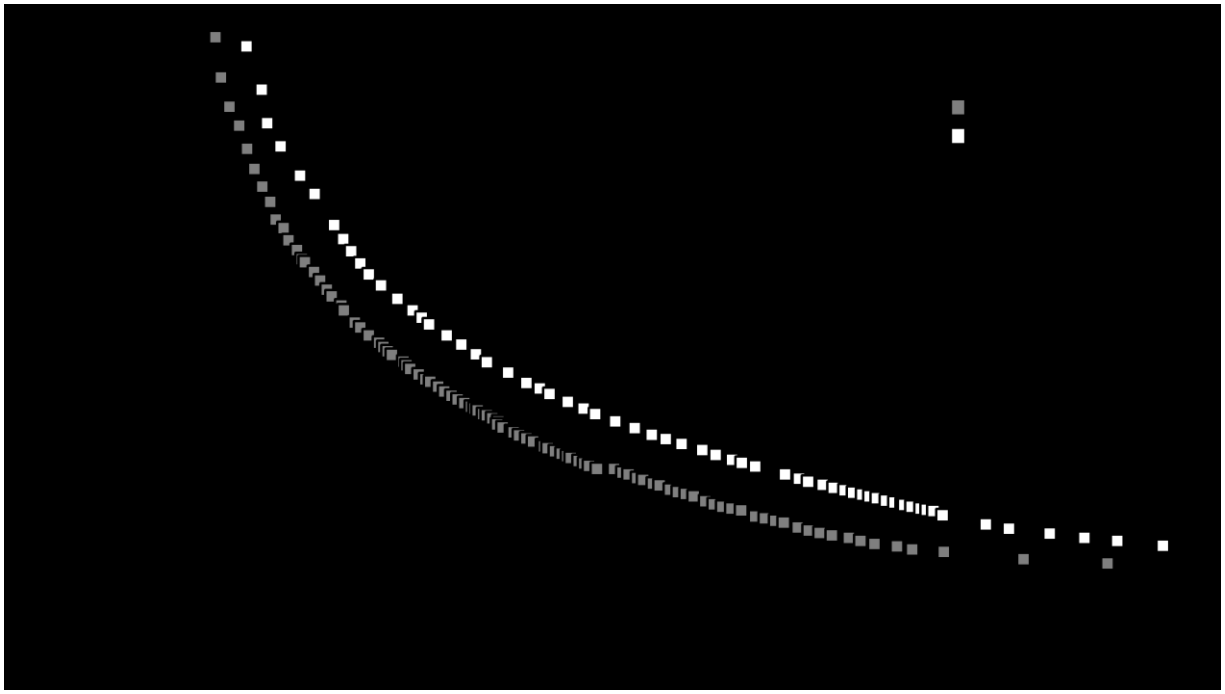

**Fig. A.3:** Binodal curves representation for the ternary systems composed of [PEG 1500] + salt + water, at 298 (± 1) K.

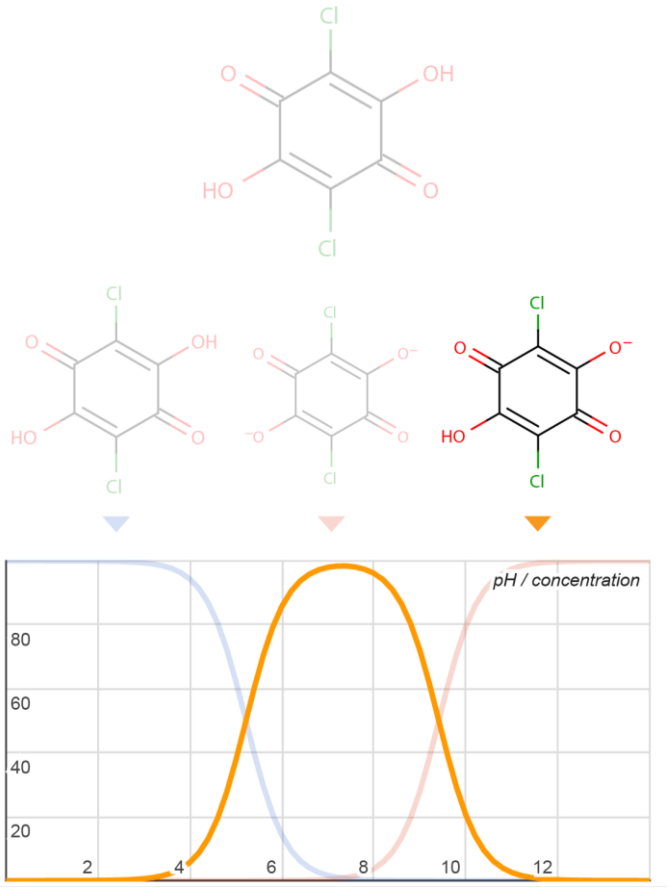


**Fig. A.4:** Speciation curve of CA as a function of pH. This content was adapted from the Chemspider chemical database [1].

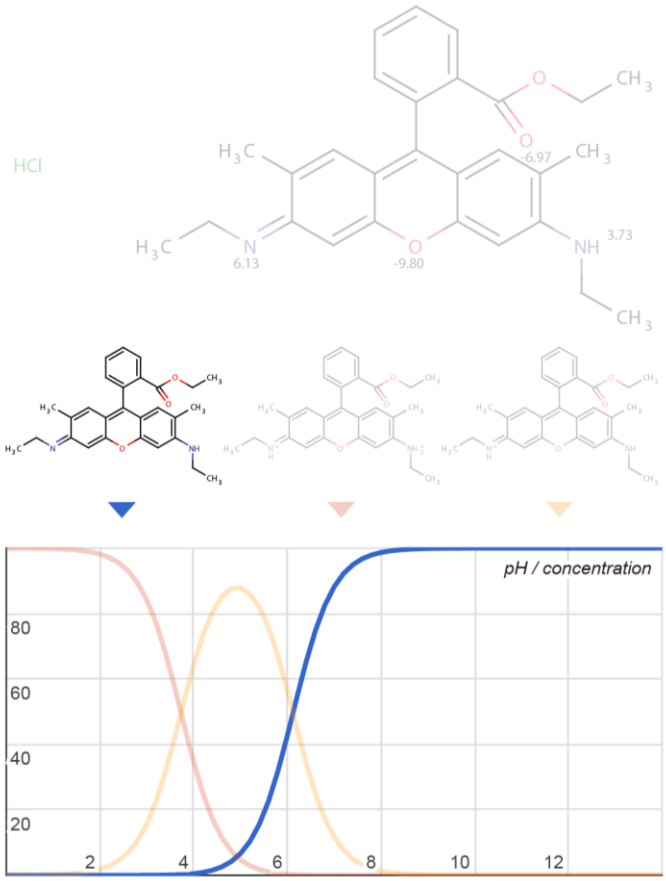


**Fig. A.5:** Speciation curve of R6G as a function of pH. This content was adapted from the Chemspider chemical database [1].

Reference:

[1] Chemspider, The free chemical database, http://www.chemspider.com/. Accessed at 19-11-2013.

## ARTIGO IV

# Lipase purification using ionic liquids as adjuvants in aqueous two-phase systems

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## Abstract

Aqueous two-phase systems (ATPS) are an efficient, environmentally friendly, and "biocompatible" separation process, which allows the recovery of enzymes with high purity levels. These systems are usually based on polymers and salts and recently, to overcome the low polarity difference between the phases of the polymeric systems, ATPS based on ionic liquids (ILs) have been successfully used for the separation of various biomolecules, including enzymes from the fermentation media. However, due to the relatively high price of ILs with best extraction performance, they are here applied as additives or adjuvants in the formation of ATPS. This work discusses the use of imidazolium-based ILs as adjuvants (5 wt%) in ATPS of polyethylene glycol systems (1500, 4000, 6000 and 8000 $g.mol^{-1}$) with potassium phosphate buffer at pH 7, in the extraction and purification of a lipase produced by submerged fermentation by *Bacillus* sp. ITP-001. An initial optimization study was carried with the commercial lipase B from *Candida antarctica* (CaLB). The main results indicate that it is possible not only to further purify the commercial CaLB (*Purification Factor* ≈ 5.2) but principally, the lipase from *Bacillus* sp. ITP-001 for which a *Purification Factor* of 245 was achieved with an imidazolium with a hexyl alkyl chain. The purification factor is a consequence of the favorable interactions between the ILs and the contaminant proteins that migrate for the opposite phase of enzyme.

***Keywords*:** aqueous two-phase systems, ionic liquids, adjuvant, lipase, fermentation broth, purification

## Introduction

Lipases, triacylglycerol ester hydrolases (EC 3.1.1.3), especially of microbial origin, occupy a place of prominence among biocatalysts owing to their novel and multifold applications in

organic synthesis, detergent formulation, nutrition, use as biosensor, bioremediation, among others (Björkling et al., 1991; Hasan et al., 2006; Macrae and Hammond, 1985). Most commercial applications do not require lipase preparations; a certain degree of purity, however, enables efficient and successful usage. Further, purified lipase preparations are required in industries employing the enzymes for the biocatalytic production of fine chemicals, pharmaceuticals and cosmetics (Hasan et al., 2006; Saxena et al., 2003). The main constraints to the production of highly pure enzymes are the different steps necessary to purify it, which are in general difficult, because they may induce the deactivation of the enzyme, or they may require a high consumption of energy and chemicals. The downstream processing and purification represent 20 – 60% of the final cost of the enzyme production, and up to close to 80% in the more demanding scenarios (Martínez-Aragón et al., 2009; Ruiz-Ruiz et al., 2012). In this sense, significant efforts have been focused on the development of new or adapted technologies to perform the purification of enzymes, with lower costs, but still efficient, sustainable and biocompatible with the enzyme conformational structure (Martínez-Aragón et al., 2009). In this context, aqueous two-phases systems (ATPS) have been proposed as alternative extraction methodologies for the extraction/purification of enzymes and other molecules, such as proteins, genetic material, biopharmaceuticals, cells and organelles (Asenjo and Andrews, 2012; Mazzola et al., 2008; Molino et al., 2013; Rito-Palomares, 2004). The ATPS is a very mild method for the protein extraction or even purification, making possible to avoid the denaturation or loss of biological activity. This characteristic is usually attributed to the high water content and low interfacial tension of the systems which protects the proteins (Kula et al., 1982). Despite the well-known advantages offered by these systems, ATPS are typically composed of common polymers (namely polyethylene glycol PEG (Barbosa et al., 2011; Maciel et al., 2014; Souza et al., 2010; Zhou et al., 2013), dextran (Gündüz and Korkmaz, 2000) being their application limited to separate a large variety of molecules (hydrophobic and

hydrophilic), normally due to the limited range of polarities of the coexisting phases. Recently, to overcome the demand for more polar solvents, the use of ionic liquids (ILs) in ATPS is gaining interest (Gutowski et al., 2003). One of the main advantages of the application of ILs in the formation of ATPS is the possibility of to manipulate their physicochemical properties (Naushad et al., 2012) by the proper combination/manipulation of the cation/anion/alkyl chain of the ILs (Perumalsamy et al., 2007). Due to their advantages, these systems have been extensively studied and applied in the extraction of a wide variety of compounds such as amino-acids (Claudio et al., 2010; Neves et al., 2009; Pereira et al., 2010; Ventura et al., 2009; Zafarani-Moattar and Hamzehzadeh, 2011), drugs (Li et al., 2005b), phenolic compounds (Claudio et al., 2010), alkaloids (Freire et al., 2010; Li et al., 2009; Li et al., 2005a; Louros et al., 2010; Shiri et al., 2013), antibiotics (Li et al., 2009; Liu et al., 2005), anti-inflammatory compounds (e Silva et al., 2014), proteins (Dreyer and Kragl, 2008; Oppermann et al., 2011; Pei et al., 2010; Sheikhian et al., 2013), enzymes (Cao et al., 2008; Deive et al., 2012; Dreyer and Kragl, 2008; Ventura et al., 2011a) and natural colorants (Ventura et al., 2013).

For enzymes, the capability to keep the catalytic activity is important for the success of the extraction process and it is therefore essential that the hydrophilic ILs and their aqueous solutions possess balanced IL-enzyme interactions, which means a strong capacity to dissolve the enzyme but not too strong that would disrupt their structure and/or interact with their active sites, causing the deactivation of the enzyme (Freire et al., 2012a). Hydrophilic ILs were previously applied in the preparation of ATPS and their use as extraction systems for the separation of four different lipases; *Thermomyces lanuginosus* lipase (Deive et al., 2011), CaLB (Ventura et al., 2011a), CaLA (Deive et al., 2012), and the *Bacillus* sp. ITP-001 lipase (Ventura et al., 2012). In all cases, the imidazolium family presented the best results (Deive et al., 2011; Deive et al., 2012; Ventura et al., 2012; Ventura et al., 2011a). The use of more hydrophilic ILs, including different cation families and anions, is still scarce regarding their application as

separation agents and solvents. If the application of ILs as phases promoters, when conjugated with different inorganic or organic salts, or even with polymers, to promote the formation of ATPS, normally requires the use of large concentrations of salts or polymers, making the extraction process more expensive and less sustainable. The use of small quantities of ILs as adjuvants (Pereira et al., 2010) appears as an alternative to overcome this difficulty. Pereira et al. (2010) have demonstrated that the incorporation of 5 wt% of an IL in a polymer + salt common ATPS was able to modify the polarity of the aqueous phases, leading to a more efficient and controllable separation process. Recently, Souza et al. (2014a) have also described that the presence of a small concentration of different ILs of the type [$C_n$mim]Cl, although presenting a minor effect upon the ATPS formation, had a major impact in the PEG-rich phase characteristics and thus, in the extraction parameters (*i.e.* higher partition coefficients and extraction efficiencies for two dyes). In addition, Almeida et al. (2014) used ILs as adjuvants in conventional PEG + $Na_2SO_4$ ATPS providing enhanced extraction efficiencies for the extraction of different antioxidants (vanillic and syringic acids). Currently, there is no report in literature on the use of ILs as adjuvants in a polymer + salt ATPS in systems with potassium phosphate, principally to be applied in the purification of enzymes. In this context, this work concentrates efforts on the application of ATPS based in four polymers (PEG 1500, PEG 4000, PEG 6000 and PEG 8000) with the potassium phosphate buffer ($K_2HPO_4/KH_2PO_4$) at pH 7, using four imidazolium-based ILs as adjuvants (1-alkyl-3-methylimidazolium chloride [$C_n$mim]Cl, n = 2, 4, 6, 8) at 5 wt%. These allowed the study of various conditions, namely the molecular weight of the polymer, the alkyl chain length of the ILs and the high molecular weight of the polymer, that were investigated in terms of their effect on the purification of the commercial lipase B from *Candida antartica* (CaLB) that is here used as a model enzyme to evaluate the purification performance of these new ATPS. Subsequently, representative ATPS using ILs as adjuvants were employed to study the purification performance of *Bacillus* sp. ITP-

001 lipase produced by submerged fermentation. ATPS using ILs as adjuvants have significantly higher capacity to purify lipases, when compared with the common ATPS based in polymers + salts (Barbosa et al., 2011) and ILs + salts (Ventura et al., 2012). These systems are promising alternative extraction systems to be applied as downstream processes for other enzymes or biopharmaceuticals.

## Materials and methods

### Materials

The present study was carried out using different polyethylene glycol (PEG) polymers of average molecular weights 1500, 4000, 6000 and 8000 $g.mol^{-1}$ (abbreviated as PEG 1500, PEG 4000, PEG 6000, and PEG 8000, respectively), supplied by SIGMA-Aldrich and used as received. Aqueous solutions of potassium phosphate buffer at pH 7 was used (Ventura et al., 2011b). The salts used in the preparation of the potassium phosphate buffer $K_2HPO_4$ (purity ≥ 98 wt%) and $KH_2PO_4$ (purity ≥ 99.5 wt%) were purchased from SIGMA-Aldrich. The ILs studied were the 1-ethyl-3-methylimidazolium chloride ([$C_2$mim]Cl), 1-butyl-3-methylimidazolium chloride ([$C_4$mim]Cl), 1-hexyl-3-methylimidazolium chloride ([$C_6$mim]Cl) and 1-octyl-3-methylimidazolium chloride ([$C_8$mim]Cl). All ILs were purchased from Iolitec (Ionic Liquid Technologies, Germany) with mass fraction purities higher than 98%, being their chemical structures depicted in Figure 1. The protein bovine serum albumin (BSA, purity ≥ 97%) was obtained from Merck. The model enzyme used, *Candida antarctica* lipase B (here abbreviated as CaLB), was kindly offered by Novozymes A/S, Bagsværd, Denmark and the enzyme from *Bacillus* sp. ITP-001 was obtained by a submerged fermentation using $MgSO_4 \cdot 7H_2O$ (purity ≥ 98%) from Panreac, Triton X-100 from Fisher Scientific, $NaNO_3$ (purity ≥ 99.5%), yeast extract, peptone, and starch purchased from Himedia®. The ammonium

sulphate (P.A.) was obtained from Synth (Brazil) and the coconut oil was purchased at a local market (Aracaju - Sergipe, Brazil). SDS-PAGE Molecular Weight Standards and the marker molecular weight full-range (VWR) were used as protein standards and were purchased at GE Healthcare Life Sciences.

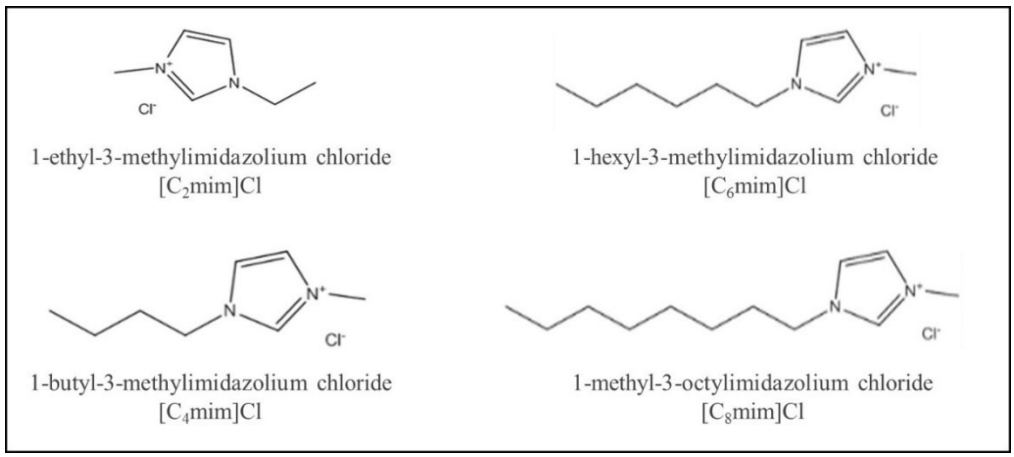


**Figure 1:** Chemical structure, full name and abbreviation of all ILs investigated.

**Production of lipase by *Bacillus* sp. ITP-001**

***Fermentation conditions***

The lipolytic enzyme was produced by *Bacillus* sp. ITP-001 by submerged fermentation. The microorganisms were isolated from an oil contaminated soil and stored at the *Instituto de Tecnologia e Pesquisa* – ITP (Aracaju - Sergipe, Brazil). The strain was cultivated in 500 mL Erlenmeyer flasks containing 200 mL of a proper medium with the following composition (%, w/v): $KH_2PO_4$ (0.1), $MgSO_4 \cdot 7H_2O$ (0.05), $NaNO_3$ (0.3), yeast extract (0.6), peptone (0.13), and starch (2.0) as the carbon source. The fermentation conditions were an initial pH around 7, the incubation temperature equal to 37º C, and the stirring speed at 1,700 rpm. After 72 h of cultivation, coconut oil (4 %, w/v) and Triton X-100 were added as inductors as described by Feitosa et al. (2010).

***Pre-purification steps***

The fermented broth was centrifuged at 10,000 rpm for 30 min, the biomass was discharged and the supernatant was used to determine the enzymatic activity and the total protein concentration, just in the final step of the fermentation process. The protein contaminants in the cell-free fermented broth were precipitated using ammonium sulphate at 80 % (w/v) and room temperature, and then the broth was centrifuged at 10,000 rpm for 30 min to separate the aqueous solution from the precipitate (mainly composed by the denatured contaminant proteins). Following the proteins denaturation, the aqueous solution obtained was dialyzed using a MD 25 dialysis bag (cut-off: 10,000-12,000 Da) against ultra-pure water for 24 h at 4 ºC. The dialyzed solution (dialysate) containing lipase from *Bacillus* sp. ITP-001 was then used in the preparation of the ATPS under study.

***Preparation of the ATPS***

The composition of the mixture points selected for the extraction experiments considering the model lipase CaLB were: PEG 1500 (15 wt%) + potassium phosphate buffer (15 wt%) + [$C_n$mim]Cl (n = 2, 4, 6 and 8); PEG 4000 (10 wt%) + potassium phosphate buffer (13 wt%) + [$C_n$mim]Cl (n = 2, 4, 6 and 8); PEG 6000 (10 wt%) + potassium phosphate buffer (12 wt%) + [$C_n$mim]Cl (n = 2, 4, 6 and 8); and PEG 8000 (10 wt%) + potassium phosphate buffer (12 wt%) + [$C_n$mim]Cl (n = 2, 4, 6 and 8)]. These were chosen to minimize or avoid the denaturation or unfold of the target enzyme at the interface and during the experiments. Considering the optimization step, all systems contained approximately 5 wt% of CaLB, and, when present, 5 wt% of IL (Table A.1 in Supporting Information). The potassium phosphate buffer solution ($K_2HPO_4$/$KH_2PO_4$) was prepared by the addition of dibasic ($K_2HPO_4$) and monobasic ($KH_2PO_4$) potassium phosphate at a ratio of 1.087 (w/w) and pH 7.

Considering the study of extraction of a lipase produced via fermentation (by the *Bacillus* sp. ITP-001) and purified by applying ATPS, the extraction systems were prepared by adding 15 wt% of PEG (1500, 6000 or 8000), 15 wt% of $K_2HPO_4/KH_2PO_4$ (potassium phosphate buffer) + 5 wt% of each $[C_nmim]Cl$ (n = 2, 4, 6, 8) - Table A.2 in Supporting Information. In this particular case, the potassium phosphate buffer was prepared with the dialysate solution, where the lipolytic lipase from *Bacillus* sp. ITP-001 is concentrated. Then, the buffer was directly used in the preparation of the extraction systems in the proportions previously mentioned. The mixture points selected form two immiscible aqueous phases which is confirmed by the ternary phase diagrams published elsewhere (Souza et al., 2014a).

Each mixture was prepared gravimetrically within $\pm 10^{-4}$ g, vigorously stirred and left to equilibrate for at least 12 h and at 25.0 (± 0.1) ºC. After this treatment, both phases became clear and transparent being then the interface well defined. The phases were carefully withdrawn using a pipette for the top phase and a syringe with a long needle for the bottom phase. The volumes and weights were determined in graduated test tubes (the total mass of each extraction system prepared was 5 g).

The partition coefficients of the main contaminant proteins ($K_P$) and the enzyme ($K_E$) are defined by Eqs. (1) and (2).

$$K_P = \frac{C_T}{C_B} \quad (1)$$

$$K_E = \frac{EA_T}{EA_B} \quad (2)$$

where $C_T$ and $C_B$ are, respectively, the total protein concentration ($mg.mL^{-1}$) in the top and bottom phases, and $EA_T$ and $EA_B$ represent the enzyme activity ($U.L^{-1}$) in the top and bottom phases, respectively.

In order to evaluate the purification process, the specific enzyme activity (SA, $U.mg^{-1}$ protein) calculated by Eq. (3), the volume ratio between the top and bottom phases ($R_v$), the contaminant

protein recovery having into account the top phase ($R_{PT}$, %), the enzyme recovery in the bottom phase ($R_{EB}$, %), and the purification factor (*PF* - fold) were calculated accordingly to Eqs. (4) to (6).

$$SA = \frac{EA}{C} \tag{3}$$

$$R_{PT} = \frac{100}{1 + \left({}^{1}/_{R_V K_P}\right)} \tag{4}$$

$$R_{EB} = \frac{100}{1 + R_V K_E} \tag{5}$$

$$PF = \frac{SA}{SA_i} \tag{6}$$

where C is the total protein concentration ($mg.mL^{-1}$). The purification factor (*PF*) was calculated by the ratio between the *SA* in the top or bottom phases (which is dependent of the phase in which the enzyme is concentrated) and the specific activity (SA*i*) of lipase from *Bacillus* sp. ITP-001 after the centrifugantion.

***Enzyme assay***

The lipolytic activity was measured according to a method proposed by Soares et al. (1999). The substrate was prepared by mixing 50 mL of olive oil with 50 mL of Arabic gum solution (7 %, w/v). The reaction mixture, containing 5 mL of the oil emulsion, 2 mL of sodium phosphate buffer (100 mM and pH 7) and 1 mL of enzyme extract was incubated in a thermostated bath reactor for 5 min at 37 °C. A blank/control titration was done on a sample where the enzyme was replaced by distilled water. After 5 min of reaction, an aliquot of ≈ 0.33 g was taken and added to 2 mL of a solution composed of acetone–ethanol–water (1:1:1). The exact weight of each aliquot was determined at the end of the addition procedure. The fatty acids produced were titrated with a potassium hydroxide solution (40 mM) in presence of phenolphthalein as indicator. One unit (U) of enzyme activity is defined as the amount of

enzyme that produces 1 μmol of free fatty acid per minute (μmol.min$^{-1}$) under the assay conditions (37 °C, pH 7 and 100 rpm).

***Protein Assay***

Total protein concentration was determined by the Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as standard, using a Shimadzu PharmaSpec UV-1700, of 595 nm, and a calibration curve previously established for the standard protein BSA. To eliminate the influence of the ILs presence on the protein concentration analysis, one control system for each extraction point but without enzyme was prepared under the same process conditions.

***SDS-PAGE electrophoresis***

Electrophoresis was performed with the Mini-PROTEAN II System (BioRad, USA) using polyacrylamide gels (stacking: 4 % and resolving: 20 %) with a running buffer consisting in 250 mM Tris HCl, 1.92 M glycine, 1 % SDS as described by Laemmli (1970). Gels were stained with Coomassie blue R-250. SDS-PAGE Molecular Weight Standards, Marker molecular weight full-range (VWR), were used as protein standards. All gels were analyzed using the Image Lab 3.0 (BIO-RAD) analysis tool.

## Results and discussion

### Optimization Approach

This work studies the use of ATPS with ILs as adjuvants for the purification of a lipolytic enzyme from the fermentation broth, a lipase produced by submerged fermentation from *Bacillus* sp. ITP-001. The initial optimization was carried using the commercial CaLB with a high purity level, aiming at understanding the most important mechanisms behind the migration

of the enzyme between the two aqueous phases. The success of the application of ATPS as extraction processes is largely dependent on the ability to manipulate the properties of the aqueous phases to obtain the appropriate migration of the (bio)molecules of interest, aiming at the specific selectivity in the migration of both target enzyme and contaminant proteins. In this work, several ATPS in the form PEG + $K_2HPO_4/KH_2PO_4$ + water + $[C_nmim]Cl$; n = 2, 4, 6 and 8 (when present) were investigated. The buffer was used in all experiments, because it made possible to keep the pH constant during the entire experiments, allowing to keep the enzyme structure and charge (e Silva et al., 2014; Pereira et al., 2013). To control the migration of the biomolecules in ATPS based in ILs, different parameters can be considered, from the simpler: different ILs chemical structures, the amount of IL, salt, and polymer and several molecular weights of the polymer (for polymeric systems); to the more complex such as the temperature and pH and the addition of additives, electrolytes or in the particular case of this work, adjuvants. Thus, in the first section of results, this work illustrates the principal effects promoted by the presence/absence of several ILs and different polymer molecular weights in the migration of the commercial CaLB.

***Effect of the ionic liquid and its chemical structure***

The partition of CaLB was analyzed considering different ATPS based in PEG 1500 + $K_2HPO_4/KH_2PO_4$ (pH 7) + 5 wt% $[C_nmim]Cl$; *n* = 2, 4, 6, 8 (when present) + water + 5 wt% of CaLB aqueous solution. Since this commercial enzyme from Novozymes is already pure (Ventura et al., 2011a), the expectable purification in the optimization section is obviously limited. However, and following the same approach of Ventura et al. (2011a), the purification factor was also calculated. Figure 2 shows the results of the partition coefficients for the enzyme ($K_E$) and the contaminant proteins ($K_P$), and the purification factor (*PF*) for CaLB (details about

the recovery of CaLB and the contaminant proteins are described in Table A.3 in Supporting Information).

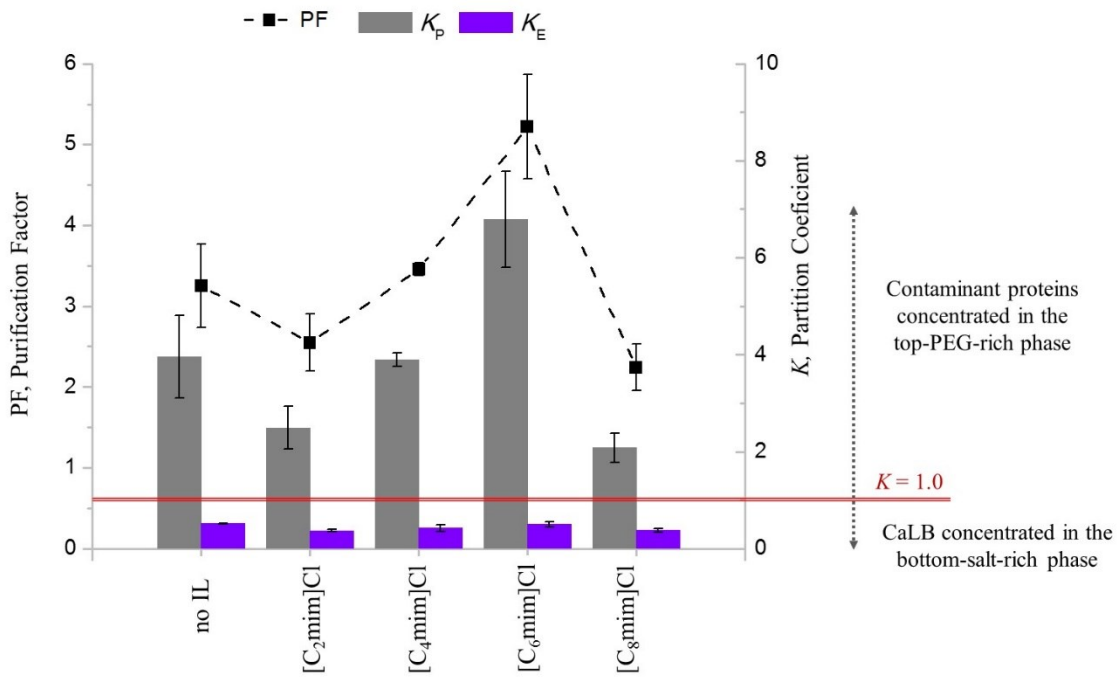


**Figure 2:** Results found for the purification factor (PF), partition coefficient of the contaminant proteins ($K_P$) and CaLB ($K_E$) by applying systems based in PEG 1500 + 5 wt% of [$C_n$mim]Cl (n = 2, 4, 6 and 8) + water + $K_2HPO_4/KH_2PO_4$, at pH 7 and 25 (± 0.1) ºC and atmospheric pressure.

The results depicted in Figure 2 show the partition of CaLB and the contaminant proteins for opposite phases ($K_E < 1$ and $K_P > 1$). CaLB is thus more concentrated in the salt-rich phase. This can be easily justified by the fact that at pH 7, CaLB is negatively charged, isoelectric point p$I$ = 6 (Forciniti et al., 1991; Shang et al., 2004), resulting in the increase of its affinity for the more hydrophilic phase (Barbosa et al., 2011; Ventura et al., 2011a). However, the contaminant proteins (as evidenced in a previous work of ours (Ventura et al., 2011a), by an eletrophoresis analysis done to the commercial lipase), are preferentially migrating to the PEG-rich phase ($K_P > 1$). In fact, the contaminant proteins are migrating for the PEG layer closely

following the migration of the ILs used, as recently described (Almeida et al., 2014; Souza et al., 2014a). This behavior seems to be justified by the stronger interactions between the contaminant proteins and the polymeric phase (already shown in (Souza et al., 2014a)), not only due to the presence of the polymer (identified by the $K_P > 1$ for the system without the presence of IL), but also due to the presence of the distinct ILs, situation easily observed by the same tendency described by the migration of the contaminant proteins ($K_P$ results here described) and each of the ILs for the polymer layer ($K_{IL}$ described in literature (Souza et al., 2014a)). It seems that the use of these ATPS conjugated with the ILs are actually more efficient in the manipulation of the contaminant proteins than in the control of the CaLB migration. The tendency of the contaminant proteins to migrate for the PEG-rich phase accordingly to the results of Figure 2 is $[C_8mim]Cl < [C_2mim]Cl < [C_4mim]Cl <$ no IL $< [C_6mim]Cl$. Based on the $K_P$ data, it is suggested that more than just the hydrophobic interactions are controlling their migration, also electrostatic interactions, van der Waals forces and hydrogen-bonding are playing a significant role, due to the presence of ILs (Pereira et al., 2010; Souza et al., 2014a). Finally, the decrease observed for $[C_8mim]Cl$ relative to $[C_6mim]Cl$ seems to be related with the possible formation of aggregates by the IL' self-aggregation, thus changing the balance of interactions acting in the ATPS bulk (Freire et al., 2012b). The negative effect of the presence of IL's aggregates was already shown in other works (Passos et al., 2013), but it should be clarified that this behavior only happens in certain processing conditions and for specific molecules.

The values for the purification factor were also depicted in Figure 2. The dependence of $K_P$ with *PF* was more clearly observed when ILs are used as adjuvants. The purification factor is the last parameter to be taken into account, since the main purpose of this work is to identify the best systems to promote the purification of a lipolytic enzyme from the fermentation broth, separating them from the remaining contaminants (mainly other proteins also produced in the

submerged fermentation). From a careful analysis of the *PF* data, it is concluded that the best system is the ATPS based in [$C_6$mim]Cl. The presence of [$C_6$mim]Cl increases the *PF* from 3.25 ± 0.65 (without IL) to 5.22 ± 0.65 (with IL). These results are mainly justified by the higher migration of the contaminant proteins for the PEG-rich phase in presence of the IL, while CaLB is still accumulated in the salt-rich phase. Again, we call the attention for the fact that this enzyme is a commercial sample which purity level is already high, and even then, it was possible to increase a *PF* of 5.22 ± 0.65, a clear sign of the capacity of these systems to improve the enzyme purity level even when compared with the common ILs-based ATPS composed of 25 wt% [$C_8$mim]Cl and 30 wt% of $K_2HPO_4$/$KH_2PO_4$ (pH 7) - *PF* = 2.6 ± 0.1 (Ventura et al., 2011a).

***Effect of the PEG molecular weight***

Although it has been shown the relevance of the use of ILs as adjuvants for the control of the contaminant proteins partition, the polymer molecular weight plays also an important role in the partition phenomenon. In this case, the partition of CaLB was analyzed considering different ATPS based in PEG (1500, 4000, 6000 and 8000) + $K_2HPO_4$/$KH_2PO_4$ (pH 7) + IL [$C_n$mim]Cl; n = 2, 4, 6, 8 (when present) + water + CaLB aqueous solution (Table A.1). The principal results considering the recovery of CaLB and the contaminant proteins for the bottom and top phases, respectively, are presented in Figure 3.

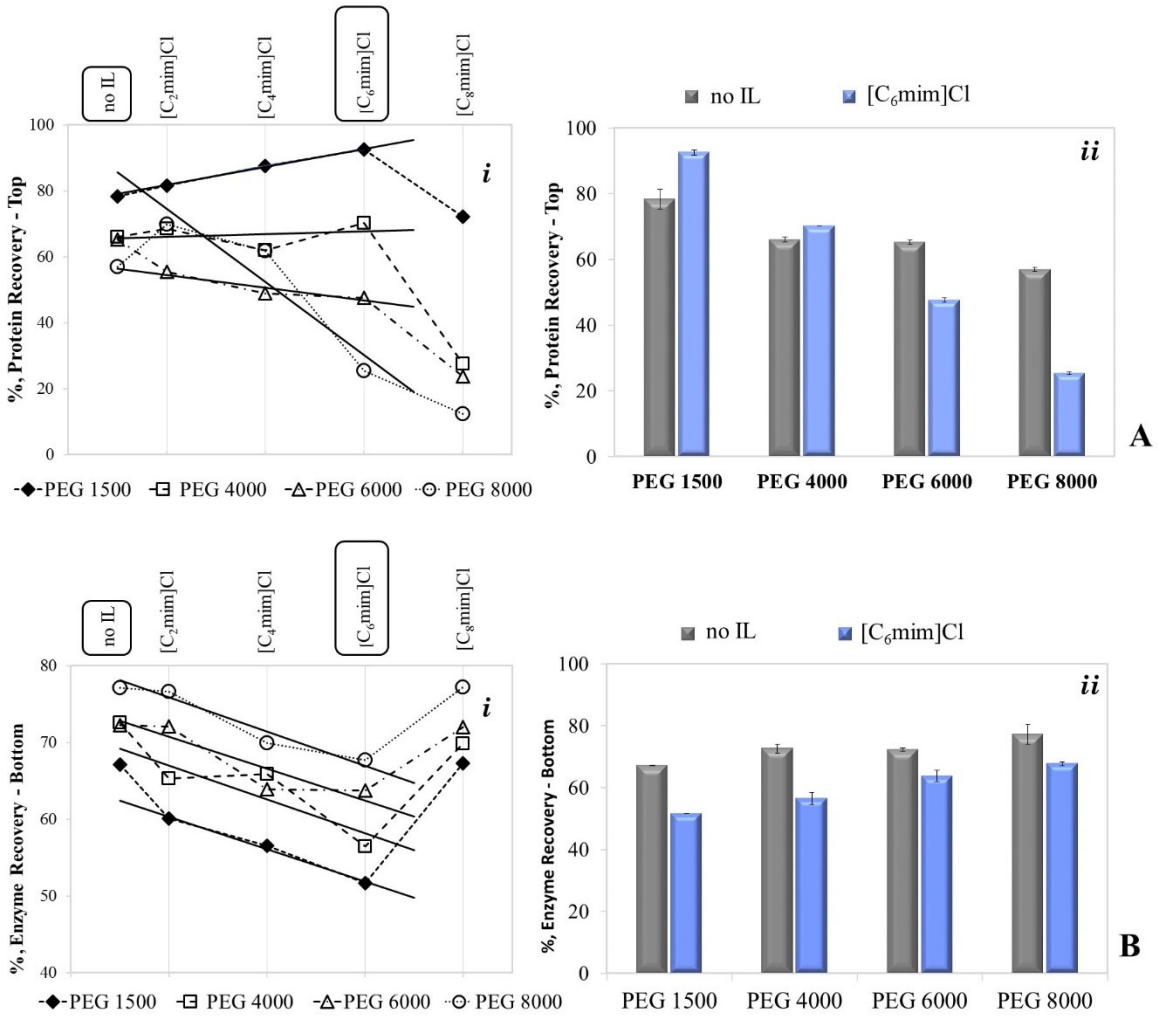


**Figure 3.** Results showing the effect of different PEG molecular weights in the recovery of the contaminant proteins in top phase **(A)** and the enzyme in bottom phase **(B)**: ***i)*** with the elongation of the IL' alkyl chain and ***ii)*** by comparing systems with and without the presence of [$C_6$mim]Cl.

The profiles presented in Fig. 3A demonstrate the effect of the polymer molecular weight increase conjugated with the elongation of the ILs' alkyl chain. The trend lines in Fig. 3A(*i*) suggest an inversion on the recovery of the contaminant proteins from the top to the bottom phase, with the simultaneous increase in both the polymer molecular weight and the IL alkyl chain length. The results from Fig. 3A(*ii*) suggest that most contaminant proteins are concentrated in the top phase, and their partition decreases with the increase of the polymer

molecular weight, by the following order: PEG 1500 > PEG 4000 > PEG 6000 > PEG 8000. It can be concluded that the presence of ILs is more advantageous regarding the separation of the CaLB and the contaminant proteins when polymers of lower molecular weight are present (Souza et al., 2014a). Actually, and in the particular case of [$C_6$mim]Cl, the extraction of the contaminant proteins decreases considerably when PEG 6000 or PEG 8000 [Fig. 3A(*ii*)] are used. These results can be justified by the differences regarding the intrinsic viscosity of the two phases, as reported by Kirincic and Klofutar (1999). The authors show that the increase of the molecular weight of PEG increased the viscosity of the aqueous solution of PEG from 4.41 $cm^3.g^{-1}$ (PEG 400) to 14.15 $cm^3.g^{-1}$ (PEG 4000) and 42.02 $cm^3.g^{-1}$ (PEG 2000). The migration of CaLB was also evaluated (Figure 3B) and in general, the results showed the preferential enzyme accumulation in the salt-rich (bottom) phase, being the contaminant proteins accumulated in the opposite phase. The partition tendency observed for CaLB is not as pronounced, when compared with the tendency found for the contaminant proteins. However, it is possible to identify an increase in the enzyme recovery *at circa* 20% with the increase in the molecular weight of PEG [from PEG 1500 to PEG 8000, Fig. 3B(*i*)].

To confirm the tendencies observed and the selective separation of CaLB from the contaminant proteins, despite the use of a commercial lipase in the optimization step, a electrophoresis analysis was carried out being the main results depicted in Figure 4. The electrophoresis gel (Figure 4) is divided in 4 main lanes, being Lane A the molecular mass standard, Lane B the commercial CaLB and the remaining Lanes represent the bottom phases of two distinct ATPS applied in the optimization step.

Analyzing carefully Lane B, it is clearly seen the presence of the principal enzyme CaLB *at circa* 35 kDa and two other bands less pronounced representing two contaminant protein (numbers 2 and 3), showing that this commercial enzyme is of a limited purity. Lanes C and D

represent the bottom phases of ATPS with IL [$C_2$mim]Cl and [$C_6$mim]Cl, respectively. Specifically in Lane C it can be seen that there is a small band additional (5), which is not found in D. In this case, it is evident that the ATPS using IL ([$C_6$mim]Cl) are more efficient for the isolation of enzymes in the bottom phase of system, since only a band (6) was found in Lane D. Thus, the observations by SDS-PAGE support the purification results previously reported.

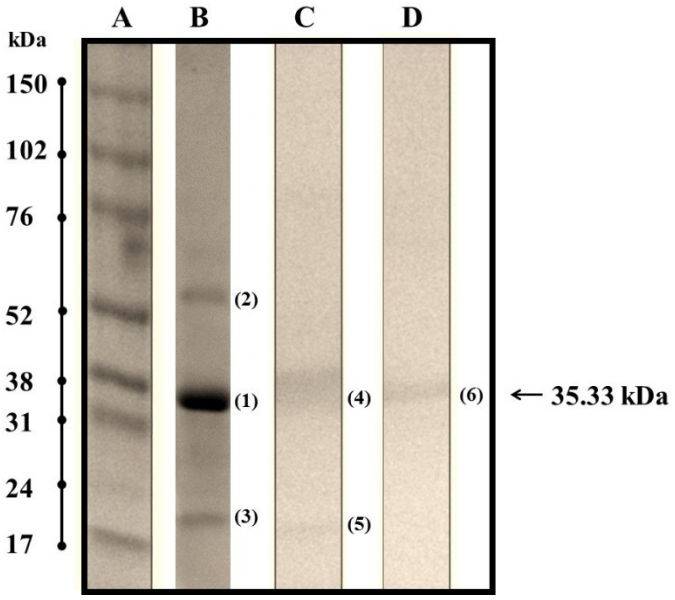


**Figure 4:** Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) patterns of CaLB. Lane A: molecular mass standard (17–150 kDa), Lane B: commercial CaLB, Lanes C-D: CaLB purified from 2 ATPS, namely: PEG 1500 + 5 wt% of [$C_2$mim]Cl + water + $K_2HPO_4$/$KH_2PO_4$ Lane C bottom phase; PEG 1500 + 5 wt% of [$C_6$mim]Cl + water + $K_2HPO_4$/$KH_2PO_4$ Lane D bottom phase.

**Production and pre-purification of lipase from *Bacillus* sp. ITP-001**

Having evaluated the purification capacities of these new ATPS, they were further employed in order to demonstrate the purification of a real matrix of lipase from *Bacillus* sp. ITP-001, produced by submerged fermentation. Also in here, two phenomena were considered in the analysis, namely the migration of the target enzyme and the main contaminants (normally proteins) present in the fermentation media. Moreover, two distinct approaches were also

applied, namely *i)* the use of a pre-purification step before the use of ATPS and *ii)* the direct application of ATPS to extract and purify the lipase without a pre-purification step. This process is described in Figure 5, in which the process diagram is presented from the production to the final step of purification considering the application of ATPS using ILs as adjuvants. Table I reports several parameters, namely the enzymatic activity (*EA* - $U.mL^{-1}$), the total protein concentration (*C* - $mg.mL^{-1}$), the specific activity (*SA* - $U.mg^{-1}$) and the purification factor (*PF* - fold) obtained as output of each step, in particular the production, in which the fermentation broth is obtained and at the end of the pre-purification step, by the obtaining the dialysate (*Route* ***i***). In *Route* ***i*** the salt $(NH_4)_2SO_4$ was used to precipitate some of the contaminant proteins (precipitation step described in the process diagram), the lipase is concentrated in the supernatant and a large amount of contaminant proteins were removed. Following the precipitation process, the supernatant was dialyzed aiming at removing the low molecular weight compounds, including inorganic salts from the fermentation and the precipitation process. In this case, the purification factor of the dialysate was around to 17.16 fold, in agreement with the results previously reported by us for this pre-purification step (Barbosa et al., 2011; Souza et al., 2014b; Souza et al., 2014c; Ventura et al., 2012). In the next section, *Routes* ***i*** and ***ii*** will be addressed taking into account only the step of purification by ATPS.

**Table I:** Purification factor, enzymatic activity, specific activity, and protein concentration at the end of each step of the production and pre-purification of lipase produced by *Bacillus* sp. ITP-001.

| **Steps** | **Process** | ***EA*** **($U.mL^{-1}$)** | ***C*** **($mg.mL^{-1}$)** | ***SA*** **($U.mg^{-1}$)** | ***PF*** **(fold)** |
|---|---|---|---|---|---|
| **Production** | Fermentation | 9,430.7 | 1.16 | 8,129.2 | – |
| **Pre-purification** | Dialysis | 9,287.8 | 0.07 | 139,473.4 | 17.16 |

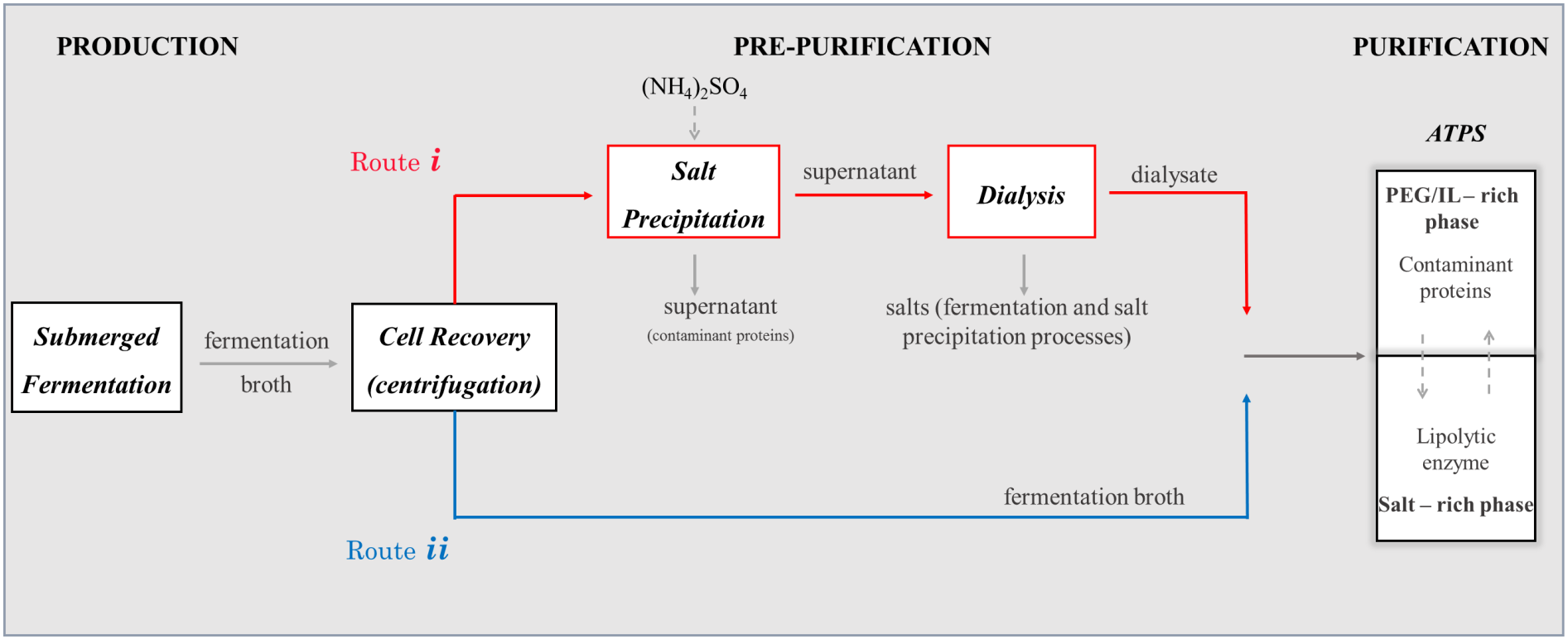


**Figure 5:** Flow chart of the two-route approach for the production and purification of the lipolytic enzyme produced by *Bacillus* sp. ITP-001 via submerged fermentation: Route ***i*** – with pre-purification steps, including precipitation with $(NH_4)_2 SO_4$ followed by dialysis; Route ***ii*** – does not include any step of pre-purification.

***Purification of lipase using ATPS***

After the optimization studies carried out using the commercial CaLB, the ATPS representing the best extraction conditions (higher purification factors) were applied to the purification of an extracellular lipolytic enzyme produced by *Bacillus* sp. ITP-001. The composition of the extraction systems used in this part of the study were composed by 15 wt% of PEG (1500, 4000 and 8000) + 15 wt% of $K_2HPO_4/KH_2PO_4$ + 5 wt% of each IL. However, in this particular case the phosphate buffer was prepared with the dialysate solution (where the lipolytic lipase from *Bacillus* sp. ITP-001 is accumulated) and it was directly introduced in the preparation of all ATPS under study. The selected ATPS using ILs as adjuvants have revealed a great performance in the purification of the lipolytic lipase produced from *Bacillus* sp. ITP-001 as shown in Table II.

**Table II:** Recovery parameters for the contaminant proteins and lipase from *Bacillus* sp. ITP-001, partition coefficients and purification factor achieved in Route ***i*** and ***ii***), using PEG (1500, 4000 and 8000) + $K_2HPO_4/KH_2PO_4$ (prepared with the fermentation broth containing the target lipase) + IL (when present) + water, at 25 (± 0.1) ºC and atmospheric pressure.

| Route | Polymer | ATPS | $R_{EB} \pm \sigma$ | $R_{PT} \pm \sigma$ | $K_E \pm \sigma$ | $K_P \pm \sigma$ | $PF \pm \sigma$ |
|---|---|---|---|---|---|---|---|
| ***i*** | **PEG 1500** | no IL | 79.23 ± 0.12 | 56.69 ± 0.80 | 0.209 ± 0.004 | 1.07 ± 0.04 | 175.61 ± 2.36 |
| | | $[C_2mim]Cl$ | 77.19 ± 0.26 | 49.61 ± 0.94 | 0.269 ± 0.003 | 0.92 ± 0.03 | 169.34 ± 0.81 |
| | | $[C_4mim]Cl$ | 77.48 ± 0.53 | 54.09 ± 1.18 | 0.231 ± 0.008 | 0.95 ± 0.04 | 220.04 ± 7.68 |
| | | $[C_6mim]Cl$ | 76.62 ± 2.52 | 62.81 ± 0.39 | 0.237 ± 0.026 | 1.32 ± 0.01 | 244.99 ± 9.52 |
| | | $[C_8mim]Cl$ | 90.16 ± 0.53 | 37.40 ± 0.04 | 0.109 ± 0.006 | 0.60 ± 0.003 | 100.76 ± 2.96 |
| | **PEG 4000** | $[C_2mim]Cl$ | 90.14 ± 0.09 | 53.16 ± 0.21 | 0.132 ± 0.002 | 1.37 ± 0.01 | 181.32 ± 2.17 |
| | | $[C_6mim]Cl$ | 79.29 ± 1.59 | 59.33 ± 0.08 | 0.233 ± 0.019 | 1.33 ± 0.02 | 254.03 ± 1.01 |
| | **PEG 8000** | $[C_2mim]Cl$ | 89.69 ± 0.90 | 53.31 ± 0.61 | 0.134 ± 0.014 | 1.33 ± 0.02 | 160.58 ± 1.94 |
| | | $[C_6mim]Cl$ | 80.89 ± 0.84 | 60.96 ± 0.29 | 0.266 ± 0.012 | 1.49 ± 0.01 | 222.86 ± 2.02 |
| ***ii*** | **PEG 1500** | no IL | 83.93 ± 0.41 | 88.69 ± 0.13 | 0.175 ± 0.05 | 7.12 ± 0.08 | 73.05 ± 2.48 |
| | | $[C_6mim]Cl$ | 77.60 ± 0.19 | 94.31 ± 0.28 | 0.152 ± 0.02 | 8.90 ± 0.25 | 103.47 ± 1.23 |

Concerning *Route **i***, the *PF* achieved for the lipase from *Bacillus* sp. ITP-001 was increased from 175.6 ± 2.4 from the common ATPS (without any IL) to 245.0 ± 9.5 using the ATPS with [$C_6$mim]Cl. Additional tests were performed for the *Route **i*** purification approach regarding the variation on the polymer molecular size conjugated with [$C_2$mim]Cl and [$C_6$mim]Cl (Table II). Regarding the effect of the polymer molecular weight, the results seem to corroborate the data previously found in the optimization step by using the commercial CaLB, despite the fact that the purification results achieved for the lipase produced by *Bacillus* sp. ITP-001 are much more significant due to the completely different level of purity of the lipase samples (the fermentation broth has a higher content of contaminant proteins). The same tendencies of purification were already demonstrated in previous studies from our group, in which the same lipolytic enzyme (from *Bacillus* sp. ITP-001) was focused, but by applying simpler ATPS based in ILs and salts as separation agents, namely using 25 wt% of [$C_8$mim]Cl and 30 wt% of the same buffer (pH 7) used in this work. However, a new fact with utmost importance arises in this work, the use of ATPS composed of ILs as adjuvants allowed much higher purification factors (*Route **i***: *PF* = 245.0 ± 9.5,) when compared with the IL based ATPS for which Ventura et al. reported (*PF* = 51 ± 2) (Ventura et al., 2012) and the common polymeric ATPS (*PF* lower than 30, taking into account the differences in some conditions namely the pH) (Barbosa et al., 2011). Table III shows a comparison between the *PF* of all approaches previously described in the literature, with the results presented in this work for the purification of lipase from *Bacillus* sp. ITP-001.

As shown in the diagram of the purification process (Figure 5), two distinct routes were contemplated, one with a pre-purification step (*Route **i*** already discussed) and the other described without any pre-purification step, named *Route **ii***, being these results also presented in Table 2. These final tests were performed using the system PEG 1500 + $K_2HPO_4$/$KH_2PO_4$ + water without any IL, representing *PF* of 175.6 ± 2.4 and 73.1 ± 2.5 for Routes ***ii*** and ***i***, respectively, and with the presence of [$C_6$mim]Cl, which is represented by *PF* of 103.5 ± 1.2

and 245.0 ± 9.5 for *Routes* ***ii*** and ***i***, respectively. In this sense, the main results indicate that the lipase purification is reduced in about 40%, comparing *Routes* ***ii*** and ***i***. Although with values 40% smaller in the purification of lipase, the second approach of purification without pre-purification step may be considered as an alternative for downstream processes of industrial sectors which do not require a high degree of purification of the lipase, since this will be decreasing significantly the economic impact of the purification process.

**Table III:** Aqueous two-phase systems applied to purification of lipase from *Bacillus* sp. ITP-001.

| ATPS | | | Mass Fraction Composition / (wt%) | | | *PF* | Reference |
|---|---|---|---|---|---|---|---|
| ***w1*** | ***w2*** | ***w3*** | ***w1*** | ***w2*** | ***w3*** | | |
| PEG 1500 | $H_2HPO_4/KH_2PO_4$ | $[C_6mim]Cl$ | 15 | 15 | 5 | 245.0 | |
| PEG 8000 | $H_2HPO_4/KH_2PO_4$ | NaCl | 20 | 18 | 6 | 201.5 | Barbosa et al., 2011 |
| THF | [Ch][Bit] | - | 20 | 20 | - | 136.8 | Souza et al., (2014b) |
| THF | $H_2HPO_4/KH_2PO_4$ | - | 40 | 30 | - | 103.9 | Souza et al., (2014c) |
| $[C_8mim]Cl$ | $H_2HPO_4/KH_2PO_4$ | - | 25 | 30 | - | 51.0 | Ventura et al., 2012 |

## Conclusion

The formation of ATPS composed of different polyethylene glycols PEG 1500, 4000, 6000 and 8000 and various ILs as adjuvants (5 wt %) of the type $[C_nmim]Cl$ (n = 2, 4, 6, 8) was studied to analyze the effect of the IL cation alkyl side chain length and the molecular weight of PEGs, upon the purification of lipases. Quaternary systems were used for the purification of lipases, by starting with an optimization study and then applying the best systems (with the highest purification factors; *PF* = 5.22 ± 0.65) to the purification of a lipolytic enzyme produced in a submerged fermentation by the *Bacillus* sp. ITP-001. High purification factors were achieved (*PF* = 245.0 ± 9.5) regarding the purification of the lipase from *Bacillus* sp. ITP-001). These

higher purification factors were achieved, principally by the manipulation of the migration of the contaminant proteins by the competition of interactions acting as driven forces of the partitioning phenomena. The use of two different approaches regarding the process of purification of the lipolytic enzyme from the fermentation broth were tested and it was here proved that the use of ATPS using ILs as adjuvants has a tremendous advantage in terms of purification performance when compared with the common polymeric and IL based ATPS, even when no pre-purification steps are implemented.

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# Supporting Information

## Purification of lipase using ionic liquids as adjuvants in aqueous two-phase systems

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**Table A.1:** The volume ratio ($R$v) with the respective standard deviations and mass fraction compositions of the ATPS for CaLB purification at 25 (± 0.1) ºC and atmospheric pressure.

| ATPS | | Mass Fraction Composition / (wt%) ± $\sigma$ | | | | $R$v ± $\sigma$ |
|---|---|---|---|---|---|---|
| | | PEG | Salt | IL | CalB | |
| PEG 1500 | no IL | 15.02 ± 0.02 | 14.95 ± 0.04 | | 5.15 ± 0.05 | 0.93 ± 0.03 |
| | [$C_2$mim]Cl | 14.96 ± 0.03 | 14.62 ± 0.64 | 5.03 ± 0.05 | 5.16 ± 0.05 | 1.79 ± 0.02 |
| | [$C_4$mim]Cl | 15.05 ± 0.04 | 14.90 ± 0.10 | 5.05 ± 0.04 | 5.06 ± 0.10 | 1.83 ± 0.06 |
| | [$C_6$mim]Cl | 15.09 ± 0.04 | 15.15 ± 0.22 | 5.00 ± 0.01 | 5.14 ± 0.17 | 1.87 ± 0.01 |
| | [$C_8$mim]Cl | 15.05 ± 0.06 | 15.02 ± 0.06 | 5.03 ± 0.01 | 5.11 ± 0.15 | 1.54 ± 0.06 |
| PEG 4000 | no IL | 10.04 ± 0.04 | 13.01 ± 0.01 | | 5.16 ± 0.08 | 0.65 ± 0.03 |
| | [$C_2$mim]Cl | 10.05 ±0.06 | 13.04 ± 0.03 | 5.05 ± 0.06 | 5.11 ± 0.03 | 0.86 ± 0.02 |
| | [$C_4$mim]Cl | 10.04 ± 0.05 | 12.98 ± 0.04 | 5.10 ± 0.03 | 5.02 ± 0.02 | 0.85 ± 0.06 |
| | [$C_6$mim]Cl | 10.00 ± 0.03 | 12.99 ± 0.03 | 5.03 ± 0.04 | 5.19 ± 0.13 | 1.53 ± 0.04 |
| | [$C_8$mim]Cl | 10.01 ± 0.01 | 13.05 ± 0.07 | 5.04 ± 0.04 | 5.12 ± 0.03 | 0.82 ± 0.02 |
| PEG 6000 | no IL | 10.03 ± 0.03 | 12.03 ± 0.03 | | 5.07 ± 0.04 | 0.69 ± 0.01 |
| | [$C_2$mim]Cl | 10.00 ± 0.02 | 12.01 ± 0.01 | 5.05 ± 0.04 | 5.03 ± 0.02 | 0.73 ± 0.02 |
| | [$C_4$mim]Cl | 10.07 ± 0.09 | 12.06 ± 0.08 | 5.09 ± 0.05 | 5.06 ± 0.09 | 0.92 ± 0.01 |
| | [$C_6$mim]Cl | 10.04 ± 0.04 | 12.04 ± 0.03 | 5.08 ± 0.04 | 5.11 ± 0.12 | 1.16 ± 0.03 |
| | [$C_8$mim]Cl | 10.09 ± 0.05 | 12.06 ± 0.11 | 5.08 ± 0.09 | 5.08 ± 0.09 | 0.92 ± 0.02 |
| PEG 8000 | no IL | 10.07 ± 0.08 | 12.02 ± 0.06 | | 5.09 ± 0.09 | 0.63 ± 0.01 |
| | [$C_2$mim]Cl | 10.07 ± 0.08 | 12.03 ± 0.04 | 5.06 ± 0.05 | 5.05 ± 0.07 | 0.65 ± 002 |
| | [$C_4$mim]Cl | 10.05 ± 0.05 | 12.03 ± 0.01 | 5.12 ± 0.02 | 5.14 ± 0.09 | 0.85 ± 0.02 |
| | [$C_6$mim]Cl | 10.07 ± 0.06 | 12.05 ± 0.06 | 5.02 ± 0.01 | 5.03 ± 0.02 | 1.06 ± 0.03 |
| | [$C_8$mim]Cl | 10.08 ± 0.11 | 12.07 ± 0.04 | 5.09 ± 0.11 | 5.06 ± 0.08 | 0.79 ± 0.04 |

**Table A.2:** The volume ratio (*R*v) with the respective standard deviations and mass fraction compositions of the ATPS for purification of lipase from *Bacillus* sp. ITP-001, at 25 (± 0.1) ºC and atmospheric pressure**.**

| Route | ATPS | | Mass Fraction Composition / (wt%) ± $\sigma$ | | | | $R$v ± $\sigma$ |
|---|---|---|---|---|---|---|---|
| | | | PEG | Salt | IL | Solution of *Bacillus* | |
| ***i*** | PEG 1500 | no IL | 15.02 ± 0.02 | 14.95 ± 0.04 | | 70.03 ± 0.06 | 1.26 ± 0.01 |
| | | [$C_2$mim]Cl | 14.96 ± 0.03 | 14.62 ± 0.64 | 5.03 ± 0.05 | 65.40 ± 0.56 | 1.10 ± 0.01 |
| | | [$C_4$mim]Cl | 15.05 ± 0.04 | 14.90 ± 0.10 | 5.05 ± 0.04 | 65.01 ± 0.10 | 1.26 ± 0.08 |
| | | [$C_6$mim]Cl | 15.09 ± 0.04 | 15.15 ± 0.22 | 5.00 ± 0.01 | 64.76 ± 0.16 | 1.29 ± 0.04 |
| | | [$C_8$mim]Cl | 15.05 ± 0.06 | 15.02 ± 0.06 | 5.03 ± 0.01 | 64.90 ± 0.01 | 1.00 ± 0.00 |
| ***ii*** | PEG 1500 | no IL | 15.02 ± 0.03 | 15.07 ± 0.07 | | 69.05 | 1.09 ± 0.03 |
| | | [$C_6$mim]Cl | 15.10 ± 0.09 | 15.15 ± 0.16 | 5.16 ± 0.07 | 64.87 | 1.90 ± 0.05 |
| ***i*** | PEG 6000 | [$C_2$mim]Cl | 15.04 ± 0.02 | 15.05 ± 0.02 | 5.17 ± 0.01 | 64.77 ± 0.18 | 0.83 ± 0.02 |
| | | [$C_6$mim]Cl | 15.06 ± 0.02 | 15.02 ± 0.02 | 5.14 ± 0.04 | 64.98 ± 0.14 | 1.09 ± 0.02 |
| ***i*** | PEG 8000 | [$C_2$mim]Cl | 15.08 ± 0.08 | 15.13 ± 0.20 | 5.01 ± 0.01 | 64.79 ± 0.28 | 0.86 ± 0.02 |
| | | [$C_6$mim]Cl | 15.05 ± 0.07 | 15.08 ± 0.13 | 5.05 ± 0.04 | 64.90 ± 0.10 | 1.05 ± 0.06 |

**Table A.3:** Enzyme recovered in the bottom phase ($R_{EB}$), contaminating protein recovered in the top phase ($R_{PT}$), partition coefficient for contaminant protein ($K_P$), and enzyme ($K_E$), and purification factor (PF) with the respective standard deviations ($\sigma$). All ATPS were constituted with PEG + $K_2HPO_4/KH_2PO_4$ + IL (when present) + water + CaLB, at 25 (± 0.1) ºC and atmospheric pressure.

| ATPS | | $R_{EB} \pm \sigma$ | $R_{PT} \pm \sigma$ | $K_E \pm \sigma$ | $K_P \pm \sigma$ | PF ± $\sigma$ |
|---|---|---|---|---|---|---|
| PEG 1500 | no IL | 67.13 ± 0.08 | 78.39 ± 3.00 | 0.52 ± 0.01 | 3.96 ± 0.85 | 3.25 ± 0.51 |
| | [$C_2$mim]Cl | 60.12 ± 1.27 | 81.66 ± 2.22 | 0.37 ± 0.02 | 2.49 ± 0.44 | 2.55 ± 0.35 |
| | [$C_4$mim]Cl | 56.53 ± 0.86 | 87.61 ± 0.005 | 0.42 ± 0.07 | 3.90 ± 0.14 | 3.45 ± 0.08 |
| | [$C_6$mim]Cl | 51.68 ± 0.003 | 92.49 ± 0.83 | 0.50 ± 0.05 | 6.79 ± 0.99 | 5.22 ± 0.65 |
| | [$C_8$mim]Cl | 67.33 ± 3.02 | 72.21 ± 7.97 | 0.38 ± 0.04 | 2.08 ± 0.31 | 2.24 ± 0.29 |
| PEG 4000 | no IL | 72.62 ± 1.49 | 66.02 ± 0.79 | 0.58 ± 0.02 | 2.96 ± 0.08 | 2.51 ± 0.02 |
| | [$C_2$mim]Cl | 65.34 ± 2.58 | 68.56 ± 0.01 | 0.62 ± 0.07 | 2.54 ± 0.01 | 2.19 ± 0.09 |
| | [$C_4$mim]Cl | 65.91 ± 3.87 | 62.00 ± 0.57 | 0.61 ± 0.09 | 1.85 ± 0.02 | 1.78 ± 0.09 |
| | [$C_6$mim]Cl | 56.50 ± 1.92 | 70.17 ± 0.01 | 0.50 ± 0.04 | 1.53 ± 0.01 | 1.69 ± 0.04 |
| | [$C_8$mim]Cl | 69.83 ± 0.29 | 27.51 ± 0.49 | 0.53 ± 0.02 | 0.46 ± 0.01 | 0.96 ± 0.01 |
| PEG 6000 | no IL | 72.24 ± 0.48 | 65.19 ± 0.60 | 0.55 ± 0.01 | 2.66 ± 0.07 | 2.38 ± 0.04 |
| | [$C_2$mim]Cl | 72.07 ± 0.65 | 55.35 ± 4.96 | 0.54 ± 0.02 | 1.71 ± 0.33 | 1.63 ± 0.03 |
| | [$C_4$mim]Cl | 63.89 ± 2.72 | 48.79 ± 0.15 | 0.57 ± 0.07 | 1.04 ± 0.01 | 1.26 ± 0.06 |
| | [$C_6$mim]Cl | 63.77 ± 1.87 | 47.60 ± 0.65 | 0.47 ± 0.02 | 0.78 ± 0.01 | 1.20 ± 0.02 |
| | [$C_8$mim]Cl | 72.01 ± 2.67 | 23.62 ± 0.09 | 0.39 ± 0.05 | 0.34 ± 0.01 | 0.94 ± 0.04 |
| PEG 8000 | no IL | 77.13 ± 3.19 | 56.96 ± 0.60 | 0.48 ± 0.10 | 2.09 ± 0.01 | 2.10 ± 0.15 |
| | [$C_2$mim]Cl | 76.61 ± 0.48 | 69.84 ± 0.75 | 0.46 ± 0.01 | 3.54 ± 0.03 | 3.12 ± 0.01 |
| | [$C_4$mim]Cl | 69.95 ± 0.39 | 61.73 ± 0.58 | 0.51 ± 0.02 | 1.91 ± 0.02 | 1.93 ± 0.03 |
| | [$C_6$mim]Cl | 67.72 ± 0.59 | 25.38 ± 0.44 | 0.45 ± 0.01 | 0.32 ± 0.05 | 0.91 ± 0.02 |
| | [$C_8$mim]Cl | 77.23 ± 3.53 | 12.23 ± 0.62 | 0.38 ± 0.06 | 0.18 ± 0.01 | 0.85 ± 0.04 |

# Capítulo IV

## CONSIDERAÇÕES FINAIS

As lipases têm atraído uma especial atenção por catalisar com extrema eficiência e especificidade diversas reações de interesse industrial, entretanto a comercialização e a produção em escala industrial destas enzimas dependem significativamente das técnicas empregadas na purificação. Para viabilizar esta demanda, neste trabalho foram desenvolvidas novas abordagens para os processos de separação e/ou purificação de enzimas lipolíticas utilizando a técnica de extração líquido-líquido por meio dos SABs.

Os novos SABs foram avaliados a partir de uma variedade de compostos capazes de induzir a formação de duas fases aquosas. Uma dessas abordagens incluiu a formação de novos sistemas ternários à base de solvente orgânico (tetrahidrofurano), sais e líquidos iônicos formados pelo cátion colina. Também foram avaliados sistemas quaternários utilizando líquido iônico como um adjuvante em SABs à base de PEG + sal. Para todos estes sistemas foram determinados os seus diagramas de fases e os parâmetros empíricos que correlacionam os pontos experimentais de equilíbrio termodinâmico. Além disso, os mecanismos que conduziam à formação de duas fases aquosas imiscíveis também foram discutidos.

A partir dos resultados de separação e/ou purificação das lipases os desempenhos destes novos sistemas foram avaliados. Com o objetivo de substituir a fase rica em polímero por um solvente orgânico, o THF foi pela primeira vez utilizado para formar SABs com a adição do tampão fosfato de potássio a pH 7 ou de LIs à base de colinas. Nem sempre a utilização de enzimas em meios contendo solventes orgânicos são atraentes, em alguns casos o solvente orgânico pode desnaturar as enzimas. Em função desta problemática foi mostrado que o THF não causa efeito deletério para a lipase, e portanto pode ser utilizado como constituinte de SABs. Ao avaliar os coeficientes de partição da lipase de *Burkholderia cepacia* em sistemas formados por THF + sal, observou-se que as enzimas são preferencialmente migradas para a fase rica em sal, devido sua capacidade de hidratação induzindo a biomolécula para a fase salina à medida que a *tie-line* do sistema é aumentada. Posteriormente estes sistemas foram capazes de purificar a lipase produzida a partir de uma cepa de *Bacillus* sp. ITP-001 via fermentação submersa, embora sem alcançar à homogeneidade.

Uma classe de LIs mais sustentáveis (LI à base de sais de colinas) foram utilizados para formar SABs com tetrahidrofurano. Após estudos de otimização utilizando a lipase de *Burkholderia cepacia* como modelo de purificação da lipase extracelular, foi possível determinar as condições de dependência para uma ajustada separação. A capacidade de separação destes sistemas foi relacionada à habilidade do [Ch][Bit] em separar a lipase modelo, ao qual foi escolhido para a purificação da lipase extracelular de *Bacillus* sp. ITP-001.

Os SABs utilizando LIs como adjuvante, como uma alternativa menos dispendiosa frente aos sistemas formados exclusivamente por uma fase rica em LI foram considerados os melhores sistemas para purificar a lipase extracelular de *Bacillus* sp. ITP-001. O uso do líquido iônico $[C_6mim]Cl$ como adjuvante em SABs à base de PEG 1500/tampão fosfato de potássio (pH 7) foi selecionado como o melhor LI. Vale ressaltar que a purificação da lipase B de *Candida antarctica* (fornecida pela empresa *Novozymes*) também foi avaliada para estes sistemas quaternários em particular, apresentando valores superiores de purificação ao comparar com SABs à base de LI/sal descritos na literatura (VENTURA *et al.,* 2011). A eficiência destes sistemas contendo LIs em pequenas quantidades (5%, m/m) foi associada à capacidade em manipular a partição das proteínas contaminantes para a fase rica em PEG (oposto a das enzimas), em função principalmente de um ajustado balanço de interações entre LIs – PEG – proteínas. A compreensão destas interações foi apoiada pelo estudo da partição de dois corantes com cargas elétricas diferentes, mostrando que não somente interações hidrofóbicas são importantes para controlar a migração, mas também interações eletrostáticas, forças de van der Waals e ligações de hidrogênio ajustado pela alteração do comprimento da cadeia alquílica do LI utilizado como adjuvante.

Os sistemas formados por THF + sal ou LIs à base de colinas foram aplicados com sucesso para purificação da lipase de *Bacillus* sp. ITP-001, embora com resultados menos promissores comparados aos SABs à base de PEG/sal + $[C_6mim]Cl$. Na Tabela 7, é apresentado todos os resultados relacionados com a purificação da lipase de *Bacillus* sp. ITP-001 utilizando SABs descritos na literatura.

Tabela 7: Sistemas aquosos bifásicos aplicados à purificação da lipase de *Bacillus* sp. ITP-001.

| SAB | | | Fração mássica (m/m) | | | Fator de purificação (vezes)* | Referência |
|---|---|---|---|---|---|---|---|
| *w*1 | *w*2 | *w*3 | *w*1 | *w*2 | *w*3 | | |
| PEG 1500 | $H_2HPO_4/KH_2PO_4$ | $[C_6mim]Cl$ | 15 | 15 | 5 | 245,0 | - |
| PEG 8000 | $H_2HPO_4/KH_2PO_4$ | NaCl | 20 | 18 | 6 | 201,5 | (BARBOSA *et al.,* 2011) |
| THF | [Ch][Bit] | - | 20 | 20 | - | 130,1 | - |
| THF | $H_2HPO_4/KH_2PO_4$ | - | 40 | 30 | - | 103,9 | - |
| $[C_8mim]Cl$ | $H_2HPO_4/KH_2PO_4$ | - | 25 | 30 | - | 51,0 | (VENTURA *et al.,* 2012a) |

* *condições otimizadas.*

A eficácia para a purificação da lipase de *Bacillus* sp. ITP-001 utilizando os sistemas à base de LIs como adjuvante a partir de PEG + sal, foi em função das capacidades de interações adicionais. Além disso, estes resultados são promissores comparado aos sistemas à base de PEG + sal estudados por Barbosa et al., 2011, uma vez que utilizando PEG de menor massa molecular (PEG 1500) é possível propiciar ao processo uma menor viscosidade e um menor custo energético, vale ressaltar ainda que, menos concentrações dos constituintes foram necessários para uma melhor purificação da lipase. Em resumo, os resultados obtidos neste trabalho sugerem que estes sistemas podem ser aplicados para a purificação de diferentes lipases, não descartando a aplicação dos sistemas à base de THF ou sais de colinas, pois estes apresentaram boa capacidade para purificação, biocompatibilidade para as lipases estudadas, e são formados por constituintes considerados de baixo custo e de baixa viscosidade. Neste contexto, estes novos SABs podem ser vistos como processos alternativos à serem aplicados para a purificação de lipases viabilizado por indústrias farmacêuticas ou biotecnológicas.

## 4.1. Trabalhos futuros

Os SABs estudados neste trabalho apresentaram propriedades interessantes para serem aplicados não só para enzimas lipolíticas, mas também para uma grande variedade de compostos. Além disso, de uma forma geral, limitações associadas a propriedades físico-químicas dos constituintes limitam sua aplicação em escala industrial. Desta forma, alguns estudos adicionais devem ser considerados a fim de promover e explorar os sistemas aqui propostos:

- Avaliar outros solventes orgânicos miscíveis em água para a formação de SABs, tais como o dioxino e etanol.
- Avaliar outros LIs como adjuvantes para um melhor ajuste de purificação.
- Purificação de outras enzimas produzidas a partir de micro-organismos, tais como proteases e peroxidases.
- Purificação de compostos farmacêuticos, tais como antibióticos e antiflamatórios.
- Conduzir estes sistemas em operação contínua.
- Estudar a possibilidade de reutilização dos compostos que formam o SAB.
- Avaliar outros métodos de purificação envolvendo sistemas líquido-líquido, tais como sistema micelar.

## 4.2. Trabalhos apresentados

Apresentação de trabalhos em eventos científicos:

- X CICECO Meeting 2013.
- Summer School of the Galician Network of Ionic Liquids (REGALIS), 2013
- 5th Congress on Ionic Liquids - COIL 2013,
- XIX Simpósio Nacional de Bioprocessos, X Simpósio de Hidrólise Enzimática de Biomassa – SINAFERM 2013.
- XI Seminário Brasileiro de Tecnologia Enzimática - ENZITEC 2014.
- 2nd International Conference on Ionic Liquids in Separation and Purification Technology – ILSEPT 2014.

Publicações em revistas científicas internacionais:

- Effect of ionic liquids as adjuvants on PEG-based ABS formation and the extraction of two probe dyes. *Fluid Phase Equilibria*, v. 1, p. 1-24, 2014.

Participação em trabalhos:

- Partitioning and purification of capsaicin using aqueous two-phase systems based on sodium salts. Trabalho de conclusão de curso da aluna Nancy E. C. Cienfuegos. Em submissão ao periódico *Separation Science and Technology*.

- Ionic liquid-based aqueous biphasic systems as a versatile tool for the recovery of antioxidant compounds. Trabalho de conclusão de curso do aluno João Santos. Aceito para publicação no *Journal of Chemical Technology & Biotechnology*, 2014.

Prêmios

- Prêmio FAPITEC/SE de Divulgação Científica e Inovação Tecnológica, 2014 – Modalidade Pesquisador Júnior.

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