UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
CURSO DE ENGENHARIA DE ALIMENTOS

COMPARAÇÃO ENTRE AGITAÇÃO MECÂNICA E ULTRASSÔNICA NA SÍNTESE DE ÉSTERES DE AROMAS CATALISADA POR LIPASE

Andréa Bercini Martins

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Trabalho de Conclusão de Curso apresentado à Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Engenheiro de Alimentos.

Orientador: Prof. Dr. Rafael Costa Rodrigues

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EPÍGRAFE

"Viva como se você fosse morrer amanhã. Aprenda como se você fosse viver para sempre"

Mahatma Gandhi

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RESUMO

Ésteres de cadeia curta são compostos de aroma encontrados naturalmente em diversas frutas, obtidos por extração através de processos físicos. Contudo, esse método é muito escasso e caro para ser aplicado comercialmente. A legislação atual define como "aromas naturais" aqueles obtidos através de processos físicos ou rotas biotecnológicas. Lipases são enzimas que, sob apropriadas condições de trabalho, tem mostrado serem bons catalisadores nas reações de esterificação. Assim, neste trabalho, estudou-se o efeito do método de agitação sobre os parâmetros da reação de esterificação para síntese de acetato de butila (aroma de maçã), assim como sobre a estabilidade da enzima em ácido acético e na sua reciclagem, por reutilizações múltiplas. Um delineamento composto central e a metodologia de superfície de resposta foram utilizados para analisar os efeitos dos parâmetros de reação (temperatura, razão molar de substratro, quantidade de enzima e água adicionada) e suas respostas. As condições ótimas para o método de agitação mecânica foram: temperatura 40 ºC; razão molar do substrato 3:1 butanol:ácido acetico; quantidade de enzima 7,5 %; água adicionada 0,25 % da massa de substrato. Sob essas condições, mais de 90 % de conversão foi obtida em 2,5 h. As condições ótimas usando o ultrassom foram: temperatura 46 ºC; razão molar do substrato 3,6:1 butanol:ácido acetico; quantidade de enzima 7 %; água adicionada 0,25 % da massa de substrato. Sob essas condições, 94 % de conversão foi obtido em 2,5 h. A concentração ótima de ácido acético foi determinada como sendo 2,0 M, em comparação com 0,3 M sem ultrassom. A produtividade da enzima também foi significativamente melhorada quando compara-se ultrassom e a agitação mecânica (cerca de 7,5 vezes por ciclo). O biocatalisador pôde ser reutilizado diretamente durante 14 ciclos de reação, mantendo cerca de 70 % de sua atividade original (enquanto que a atividade foi reduzida no terceiro ciclo usando a agitação mecânica). Assim, em comparação com a tecnologia mecânica tradicional a agitação ultrassônica, além de melhorar a produtividade do processo, também aumentou a capacidade de reutilização da enzima e a sua estabilidade em ácido acético, sendo uma ferramenta poderosa para melhorar o desempenho do biocatalisador na presente reação.

Palavras-chave: Acetato de butila, Ésteres de aroma, Novozym 435, Ultrassom, Reuso da enzima.

ABSTRACT

Short chain esters are flavor compounds found naturally in various fruits, these can be extracted by physical processes. However this method is very scarce and expensive to be commercially applied. Nowadays, legislation defines as "natural flavors" those obtained by physical processes, microbiological or biotechnological routes. Lipases are enzymes that, under appropriate conditions, have shown to be a good catalyst in esterification reactions. It was studied the effect of the method of agitation on the optimal reaction parameters, as well as on the stability of the enzyme on acetic acid and the enzyme reusability for multiple reuses. A central composite design and the response surface methodology were used to analyse the effects of the reaction parameters (temperature, substrate molar ratio, enzyme content and added water) and their response. The optimum conditions for butyl acetate synthesis using the mechanical agitation method were: temperature 40 °C; substrate molar ratio 3:1 butanol:acetic acid; enzyme content 7.5 % by mass fraction of substrate wt.; added water 0.25 % by mass fraction of substrate wt.. Under these conditions, over 90 % of conversion was obtained in 2.5 h. The optimal reaction conditions for ultrasound-assisted butyl acetate synthesis were: temperature 46 °C; substrate molar ratio 3.6:1 butanol:acetic acid; enzyme content of 7 % by mass fraction of substrate wt.; added water 0.25 % by mass fraction of substrate wt., slightly different to those found using mechanical agitation. Under these conditions over 94 % of conversion was obtained in 2.5 h. The optimal acid concentration for the reaction was determined to be 2.0 M, compared to 0.3 M without ultrasounds. Enzyme productivity was also significantly improved when comparing ultrasound and standard stirring systems (around 7.5-fold by batch). The biocatalyst could be directly reused for 14 reaction cycles keeping around 70 % of its original activity (while activity was reduced to zero percent in the third cycle using standard stirring systems). Thus, compared to the traditional mechanical agitation, ultrasound technology besides improving the process productivity also enhanced the enzyme reusability, and stability in acetic acid being. Ultrasound technology has shown to be a powerful tool to improve biocatalyst performance in this reaction.

Keywords: Butyl acetate, Flavor esters, Novozym 435, Ultrasound, Enzyme reuse.

1. INTRODUÇÃO

Aromas são substâncias amplamente usadas na indústria de alimentos como aditivos alimentares. Estes são aplicados com o objetivo de melhor as características organolépticas dos alimentos. Dentre essas susbtâncias, alguns compostos químicos se destacam como é o caso dos ésteres, os terpenos, as lactonas, os ácidos graxos, as pirazinas, por contribuirem na formação de aromas em alimentos. Em relação aos

ésteres, os de baixo peso molecular são comumente usados na fabricação de aromas e fragâncias por causa do seu odor frutado (ALVAREZ-MACARIE e BARATTI, 2000).

A esterificação é uma reação que ocorre entre um ácido e um álcool formando éster e água. Essa reação em condições normais é lenta, mas o uso de biocatalisadores, como as lipases, aumenta a velocidade da reação (RAJENDRAN *et al.*, 2009). Estudos sobre a capacidade das enzimas em catalisar reações de síntese tem sido realizados devido as vantagens que esse método propicia frente aos métodos convencionais (SERRA *et al.*, 2005). Os esteres obtidos por métodos enzimáticos são considerados pela legislação atual como “aromas naturais".

A reação enzimática de esterificação é realizada comumente através da mistura do ácido e álcool, correspondentes ao aroma que se deseja produzir, adicionando-se a enzima e mantendo sob agitação. Dentre os métodos de agitação, o mais utilizado é a agitação mecânica, devido ao seu baixo custo e eficiência. Contudo, diferentes métodos têm sido estudados em busca de alternativas que aumentem o efeito positivo da homogeneização do meio reacional.

Neste contexto, a energia ultrassônica tem se mostrado uma alternativa tecnológica e economicamente viável para aplicações em biotecnologia (SINISTERRA, 1992). Ela é considerada uma tecnologia "verde" por causa de sua alta eficiência, o seu desempenho econômico e baixos requisitos de instrumentais. Essas características reduzem significativamente o tempo de processamento em comparação com outras técnicas convencionais de agitação, como é o caso da agitação mecânica (ROKHINA *et al.*, 2009).

Os aromas influenciam diretamente no sabor dos alimentos. Atualmente, em meio a um mercado tão competitivo e com grande diversidade de opções, a indústria precisa estar preparada e apresentar inovações, pois são os diferenciais que vão auxiliar no sucesso e na aceitação de um produto por parte do consumidor.

Além disso, observa-se um aumento do conhecimento dos consumidores sobre as características nutricionais e os efeitos à saude vinculados ao consumo de determinados ingredientes e insumos utilizados nos produtos industrializados (POLÔNIO e PERES, 2009). Nesse contexto, os aditivos naturais têm recebido especial atenção por parte destes consumidores, uma vez que passam a idéia de produto

saudável, menos “industrializado”, enquanto os aditivos sintéticos e artificiais apresentam um caráter negativo. Todos esses fatores têm estimulado as empresas alimentícias a buscar por ingredientes naturais. Logo é de suma importância a inovação não somente de novos aromas, mas sim de novas tecnologias para seu desenvolvimento.

As tendências à substituição de aditivos que são considerados artificiais, na produção de alimentos industrializados, assim como o crescente uso da biotecnologia, criaram um nicho na indústria de aditivos: ésteres de aromas por síntese enzimática. Desta forma, esse trabalho tem como **objetivo geral** investigar a influência de dois diferentes métodos de agitação na reação de esterificação para síntese de acetato de butila, catalisada pela lipase B de *Candida antarctica*, imobilizada comercialmente, disponível com o nome de Novozym 435, e como **objetivos específicos**:

- Otimizar a reação de síntese do éster acetato de butila,
- Avaliar a influencia e a interação entre os parâmetros de reação: temperatura, relação molar do substrato, quantidade de enzima e de água adicionada,
- Estudar o efeito do método de agitação sobre os parâmetros reacionais ótimos,
- Avaliar a estabilidade da enzima em ácido acético e
- Avaliar a estabilidade operacional da enzima em múltiplos reusos.

Este trabalho está composto por diferentes partes. A revisão bibliográfica aborda diversos tópicos relevantes para a compreensão dos temas. Os materiais e métodos, e resultados e discussão serão apresentados na forma de artigos científicos. Por fim, apresentam-se considerações finais e sugestões para trabalhos seguintes.

2. REVISÃO BIBLIOGRÁFICA

2.1. Aromas

Aromatizantes são substâncias amplamente utilizadas na indústria de alimentos como aditivos alimentares. Segundo a Portaria nº 540, de 27 de outubro de 1997, entende-se por aditivo alimentar "qualquer ingrediente adicionado intencionalmente aos alimentos, sem propósito de nutrir, com o objetivo de modificar as características físicas, químicas, biológicas ou sensoriais, durante a fabricação, processamento, preparação, tratamento, embalagem, acondicionamento, armazenagem, transporte ou manipulação de um alimento". Os aditivos são usados para melhorar as características organolépticas do produto e assim torná-los mais atrativos para o consumidor, mas também por possuírem características tecnológicas e de conservação, aumentando a estabilidade microbiológica e a vida de prateleira do produto (DAMONDARAM, 2010).

A RDC nº 2, de 15 de janeiro de 2007, caracteriza os aromatizantes como "substâncias ou misturas de substâncias com propriedades odoríferas e/ou sápidas, capazes de conferir ou intensificar o aroma e/ou sabor dos alimentos". Essa mesma portaria classifica os aromatizantes como naturais ou sintéticos. São considerados como aromas naturais aqueles obtidos através de métodos físicos, microbiológicos ou enzimáticos, e como sintéticos os sintetizados por processos químicos. As misturas de aromas, os aromas de reação ou de transformação e os de fumaça serão considerados naturais ou sintéticos, dependendo da natureza de suas matérias-primas ou dos processos de elaboração. Os aromatizantes podem ser comercializados na forma sólida (pós, granulados ou tabletes), líquida (soluções ou emulsões) ou pastosa. A Figura 1 mostra o esquema de classificação dos aromas, segundo a legislação vigente.

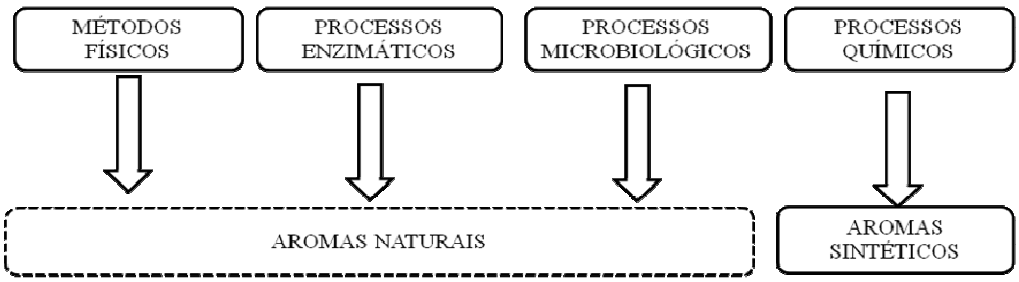

Figura 1 – Esquema da classificação dos aromas

Os aromas são produtos químicos orgânicos altamente voláteis, uma vez que possuem baixo peso molecular, já que são compostos por moléculas de cadeia curta (BICAS *et al.*, 2010). Tais compostos são os princípios sensoriais de muitos produtos e podem ser responsáveis pela criação de novos sabores, além de reforçar, substituir ou mascarar os aromas já presentes (BERGER, 2009).

A maior parte dessas substâncias pode ser classificada de acordo com as suas estruturas químicas. Alguns destes grupos são: lactonas, terpenos, ésteres, ácidos graxos, e pirazinas (LONGO e SANROMÁN, 2006).

As lactonas são ésteres cíclicos de hidroxiácidos (Figura 2) e são encontrados em diversos alimentos, contribuindo para o gosto e sabor em frutas, coco, manteiga e cremes. Contudo, podem ser responsáveis por odores indesejáveis como o de ranço em alguns alimentos. Embora a síntese química ainda seja o método mais prático de produção, este composto pode ser naturalmente sintetizado por fungos como: *Tyromyces sambuceus* e *Cladosporium suaveolens*, e por leveduras como *Candida tropicalis* e *Yarrowia lipolytica* (JONG e BIRGMINGHAM, 1993).

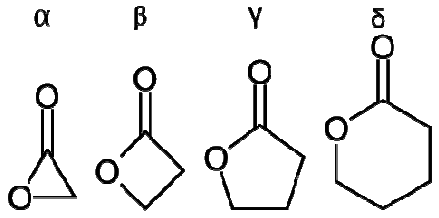

Figura 2 – Lactonas

Os terpenos (Figura 3) são muito comuns na natureza, principalmente em plantas como constituintes de óleos essenciais. Geralmente, são obtidos por processos físicos (destilação por arraste com vapor de água, destilação a pressão reduzida ou outro método adequado de extração). Tais compostos são hidrocarbonetos, compostos de unidades de isopropeno, e podem ser cíclicos, com cadeia aberta, saturado ou insaturado e oxidados (LONGO e SANROMÁN, 2006). A maioria dos terpenos podem ser obtidos de culturas microbianas, sintetizados por fungos que pertencem as espécies: ascomycetes e basidiomicetos, como a *Ceratocystis*, que vem sendo objeto de intensiva

pesquisa. Contudo, esforços significativos têm sido feitos no estudo de enzimas relacionadas com a biossíntese dos terpenos (BICAS *et al.*, 2010).

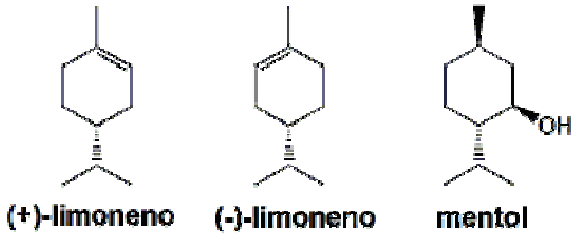

Figura 3 – Terpenos

Os ésteres são muito utilizados como aromatizantes, sendo apreciados pelo aroma frutado que estes fornecem. Eles são utilizados em produtos que possuem sabor de frutas (bebidas, geléias, doces), mas também encontram-se aplicados em produtos lácteos, como os iogurtes. Produzidos como os primeiros aromas sintéticos há um século, estes compostos também podem ser sintetizados através de rotas microbiológicas e enzimáticas (JONG e BIRGMINGHAM, 1993).

Outro grupo de aromas são os ácidos carboxílicos, as bactérias, que são capazes de oxidar álcoois para o correspondente ácido, despertam interesse para a produção dos mesmos. Os ácidos carboxílicos são relativamente baratos e de grande importância para a indústria de aromas, seja pelo seu intenso aroma ou como substrato para as reações enzimáticas de síntese de ésteres de aromas. Dentre estes pode-se citar o ácido butírico e o ácido propiônico (SCHRADER *et al.*, 2004).

Pirazinas são compostos heterocíclicos contendo nitrogênio que são conhecidos pelo aroma típico em alimentos tostados. Normalmente são formados através de reações químicas como o escurecimento não enzimático (Reação de Maillard), que ocorre quando, aquecidos acima de 100 ºC, um grupo carbonila e um grupo amilo condensam formando diferentes compostos. As pirazinas têm sido isolados de vegetais e associados a características sensoriais de alimentos como: pipoca, batatas, pimentão (JONG e BIRGMINGHAM, 1993). Com a atual demanda por produtos pré-prontos, a quantidade de alimentos que necesitam apenas de aquecimento por microondas vem aumentando exponencialmente. Assim para esses alimentos, que não passam pelo processo de

cozimento, não favorecendo a formação de pirazinas naturais, tem-se a necessidade de usar essas substâncias como aditivos (LONGO e SANROMÁN, 2006).

Segundo dados da Associação Brasileira da Indústria e Comércio de Ingredientes e Aditivos para Alimentos (ABIAM), os aromatizantes são responsáveis por grande parte do faturamento do setor. Estima-se que, em 2008, o mercado de aditivos atingiu cerca de dois bilhões de reais, sendo os aromas responsáveis por cerca de 40 % a 50 % deste montante (ABIAM, 2008). Mundialmente, os aromatizantes representam 25 % do total do mercado de aditivos, e essa porcentagem vem aumentando a cada ano, com faturamentos que passam de sete bilhões de dólares ao ano (DUBAL *et al.*, 2008).

Cada produto tem o seu aroma típico, contudo não é apenas uma substância volátil que o representa, mas sim o resultado da combinação de dezenas dessas substâncias, com diferentes propriedades físico-químicas. Entretanto, em alguns alimentos existe um ou mais componentes que sozinhos lembram a identidade do produto (VEGA e FLORENTINO, 2000). Além disso, são neutros em relação à base podendo ser usados para diferentes fins, como é o caso do aroma de pêssego, por exemplo, que pode tanto ser usado em iogurtes, quanto em amaciantes para roupas.

Encontra-se na literatura o desenvolvimento de métodos para medir emoções associadas com os alimentos (KING e MEISELMAN, 2010; CARDELLO *et al.*, 2012; KING *et al.*, 2013). Os autores King *et al.* (2013) abordam através de questionários e diversos testes, o estudo das emoções provocadas em um grupo de consumidores e observam que o contexto: nome do aroma, o aroma e o *flavor*, tiveram considerável efeito na resposta emocional dos consumidores.

2.2. Ésteres de aromas

Ésteres de ácidos graxos de cadeia curta são comumente usados na fabricação de aromas e fragâncias por causa do seu odor frutado (ALVAREZ-MACARIE e BARATTI, 2000). A Tabela 1 apresenta alguns exemplos de ésteres e seus respectivos aromas.

Tabela 1 – Ésteres sintetizados por esterificação enzimática

Éster	**Aroma**	**Referência**
Acetato de isoamila	Banana	TORRES *et al.*, 2009
Butirato de isoamila	Banana	POLAINA *et al.*, 2007
Propionato de isoamila	Banana	POLAINA *et al.*, 2007
Acetato de butila	Maçã	OZYILMAZ e GEZER, 2010
Butirato de butila	Abacaxi	POLAINA *et al.*, 2007
Valerato de etila	Maçã verde	OZYILMAZ e GEZER, 2010
Acetato de hexila	Pera	HORCHANI *et al.*, 2012
Acetato de 2-feniletil	Rosas	KUO *et al.*, 2012

Estes compostos podem ser provenientes de métodos tradicionais que incluem processos físicos (extração direta da sua fonte natural) ou processos químicos. Contudo, o primeiro método é caro, dispendioso e não ecológico. Essas características negativas devem-se ao fato de que são encontrados em baixas concentrações nos produtos de interesse, dependem de fatores externos de difícil controle (como as mudanças nas condições climáticas) e o processo de extração produz elevadas quantidades de resíduos químicos. Já os aromas produzidos pelo segundo método não podem ser classificados como naturais, sendo classificados como artificiais e assim são menos apreciados pelos comsumidores (ALVAREZ-MACARIE e BARATTI, 2000).

Portanto, a procura por métodos não convencionais tem estimulado a indústria dos aromas a buscar por alternativas tecnológicas, junto a isso tem se o crescente interesse dos consumidores por processos ecológicos e a preferência por produtos "naturais". Esses fatores propiciam o desenvolvimento de um grande nicho no mercado: aromas de origem biotecnológica (DUBAL *et al.*, 2008), já que legislação atual classifica os aromas obtidos por este método de síntese, como naturais (BRASIL, 2007). Uma das principais vias alternativas é o uso de lipases como catalisadores da reação de esterificação para a obtenção de aromas naturais.

2.3. Lipases

Lipases são biocatalisadores versáteis que são usados nas reações de hidrólises de óleos e gorduras. Entretanto, sob condições reacionais específicas, também são capazes de catalisar as reações de esterificação, transesterificação e interesterificação (RAJENDRAN *et al.*, 2009). Devido a sua versatilidade de aplicações, essas enzimas são usadas para diversos propósitos biotecnológicos, como catalisadores na reação de síntese de materiais biopoliméricos (YU *et al.*, 2012), na produção de biodiesel (FAN *et al.*, 2012), na indústria de alimentos, na indústria de papel e em diversas sínteses químicas (SHARMA *et al.*, 2001).

Essas enzimas podem ser facilmente sintetizadas por diversos tipos de micro-organismos. Enzimas obtidas a partir de fungos e bactérias tem um grande potencial como biocatalisadores industriais, uma vez que estes são fáceis de serem produzidos por fermentação e são de fácil purificação (POLAINA *et al.*, 2007). A maioria das lipases utilizadas como catalisadores em sínteses orgânicas são de origem microbiana, como *Candida rugosa, Pseudomonas fluorescens, Rhizopus oryzae, Burkholderia cepacia, Aspergillus niger, Thermomyces lanuginosus,* e *Rhizomucor miehei* (AL-ZUHAIR, 2007).

A utilização de lipases apresenta diversas vantagens como o fato de não necessitarem de condições extremas de reação, apresentarem alta eficiência catalítica, alto grau de especificidade e a capacidade de acelerar reações químicas específicas sem a formação de subprodutos indesejáveis. Estes são aspectos que contribuem para o emprego acelerado desses biocatalisadores em todos os campos da indústria (HASAN *et al.*, 2006). Assim, observa-se que o mercado mundial de enzimas movimenta cerca de um bilhão de dólares por ano (DUBAL *et al.*, 2008).

Estas enzimas são muito estáveis em solventes orgânicos, proporcionando uma condição de baixa atividade de água, necessária na reação de esterificação (JAEGER e EGGERT, 2002). Em particular, a aplicação dessas enzimas para a síntese de ésteres de

cadeia curta tem atraído o interesse de uma ampla variedade de campos industriais, como o de alimentos, o farmacêutico e de cosméticos (TORRES *et al.*, 2009).

Algumas lipases comerciais se destacam por sua eficaz capacidade em catalisar reações de esterificação, como é o caso das enzimas: Novozym 435 e Lipozyme RM-IM. Ambas são distribuídas internacionalmente pela empresa Novo Nordisk, Dinamarca. A primeira é a isoforma B da lipase de *Candida antarctica* e imobilizada em uma resina macroporosa. Já a segunda é a lipase de *Rizomucor miehei* e imobilizada em uma resina de troca aniônica (YAHYA *et al.*, 1998). Diversos autores relatam a eficiência dessas duas enzimas (GUBICZA *et al.*, 2000; CHIANG *et al.*, 2003; CHANG *et al.*, 2006; BEZBRADICA *et al.*, 2007; MARTINS *et al.*, 2011; FRIEDRICH *et al.*, 2012; GRAEBIN *et al.*, 2012; LORENZONI *et al.*, 2012).

2.4. Enzimas imobilizadas

Várias estratégias têm sido utilizadas para melhorar o desempenho das lipases em solventes orgânicos. Entre elas encontram-se a otimização do solvente, o controle do teor de água pelo uso de peneiras moleculares, a engenharia de proteínas e a imobilização de enzimas (DEBULIS e KLIBANOV, 1993).

Um dos principais problemas em utilizar enzimas livres em meios orgânicos é a desnaturação e, também, a agregação das enzimas, que impede a homogeneidade do meio reacional, pois estas se aderem às paredes do reator. Este problema pode ser resolvido através da utilização de enzimas na forma imobilizada (OZYILMAZ e GEZER, 2010). Por isso observa-se um crescente aumento na utilização da técnica de imobilização de enzimas dentro do campo da biotecnologia aplicada.

As enzimas imobilizadas aumentam o número de moléculas por unidade de área, aumentando a eficiência da reação. Além disso, as vantagens incluem também a reutilização da enzima, permitindo o uso contínuo; o aumento das propriedades cinéticas, melhorando controle do processo catalítico; o aumento da estabilidade; eliminação da contaminação microbiana mesmo as enzimas de microrganismos não-"GRAS" e um produto livre de enzima (POLAINA *et al.*, 2007; GARCIA-GALAN *et al.*, 2011).

A imobilização das lipases tem sido feita por vários métodos. Esses podem ser classificados em duas categorias básicas: imobilização por ligação em suportes e encapsulamento (BUCHHOLZ *et al.*, 2005). Entre os suportes mais utilizados estão principalmente materiais porosos -o que facilita a fixação da enzima ao suporte - argilosos ou cerâmicos. Como exemplo tem-se a Celita, que consiste em terra diatomácea altamente porosa composta de sílica e outros dióxidos inorgânicos (KARRA-CHAABOUNI *et al.*, 2006).

Como muitos sistemas reacionais necessitam de agitação para garantir uma boa homogeneização do meio, materiais com uma elevada resistência mecânica são particularmente desejáveis em sistemas agitados. Já, em relação à presença de solventes, exigem-se materiais com elevada resistência química. Outra característica importante é a capacidade do suporte de ser regenerado com enzimas novas e ativas, uma vez que a perda de atividade enzimática é uma certeza ao longo do tempo (YAHYA *et al.*, 1998).

A Figura 4 apresenta uma representação esquemática dos principais métodos de imobilização de enzimas.

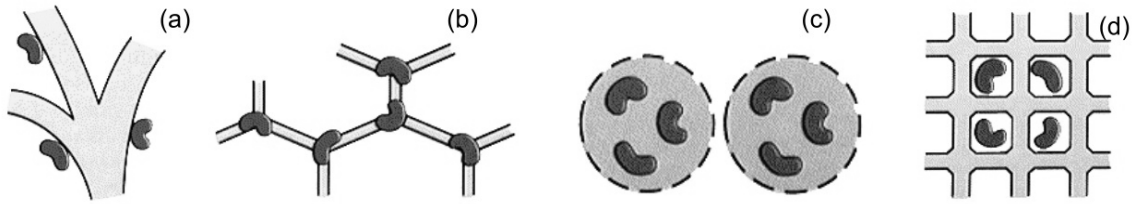

Figura 4 – Métodos de imobilização de enzimas: (a) Adsorção ou ligação covalente em suporte sólido; (b) Ligação cruzada entre a enzima e o suporte; (c) Microcápsulas; (d) Matriz poliméricas sintéticas ou naturais.

Os autores GRAEBIN *et al.* (2012) observaram as vantagens na utilização de suportes hidrofóbicos na síntese do acetato de butila. Este tipo de suporte seria capaz de impedir a acumulação de moléculas hidrofílicas, como o ácido acético, diminuindo assim a necessidade da lavagem entre os ciclos de reuso. Em outro trabalho, FRIEDRICH *et al.* (2012) relataram que variações no protocolo de imobilização causaram diferentes condições ótimas para a reação de esterificação do butirato de etila.

2.5. Reação de esterificação catalisada por lipases

Esterificação é uma reação relativamente simples que consiste na condensação do ácido carboxílico livre, sem qualquer tratamento, e o álcool de destino, formando éster e água (Figura 5). Contudo, esta reação é muito lenta, por isso a utilização de enzimas, como as lipases, aumenta a velocidade da reação (RAJENDRAN *et al.*, 2009). Essa reação acontece em sistema com teor de água muito baixo, usando diferentes solventes ou mesmo em meios sem solvente. Além disso, diversos fatores como a natureza do substrato e do solvente, temperatura e quantidade de água afetam a atividade da lipase (SERRA *et al.*, 2005; TORRES *et al.*, 2009; EISENMENGER e REYES-DE-CORCUERA, 2010).

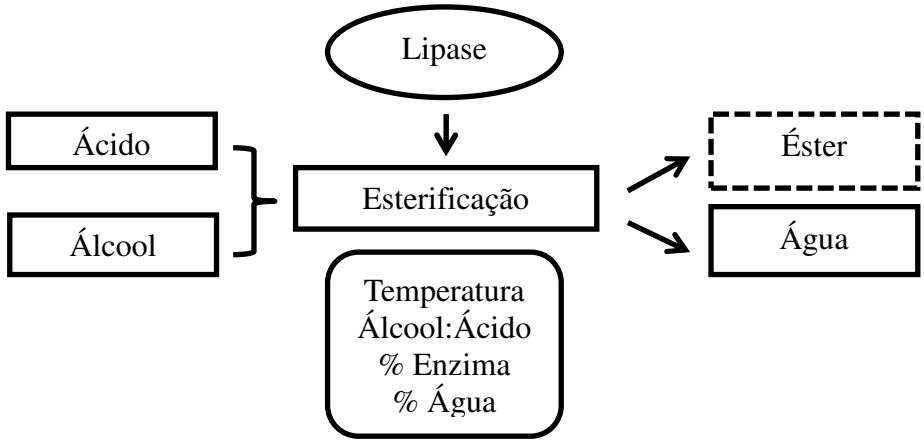

Figura 5 – Lipase como catalisador na síntese de ésteres por esterificação

Em vista disso, é de grande interesse o estudo dos parâmetros que influenciam na velocidade da reação de esterificação, como a temperatura, a razão molar do substrato, a quantidade de enzima e água adicionada, assim como a interação que estes possuem entre si.

A temperatura de reação é responsável por diversos impactos no sistema. O seu aumento pode influenciar positivamente ou negativamente. Positivamente, se considerarmos que o aumento da temperatura aumenta a velocidade da reação. Negativamente, quando se considera que o emprego de altas temperaturas, pode perturbar a estrutura terciária da enzima, o que levará a uma perda da atividade da enzima (ROMERO *et al.*, 2005).

O efeito positivo da razão molar do substrato pode ser explicado pela termodinâmica da reação. Por outro lado, uma vez que a acidificação do meio pelo ácido acético é muito tóxica para a enzima (COUTO *et al.*, 2011), é interessante utilizar um excesso de álcool a fim de melhorar os rendimentos.

A água é um dos produtos da reação de esterificação, assim observa-se que um aumento excessivo do teor de água tem efeito negativo no rendimento da conversão (GRAEBIN *et al.*, 2012). Além disso, tem sido relatado que algumas enzimas são menos ativas na presença de excesso de água (TAMALAMPUDI *et al.*, 2008; OGNJANOVIC *et al.*, 2009). No entanto, algum teor de água é necessário para que a enzima mantenha ativo seu estado tridimensional. A água também contribui para a integridade estrutural, atividade do sítio de polaridade e estabilidade da proteína (YAHYA *et al.*, 1998).

O aumento da quantidade de enzima, até certo limite, afeta positivamente a velocidade de reação. Contudo, a uma concentração muito elevada de enzima, a conversão da reação não aumenta de modo claro. Isto pode estar relacionado com: (1) a dificuldade em manter a suspensão uniforme dos biocatalisadores, (2) captura da água livre pelo suporte, conduzindo à inativação da enzima (WEI *et al.*, 2003), ou (3) o rendimento da reação é tão elevado que um novo aumento no teor de biocatalisador não proporciona um aumento significativo no rendimento de conversão final. Em reações enzimáticas um dos maiores problemas é o custo da enzima, por isso é importante atingir altas taxas de conversão usando a menor quantidade de enzima possível.

2.6. Energia ultrassônica

Utilizado em diversas áreas, o ultrassom é uma onda sonora cuja frequência é maior do que o ouvido humano pode responder. É considerada uma tecnologia "verde" por causa de sua alta eficiência, o seu desempenho econômico e seus baixos requisitos de instrumentais. Essas características reduzem significativamente o tempo de processamento em comparação com outras técnicas convencionais de agitação, como é o caso da agitação mecânica (ROKHINA *et al.*, 2009).

Até recentemente, o uso de ultrassom em aplicações químicas era incomum. Mas, com o objetivo de encontrar novos métodos e soluções para melhorar o

desempenho de bioprocessos, este tipo de energia tem se mostrado uma interessante alternativa. Alguns estudos apontam que esta tecnologia pode ser usada para aplicações biotecnológicas (SINISTERRA, 1992; KWIATKOWSKA *et al.*, 2011; BATISTELLA *et al.*, 2012).

Esta tecnologia tem sido utilizada devido à sua capacidade em aumentar a interação entre as fases através do colapso das bolhas de cavitação, e do jato de ultrassom que rompe as fases dos substratos, auxiliando a emulsificação (ROKHINA *et al.*, 2009; HOBUSS *et al.*, 2012). Por outro lado, quando o ultrassom é aplicado em soluções aquosas ou em suspensão observa-se um aumento do cisalhamento, da homogeneização e da transferência de massa (KWIATKOWSKA *et al.*, 2011; ZHENG *et al.*, 2012).

De acordo com KWIATKOWSKA *et al.* (2011) a influência do ultrassom de baixa frequência não é capaz de inativar enzimas, mas pode afetar esse catalisador em alguns processos. No entanto, nota-se que a influência do ultrassom, sobre a atividade e a estabilidade de enzimas, depende dos parâmetros de sonificação e da preparação enzimática específica. Neste sentido, alguns pesquisadores relataram a influência de ultrassom nos parâmetros cinéticos e redução do tempo de reação de esterificação (DESHMANE *et al.*, 2008; 2009). Entretanto, sua aplicação para reações catalisadas enzimaticamente é ainda pouco explorada, e não foi encontrada na literatura qualquer investigação sobre a aplicação do ultrassom na síntese de ésteres de aroma catalisada por lipases.

2.7. Metodologia de Superfície de Resposta

A determinação dos parâmetros reacionais é comumente feita variando um fator de cada vez, enquanto mantêm-se os outros constantes (KARRA-CHAABOUNI *et al.*, 2006). Contudo, esse método não é eficiente quando há interações entre diversas variáveis. Para processos complexos, que envolvem muitas variáveis, a metodologia de superfície de resposta (MSR) é considerada uma técnica estatística muito eficiente. Como principal vantagem tem se a redução no número de experimentos necessários para prover informação suficiente para resultados estatisticamente aceitáveis, tornando o

um método mais rápido e mais barato para coleta de dados do que o método clássico (GUNAWAN *et al.*, 2005).

A metodologia de superfície de resposta tem sido usada na síntese de aromas a fim de estudar os efeitos das variáveis de maior influencia no desempenho da reação, assim como a interação entre elas. FRIEDRICH *et al.* (2012) estudaram, através dessa metodologia, duas preparações enzimáticas diferentes como biocatalisador para a reação de esterificação do butirato de etila e obtiveram taxas de conversão de 85 % em 2,5 h de reação. Já PIRES-CABRAL *et al.* (2007) obtiveram taxas de 95 % de conversão usando a mesma enzima para a mesma síntese, mas em condições reacionais diferentes e em suportes diferentes.

3. RESULTADOS E DISCUSSÕES

Ao longo deste trabalho foram desenvolvidos dois artigos científicos, um para cada método de agitação: agitação mecânica e ultrassônica. A seguir estão apresentados os dois manuscritos em língua inglesa.

3.1. Rapid and high yields of synthesis of butyl acetate catalyzed by Novozym 435: reaction optimization by response surface methodology

Neste trabalho foram otimizadas as condições para a reação de esterificação entre o ácido acético e o butanol calisada pela lipase B de *Candida antarctica*, comercialmente disponível como Novozym 435. As variáveis avaliadas através do delineamento composto central e da metodologia de superfície de resposta foram a temperatura de reação, a razão molar do substrato, a quantidade de enzima e a água adicionada, tendo como resposta o rendimento em taxa de conversão. Os resultados estão apresentados no manuscrito a seguir, publicado no periódico *Process Biochemistry*, v. 46, p. 2311–2316.

Rapid and high yields of synthesis of butyl acetate catalyzed by Novozym 435: reaction optimization by response surface methodology

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Abstract

In this paper is described the optimization of the esterification reaction of butyl acetate synthesis catalyzed by *Candida antarctica* lipase B (Novozym 435). The reaction parameters temperature, substrate molar ratio, enzyme content, and added water, and their responses measured as conversion yields, were evaluated using central composite design and response surface methodology. The best acid concentration for the reaction without enzyme inactivation was determined to be 0.3 M. The optimal conditions for butyl acetate synthesis were found to be temperature of 40 °C; substrate molar ratio of 3:1 butanol:acetic acid; enzyme content of 7.5% of substrate wt.; added water 0.25% of substrate wt.. Under these conditions, over 90% of conversion was obtained in 2.5 h. Enzyme reuse was tested performing three different treatments before each batch: washing the enzyme system with either n-hexane or water, or suspending the immobilized enzyme in water for 24 h. Direct enzyme reuse or washing with water produced a rapid decrease on enzyme activity, while washing with n-hexane allowed enzyme to be reused for 6 reactions cycles keeping around 70 % of its activity. This fast and high yield of conversion represents a large improvement to previously reported results.

Key-words: lipase; Novozym 435; butyl acetate; flavor ester; RSM; enzyme reuse

1. Introduction

Flavor esters are important raw materials used in food, cosmetics and pharmaceutical industries [1]. Current international regulations classify as 'natural flavors' those obtained by direct extraction from natural sources, which are produced in small amounts and are too expensive for commercial use [2], and those obtained in biotechnological processes using enzymes or the microbial bioconversion of natural precursors. On the other hand, synthetic esters can also be obtained by direct chemical esterification of carboxylic acids with the adequate alcohol in the presence of inorganic catalysts at elevated temperatures (200–250ºC) [3-5]. This fact has stimulated research aiming at developing new biotechnological processes for the production of these valuable compounds [2].

Lipases are enzymes that catalyze the hydrolysis of oils and fats and, under appropriate working conditions, will also present catalytic activities of esterification, transesterification, and alcoholysis reactions [6-8]. Although the literature on the enzymatic esterification catalyzed by lipases [3, 5, 7] is vast, most of it reports on the reaction between a short chain alcohol and a long chain fatty acid. However, the reaction between a short chain alcohol and a short chain carboxylic acid produces short chain aliphatic esters that are small enough to be volatile thus capable of producing pleasant fruity notes [9]. Many aliphatic acetate esters are components of natural flavors, mainly fruit flavors esters, such as butyl acetate, which is an ester naturally present in apples, strawberries and pears, which has found many applications in the food industry [10].

Syntheses of flavor esters have been described as reaction systems with very low water contents, using different solvents (organic solvents, ionic liquids or supercritical

fluids) or even produced in solvent-free media [2, 10-17]. Determination of reaction parameters involved in the lipase-catalyzed flavor ester synthesis is commonly made by varying one factor at a time, while keeping the others constant [11-14, 16], a method that may be inefficient when there are interactions among several variables.

The use of response surface methodology (RSM) for optimizing lipase catalyzed esterification has been reported by some authors, with the main variables analyzed being the reaction temperature, substrate molar ratio, enzyme content and the reaction time [18-22]. Results often show that even after optimization, reaction time was generally long, taking around 8-48 h for maximal yields. This length of time is considered too long for an industrial application and represents most of the costs of enzyme applications, rendering it inappropriate for large-scale processes. Another important factor is the cost of the enzyme itself, which is a drawback for the biotechnological process compared with the chemical synthesis, thus requiring the enzyme to be reused several times, maintaining its activity as long as possible. Several strategies can be performed to recover the enzyme activity of immobilized systems after each reaction cycle as, for example, to wash the enzyme with solvent, which is usually water or an organic solvent, in order to eliminate any substance adsorbed on the support. For instance, it has been reported that the activity of immobilized lipase was almost unaltered after 7 cycles of washings with n-hexane in the transesterification reaction [23].

In this context, the aim of this work was to optimize the synthesis of apple flavor butyl acetate catalyzed by the immobilized lipase B from *Candida antarctica*, commercially available as Novozym 435, using central composite design and RSM analysis. The reaction parameters of temperature, substrate molar ratio, enzyme concentration, and water content were studied in order to establish the relationship

among them and the reaction conversion. It was also studied the effect of different treatments for recovering enzyme activity with the objective of recycling the enzyme system for multiple reuses. The tested treatments were (1) washings with water; (2) washings with hexane.

2. Materials and Methods

2.1 Materials

Lipase B from *Candida antarctica* immobilized in a macroporous resin (Novozym 435) was kindly donated by Novozymes (Spain). Acetic acid, n-butanol and other chemicals were of analytical grade and purchased from Sigma-Aldrich (Sigma, St. Louis, USA).

2.2 Reaction of esterification and analysis

N-butanol was added to acetic acid at different molar ratios into 50 mL Erlenmeyer flasks (working volume of 10 mL), followed by the addition of various amounts of water, enzyme, and n-hexane as solvent. Previously to the reaction, the immobilized enzyme was dried for 24 h at 40 °C in order to remove any adsorbed water. The mixtures of acetic acid, n-butanol, and Novozym 435 were stirred in an orbital shaker (200 rpm) at various reaction temperatures for 5 h (Table 1).

Table 1: Process variables and their levels used in CCD

Variables	Name	Coded Levels				
		-2	-1	0	1	2
X_1	Temperature (^oC)	25	31.25	37.5	43.75	50
X_2	Substrate Molar	1:1	2:1	3:1	4:1	5:1

	Ratio[a]					
X_3	Enzyme Content[b]	1	2.5	5	7.5	10
X_4	Added Water[b]	0	0.25	0.5	0.75	1

[a](butanol:acetic acid); [b](% by weight of substrate);

The progress of esterification was monitored by determining the residual acid content by titration of 1 mL of sample with NaOH (0.01 N) using phenolphthalein as indicator and 5 mL of ethanol as quenching agent. The amount of ester was calculated as being equivalent to the consumed acid. A calibration curve was performed to ensure the reliability of this acid determination using laboratory-made mixtures of acetic acid, n-butanol, and commercial butyl acetate.

2.3 Experimental design

A central composite design with four variables was carried out in order to obtain the optimal conditions for esterification reaction. The variables and their coded and uncoded values are presented in Table 1. Table 2 shows 28 treatments of the four variables, each at five levels. The design was constructed of 16 factorial points, 8 axial points (two axial points on the axis of design variable), and four replications at the central point. In each case, the yields of conversion for esterification were determined.

The second-order polynomial equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \qquad (1)$$

Where Y is the response variable, β_0 the constant, β_i, β_{ii}, β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j the

coded level of variables x_i and x_j. The above quadratic equation was used to plot surfaces for all variables.

Table 2: Experimental design and results of CCD

Treatment	X_1	X_2	X_3	X_4	Yield
1	-1	-1	-1	-1	8.8
2	-1	-1	-1	1	26.0
3	-1	-1	1	-1	93.0
4	-1	-1	1	1	53.3
5	-1	1	-1	-1	67.1
6	-1	1	-1	1	69.9
7	-1	1	1	-1	92.7
8	-1	1	1	1	84.8
9	1	-1	-1	-1	64.3
10	1	-1	-1	1	8.1
11	1	-1	1	-1	93.6
12	1	-1	1	1	91.3
13	1	1	-1	-1	79.6
14	1	1	-1	1	75.6
15	1	1	1	-1	88.8
16	1	1	1	1	91.1
17	-2	0	0	0	77.0
18	2	0	0	0	96.1
19	0	-2	0	0	70.4
20	0	2	0	0	90.7
21	0	0	-2	0	41.1
22	0	0	2	0	94.6
23	0	0	0	-2	93.0
24	0	0	0	2	92.9
25	0	0	0	0	93.4
26	0	0	0	0	92.6
27	0	0	0	0	93.6
28	0	0	0	0	93.1

2.4 Enzyme reuse

After the esterification reaction, the immobilized enzyme was separated from the reaction medium by means of simple filtration. The recovered biocatalyst was then treated by either of the following three different procedures: (1) washed with 20 mL of n-hexane; (2) washed with 20 mL of water; or (3) suspended in 20 mL of water by 24 h; all followed by drying for 24 h at 40 °C [24, 25]. Control experiments were carried out without any of the mentioned treatments, and without drying.

2.5 Statistical analysis

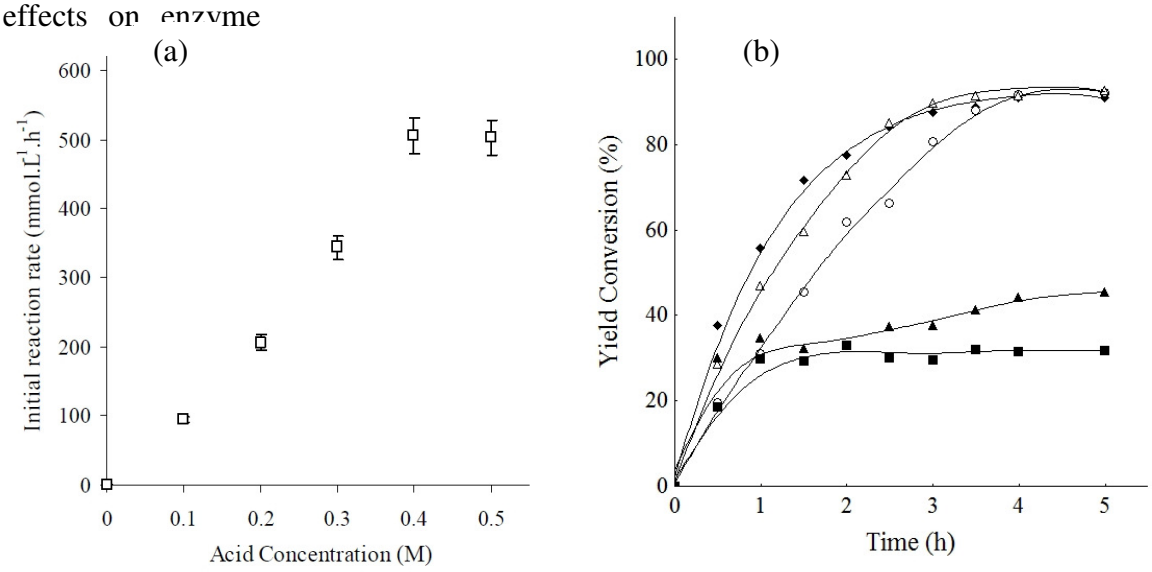
The experimental design and analysis of results were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed as analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, p(t), were determined by Student's t-test; the second order model equation significance was determined by Fisher's F-test. The variance explained by model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

3. Results and Discussion

3.1 Effect of acid concentration on enzyme activity

Experiments varying the acetic acid concentration from 0.1 to 1 M were devised and the obtained results for the initial reaction rate and the yields of conversion are presented in Fig. 1, (a) and (b), respectively. Acetic acid and its derivatives, including triacetin, have been reported to be poor substrates for lipases due to their small molecular size, which leads to the inefficient interfacial activation of the enzyme [26-28]. Results show that up to 0.4 M there was an increment in enzyme activity as the

initial reaction rate in an almost linear way, while for acetic concentrations higher than 0.5 M no reaction could be detected. However, observing the reaction kinetics (Fig. 1b), the enzyme activity decreased when using 0.4 and 0.5 M in less than 1 h of reaction. This result suggests an excessive acidification of the medium producing undesirable effects on enzyme



activity and/or stability, and that acetic acid concentration is in fact a key parameter in this reaction. The yields of conversion that were obtained in this work were higher than those obtained for the synthesis of geranyl acetate catalyzed by Novozym 435 [29], where the concentration of acetic acid used was 0.1M; and were similar to those reported for the synthesis of isoamyl acetate, where the best concentration of acetic acid was 0.3M using a lipase from *Aspergillus niger* [30]. Therefore, the highest possible concentration of acetic acid as a substrate should be around 0.3M, without exerting any negative effects on enzyme activities and stabilities.

Fig. 1: Effect of acetic acid concentration on (a) initial reaction rate and (b) yields of conversion (◆) 0.1M, (○) 0.2M, (△) 0.3M, (▲) 0.4M, (■) 0.5M. Reaction conditions: enzyme content, 10%; temperature, 30 °C; substrate molar ratio, 1:1 n-butanol:acetic acid.

3.2 Experimental design, model fitting and ANOVA

Experimental data obtained for the lipase-catalyzed synthesis of butyl acetate are shown in Table 2. The highest yield of conversion (96.08%) was obtained for treatment 18 (50 °C, 3:1 alcohol:acid, enzyme content 5%, added water 0.5%), while the less effective, with yields of conversion of only 8.07%, was the treatment 1 (31.25 °C, 2:1 alcohol:acid, enzyme content 2.5%, added water 0.25%). Most of the treatments presented yields of conversion higher than 80% within 5 h of reaction, showing that Novozym 435 presented a good activity for the butyl acetate synthesis. Comparatively, Mahapatra et al. [10] using an immobilized lipase from *Rhizopus oligosporus*, obtained approximately 55% of conversion after 28 h of reaction and a massive amount of 27.5% of enzyme. To turn this biocatalytic process suitable for large scale applications, it would be important to get high yields of conversion in short times and using as low amounts of enzymes as possible, which is compatible with the results obtained in this research, where a much more efficient conversion, in a shorter time and using less enzyme, was achieved.

Fisher's statistical test for analysis of variance (ANOVA) showed a computed *F*-value of 4.02, which is highly significant (p=0.0083). The determination coefficient (R^2 = 0.81) implies that the variation of 81% for butyl acetate synthesis is attributed to the independent variables, and can be explained by the model, while the correlation coefficient (R = 0.90) suggests a highly satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation. Linear, quadratic, and interaction terms were significant at the 5% level. Therefore, the second-order polynomial model is given by:

$$Y = 93.17 + 5.62X_1 + 10.49X_2 + 16.50X_3 - 3.66X_4 - 3.99X_1^2 - 5.48X_2^2 - 8.65X_3^2 - 2.37X_4^2 - 3.47X_1X_2 - 0.91X_1X_3 - 2.04X_1X_4 - 9.93X_2X_3 + 4.63X_2X_4 - 0.45X_3X_4 \quad (2)$$

Where Y is the percentage yield conversion, and X_1, X_2, X_3, and X_4 are the coded values of temperature, substrate molar ratio, enzyme content and added water, respectively.

3.3 Effect of parameters

The entire relationship between reaction variables and response can be better understood by examining the planned series of contour plots depicted in Fig. 2, which shows the experiments around the central point and was generated from the predicted model by keeping constant the temperature (31.25, 37.5, and 43.75 °C) and added water (0.25, 0.5 and 0.75%). Observing the contour plots it can be seen that simultaneously increasing the reaction temperature and reducing the added water, the yields of conversion were enhanced, which was confirmed by the linear effects estimated for the reaction parameters, all statistically significant ($p<0.0001$): the positive effect of the temperature (11.25) and the negative effect of added water (-7.33). However, the

substrate molar ratio (20.98) and the amount of enzyme (33.01) presented the highest effects on yields of conversion. As expected, enzyme content was the main variable affecting the synthesis of butyl acetate. In a reaction catalyzed by immobilized enzymes, where enzyme-enzyme interactions are not possible, the increase of enzyme content will, until a certain limit, positively affect the reaction rate. It is important to observe that the optimal values obtained in this work for enzyme content of reaction were around 5 to 7.5%. Other works [10, 31, 32] have all reported the use of higher enzyme contents, with longer times of reaction, obtaining lower yields of conversion. The optimal and improved reaction conditions obtained in this work reflect the importance of using RSM; all variables were studied simultaneously, allowing to identify their interactions and to define their best combinations.

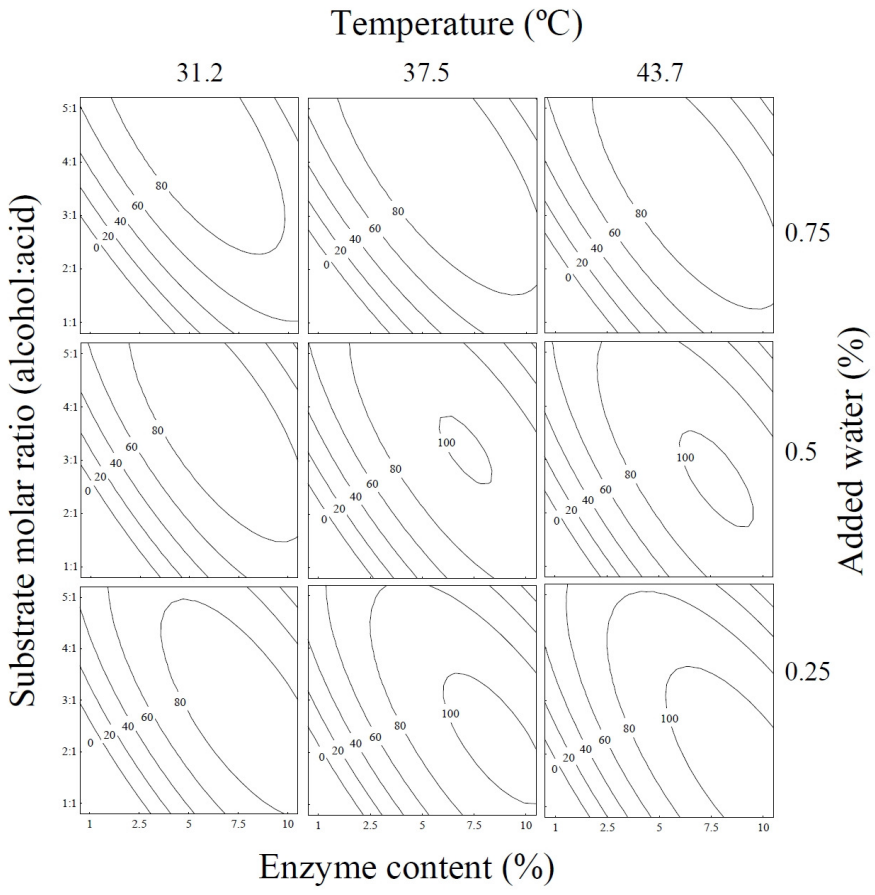


Fig. 2: Contour plots of yields of conversion in butyl acetate synthesis. The numbers inside the contour plots indicate percentage of yields of conversions (%) at given reactions conditions.

The positive effect of substrate molar ratio can be explained by the thermodynamics of the reaction. Since the acidification of the medium by acetic acid is very toxic for the enzyme [29], it is interesting to use excess of alcohol to improve the yields. It also explains the negative effect of added water. Water is one of the products of the esterification reaction and it has been reported that Novozym 435 is less active in the presence of an excess of water [33, 34]. Nevertheless, some initial water content is needed by lipases to retain their active three-dimensional conformational state. Water also contributes to the structural integrity, active site polarity, and protein stability [5].

3.4 Determination of optimal reaction conditions

The optimal conditions for lipase-catalyzed butyl acetate synthesis were determined by the response desirability profile calculated using the Statistica 7.0 software, where the optimal values of each variable were obtained for the desired response that, in this work, was the maximal yield of conversion after 5 h of reaction. The profiles for the predicted values and desirabilities of the 4 variables are shown in Fig. 3. The optimal conditions were found to be: temperature of 40 °C; substrate molar ratio of 3:1 butanol:acetic acid; enzyme content of 7.5%; and added water of 0.25%. According to Fig. 3, similar high yields of conversion could be obtained varying added water from 0 to 0.6%. Based in the experience of this lab, the optimal value for this

variable was fixed at level -1 of the CCD (0.25%). At the optimal conditions, the predicted yield of conversion was over 99%.

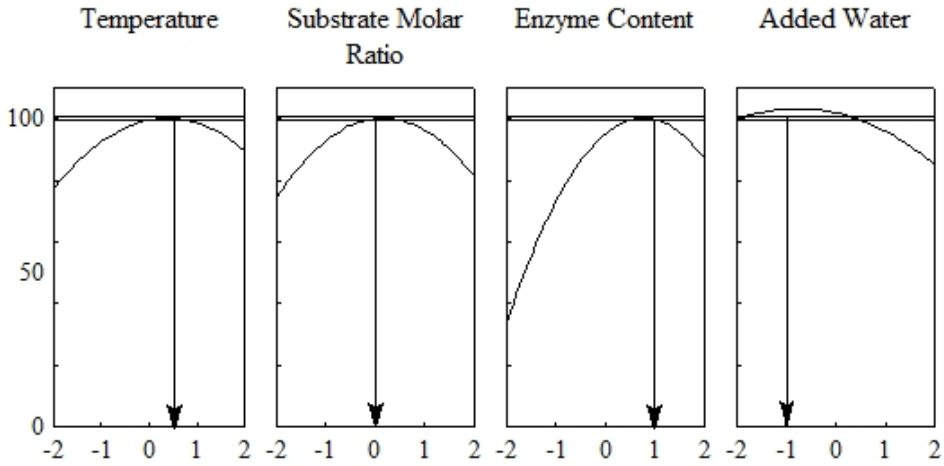


Fig. 3: Profiles for predicted values and desirability for the variables.

3.5 Kinetics of reaction and model validation

In order to validate the model, experiments were run at the optimal conditions and Fig. 4 presents the kinetics of lipase-catalyzed butyl acetate synthesis. Results show 91.5% of conversion obtained after only 2.5 h of reaction.

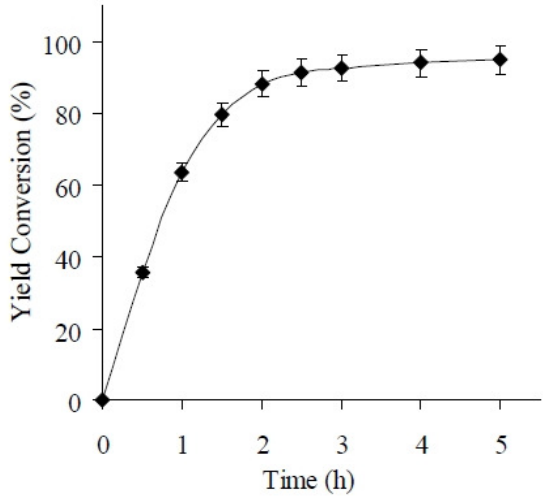


Fig. 4: Kinetics of butyl acetate synthesis under the optimal conditions.

This is relevant because the reaction time is considered an important parameter for lipase-catalyzed flavor esters production as indicator of effectiveness and economical performance. After 5 h of reaction, at the optimal conditions defined by the experimental design, the yield of conversion was 95 ± 2%, showing a good correlation between experimental results and the model statistically predicted (99%).

3.6 Enzyme reuse

In general, enzymatic reactions are challenged by the high cost of the biocatalysts. In the specific case of flavor esters, which can be chemically produced by catalysis at low costs, enzymes must be reused several times to make the biocatalytic way competitive against the chemical synthesis. Therefore, it was decided to test the best way to recover the enzyme after it was used in the synthesis of butyl acetate, according the procedures described in 2.4. Results in Fig. 5 show that the drying of the biocatalyst has an important and positive effect on the stability of enzyme. When the biocatalyst was dried, some of the volatile residues (substrates, products, solvents) that could affect the reaction were removed from the support matrix, improving the enzyme activity. Therefore, drying of the support seems to be a convenient procedure before enzyme reuse. However, when the biocatalyst was only dried without any previous treatment, the enzyme completely lost its activity after the second batch (Fig. 5), probably due to the accumulation of water produced in the reaction and the progressive accumulation of acetic acid on the support. This effect could be even more deleterious if an acidified water phase would be formed around the enzyme, since it has been reported

that the activity and stability of an enzyme rely on the pH of the solution from which it is recovered [35-37]. The washing with n-hexane was the best treatment and allowed the enzyme to retain around 70% of its activity even after 6 reaction batches.

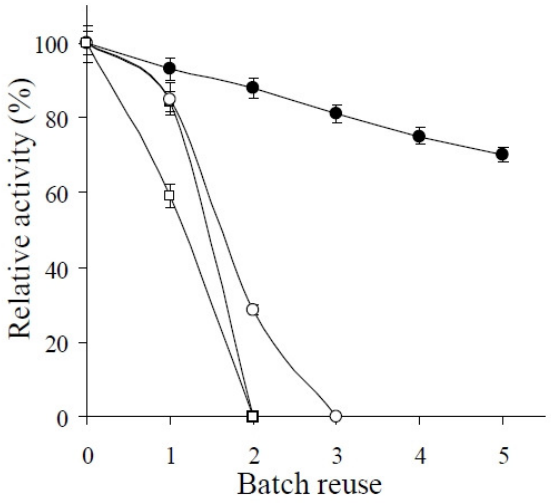


Fig. 5: Stability of Novozym 435 over repeated batches submitted to different treatments. (□, ■) Control; (○, ●) washings with n-hexane. Open symbols: without drying; close symbols: drying the biocatalyst after filtration. All reactions were carried out at the optimal conditions.

The washings with water produced faster inactivations than the ones with n-hexane, suggesting that these treatments were negative for enzyme reaction. When the water-treated samples were posteriorly washed with hexane after the second reuse, the activities were recovered and maintained to similar levels as for the n-hexane treated samples, without any significant lost. Possible explanations for these effects could be

that (1) water washings caused an increase in the amount of water adsorbed by the support; (2) the washings with water could expose the enzyme to drastic acid pH because of accumulation of the remaining acetic acid on the support; (3) the hydrolysis of esters during the water washings could lowered the biocatalyst pH, thus inactivating the enzyme. To clarify this subject, an experiment was designed where the biocatalyst was washed in two different ways after the first batch: (1) the enzyme was first washed with water and then with n-hexane; (2) reversely, the washing with n-hexane was followed by a washing with water. The first treatment caused the enzyme activity to drop 50% after the second reaction cycle, while for the second treatment the enzyme kept its activity as high as 90% after the two cycles, similarly to results obtained using only n-hexane for the washings. This result suggests that the negative effect of water washings is due to the acidification of the enzyme environment during water washings, perhaps due to the rapid hydrolysis of the ester, exposing the enzyme to very low pH values. When the biocatalyst was first washed with n-hexane, the ester and most of the remaining acetic acid were washed way from the biocatalyst pore, and the following washing with water did not produce any deleterious effects on the enzyme. When water was used first, it acidified the environment, producing enzymatic inactivation, and further washings with n-hexane were unable to revert this inactivation. Concluding, the water itself does not appear to have a negative effect on the enzyme activity, but it exposes the enzyme to a low pH value.

4. Conclusion

In this work, it was optimized the butyl acetate (apple flavor) synthesis catalyzed by Novozym 435 in an organic medium. The use of organic medium is essential to

avoid the deleterious effects caused by the acetic acid on the enzyme. It was found that 0.3 M acetic acid is the highest concentration that can be used in the reaction to obtain maximal yields of conversion, while preserving the enzyme stability. The use of CCD allowed evaluating the main variables of the enzymatic reaction; at the optimal conditions, more than 90% of conversion was obtained in the relatively short time of 2.5 h. The enzyme could be reused, keeping more than 70% of its original activity after 6 reaction cycles, by performing a simple washing procedure with n-hexane between reaction batches. The results presented in this research represent a progress in the enzymatic synthesis of flavor esters, since most of previous literature reports show smaller yields of conversions obtained after much longer times of reaction and higher amounts of enzyme. It is important to remark that even including the treatment time, our whole process time is comparable to many reaction times found in the literature.

Acknowledgments

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References

[1] Berger RG. Biotechnology of flavours-the next generation. Biotechnol Lett 2009;31:1651-1659.

[2] Serra S, Fuganti C, Brenna E. Biocatalytic preparation of natural flavours and fragrances. Trends Biotechnol 2005;23:193-198.

[3] Lortie R. Enzyme catalyzed esterification. Biotechnol Adv 1997;15:1-15.

[4] Christen P, Lopezmunguia A. Enzymes and food flavor - a review. Food Biotechnology 1994;8:167-190.

[5] Yahya ARM, Anderson WA, Moo-Young M. Ester synthesis in lipase-catalyzed reactions. Enzyme Microb Technol 1998;23:438-450.

[6] Hasan F, Shah AA, Hameed A. Industrial applications of microbial lipases. Enzyme Microb Technol 2006;39:235-251.

[7] Rajendran A, Palanisamy A, Thangavelu V. Lipase Catalyzed Ester Synthesis for Food Processing Industries. Braz Arch Biol Technol 2009;52:207-219.

[8] Jaeger KE, Eggert T. Lipases for biotechnology. Curr Opin Biotechnol 2002;13:390-397.

[9] Gillies B, Yamazaki H, Armstrong DW. Production of flavor esters by immobilized lipase. Biotechnol Lett 1987;9:709-714.

[10] Mahapatra P, Kumari A, Kumar Garlapati V, Banerjee R, Nag A. Enzymatic synthesis of fruit flavor esters by immobilized lipase from *Rhizopus oligosporus* optimized with response surface methodology. J Mol Catal B: Enzym 2009;60:57-63.

[11] Bezbradica D, Mijin D, Siler-Marinkovic S, Knezevic Z. The effect of substrate polarity on the lipase-catalyzed synthesis of aroma esters in solvent-free systems. J Mol Catal B: Enzym 2007;45:97-101.

[12] Thakar A, Madamwar D. Enhanced ethyl butyrate production by surfactant coated lipase immobilized on silica. Process Biochem 2005;40:3263-3266.

[13] Eisenmenger MJ, Reyes-De-Corcuera JI. Enhanced synthesis of isoamyl acetate using an ionic liquid-alcohol biphasic system at high hydrostatic pressure. J Mol Catal B: Enzym 2010;67:36-40.

[14] Torres S, Baigori MD, Swathy SL, Pandey A, Castro GR. Enzymatic synthesis of banana flavour (isoamyl acetate) by *Bacillus licheniformis* S-86 esterase. Food Res Int 2009;42:454-460.

[15] Yadav GD, Lathi PS. Kinetics and mechanism of synthesis of butyl isobutyrate over immobilised lipases. Biochem Eng J 2003;16:245-252.

[16] Karra-Chaabouni M, Ghamgui H, Bezzine S, Rekik A, Gargouri Y. Production of flavour esters by immobilized *Staphylococcus simulans* lipase in a solvent-free system. Process Biochem 2006;41:1692-1698.

[17] Liu SQ, Holland R, Crow V. Synthesis of ethyl butanoate by a commercial lipase in aqueous media under conditions relevant to cheese ripening. J Dairy Res 2003;70:359-363.

[18] Güvenç A, Kapucu N, Kapucu H, Aydoğan O, Mehmetoğlu U. Enzymatic esterification of isoamyl alcohol obtained from fusel oil: Optimization by response surface methodolgy. Enzyme Microb Technol 2007;40:778-785.

[19] Kumar GV, Rao MN. Enzymatic synthesis of butyl butyrate using response surface methodology. J Food Sci Technol 2004;41:560-562.

[20] Macedo GA, Pastore GM, Rodrigues MI. Optimising the synthesis of isoamyl butyrate using *Rhizopus* sp. lipase with a central composite rotatable design. Process Biochem 2004;39:687-692.

[21] Melo LLMM, Pastore GM, Macedo GA. Optimized synthesis of citronellyl flavour esters using free and immobilized lipase from *Rhizopus* sp. Process Biochem 2005;40:3181-3185.

[22] Pires-Cabral P, da Fonseca MMR, Ferreira-Dias S. Modelling the production of ethyl butyrate catalysed by *Candida rugosa* lipase immobilised in polyurethane foams. Biochem Eng J 2007;33:148-158.

[23] Rodrigues RC, Volpato G, Wada K, Ayub MAZ. Enzymatic synthesis of biodiesel from transesterification reactions of vegetable oils and short chain alcohols. J Am Oil Chem Soc 2008;85:925-930.

[24] Rodrigues RC, Bolivar JM, Palau-Ors A, Volpato G, Ayub MAZ, Fernandez-Lafuente R, Guisan JM. Positive effects of the multipoint covalent immobilization in the reactivation of partially inactivated derivatives of lipase from *Thermomyces lanuginosus*. Enzyme Microb Technol 2009;44:386-393.

[25] Rodrigues RC, Godoy CA, Filice M, Bolivar JM, Palau-Ors A, Garcia-Vargas JM, Romero O, Wilson L, Ayub MAZ, Fernandez-Lafuente R, Guisan JM. Reactivation of covalently immobilized lipase from *Thermomyces lanuginosus*. Process Biochem 2009;44:641-646.

[26] Pernas MA, Pastrana L, Fuciños P, Rúa ML. Regulation of the interfacial activation within the *Candida rugosa* lipase family. J Phys Org Chem 2009;22:508-514.

[27] Hernandez K, Garcia-Verdugo E, Porcar R, Fernandez-Lafuente R. Hydrolysis of triacetin catalyzed by immobilized lipases: Effect of the immobilization protocol and experimental conditions on diacetin yield. Enzyme Microb Technol 2011;48:510-517.

[28] Ferrato F, Carriere F, Sarda L, Verger R. A critical reevaluation of the phenomenon of interfacial activation. In: B. Rubin ,E. A. Dennis editors. Methods Enzymol vol. 286. New York: Academic Press; 1997. p. 327-347.

[29] Couto R, Vidinha P, Peres C, Ribeiro AS, Ferreira O, Oliveira MV, MacEdo EA, Loureiro JM, Barreiros S. Geranyl acetate synthesis in a packed-bed reactor catalyzed by novozym in supercritical carbon dioxide and in supercritical ethane. Ind Eng Chem Res 2011;50:1938-1946.

[30] Mhetras N, Patil S, Gokhale D. Lipase of *Aspergillus niger* NCIM 1207: A Potential Biocatalyst for Synthesis of Isoamyl Acetate. Indian J Microbiol 2010;50:432-437.

[31] Ozyilmaz G, Gezer E. Production of aroma esters by immobilized *Candida rugosa* and porcine pancreatic lipase into calcium alginate gel. J Mol Catal B: Enzym 2010;64:140-145.

[32] Pahujani S, Shukla SK, Bag BP, Kanwar SS, Gupta R. Application of lipase immobilized on nylon-6 for the synthesis of butyl acetate by transesterification reaction in n-heptane. J Appl Polym Sci 2007;106:2724-2729.

[33] Tamalampudi S, Talukder MR, Hama S, Numata T, Kondo A, Fukuda H. Enzymatic production of biodiesel from Jatropha oil: A comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. Biochem Eng J 2008;39:185-189.

[34] Ognjanovic N, Bezbradica D, Knezevic-Jugovic Z. Enzymatic conversion of sunflower oil to biodiesel in a solvent-free system: Process optimization and the immobilized system stability. Bioresour Technol 2009;100:5146-5154.

[35] Chang SW, Shaw JF, Shieh CH, Shieh CJ. Optimal formation of hexyl laurate by lipozyme IM-77 in solvent-free system. J Agric Food Chem 2006;54:7125-7129.

[36] Twu YK, Shih IL, Yen YH, Ling YF, Shieh CJ. Optimization of lipase-catalyzed synthesis of octyl hydroxyphenylpropionate by response surface methodology. J Agric Food Chem 2005;53:1012-1016.

[37] Yee LN, Akoh CC, Phillips RS. Lipase PS-catalyzed transesterification of citronellyl butyrate and geranyl caproate: Effect of reaction parameters. J Am Oil Chem Soc 1997;74:255-260.

3.2. Ultrasound-assisted butyl acetate synthesis catalyzed by Novozym 435: enhanced activity and operational stability

Neste trabalho foram otimizadas as condições para a reação de esterificação entre o ácido acético e o butanol calisada pela lipase B de *Candida antarctica*, comercialmente disponível como Novozym 435 usando a tecnologia ultrassônica como método de agitação. As variáveis avaliadas através do delinamento compostos central e da metodologia de superfície de respostas foram a temperatura de reação, a razão molar do substrato, a quantidade de enzima e a água adicionada, tendo a resposta como a taxa de conversão do ácido. Os resultados estão apresentados no manuscrito a seguir, enviado para publicação no periódico *Ultrasonics Sonochemistry.*

Ultrasound-assisted butyl acetate synthesis catalyzed by Novozym 435: enhanced activity and operational stability

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ABSTRACT

The influence of low-frequency ultrasound (40 kHz) in the esterification reaction between acetic acid and butanol for flavor ester synthesis catalyzed by the commercial immobilized lipase B from *Candida antarctica* (Novozym 435) was evaluated. A central composite design and the response surface methodology were used to analyze the effects of the reaction parameters (temperature, substrate molar ratio, enzyme content and added water) and their response (yields of conversion in 2.5 h of reaction). The reaction was carried out using *n*-hexane as solvent. The optimal conditions for ultrasound-assisted butyl acetate synthesis were found to be: temperature of 46 °C; substrate molar ratio of 3.6:1 butanol:acetic acid; enzyme content of 7 %; added water of 0.25 %, conditions that are slightly different from those found using mechanical mixing. Over 94 % of conversion was obtained in 2.5 h under these conditions. The optimal acid concentration for the reaction was determined to be 2.0 M, compared to 0.3 M without ultrasound treatment. Enzyme productivity was significantly improved to around 7.5-fold for each batch when comparing ultrasound and standard mechanical agitation. The biocatalyst could be directly reused for 14 reactions cycles keeping around 70 % of its original activity, while activity was virtually zeroed in the third cycle using the standard mixing system. Thus, compared to the traditional mechanical agitation, ultrasound technology not only improves the process productivity, but also enhances enzyme recycling and stability in the presence of acetic acid, being a powerful tool to improve biocatalyst performance in this type of reaction.

Keywords: esterification; lipase; ultrasound; enzyme reuse; enzyme stability; butyl acetate.

Highlights

- Ultrasound energy was used as mixing system in the esterification reaction of flavor esters.
- Synthesis of butyl acetate catalyzed by Novozym 435 was optimized.
- Under the optimal conditions over 94 % of conversion was obtained in 2.5 h.
- Enzyme was reused for 14 reaction cycles, keeping 70 % of its original activity.
- Enzyme stability in acetic acid was improved under ultrasound-assisted reaction.

1. INTRODUCTION

Lipases are versatile biocatalysts, mainly used to perform the hydrolysis of oils and fats and, under specific conditions, also capable of catalyzing esterification, transesterification, and interesterification reactions [1]. These enzymes can be easily obtained from microorganisms, and are very active in organic solvents, which is needed to obtain low water activity used in esterification reactions [2]. In particular, the application of these enzymes for the synthesis of short chain esters has attracted the interest of a broad range of industrial fields like foods, pharmaceuticals and cosmetics industries [3].

Lipases are of great interest for the synthesis of natural flavor esters [4]. These reactions occur between short chains alcohols and carboxylic acids. Presently, international legislation have defined that “natural” flavor substances can only be prepared by either physical processes (extraction from natural sources), or by enzymatic or microbial processes. From the consumers’ perception, compounds labeled "natural" are more readily acceptable than products labeled "nature-identical" because these are associated (and produced by) to chemical methods [5]. Thus, lipase-catalyzed esterification is the target of many current researches aiming at finding a suitable biocatalyst to make this process viable in large scales [3,6-10].

Ultrasound is a sound of a frequency higher than that the human ear can perceive, which is being used in several technical fields. It is considered a “green” technology because of its high efficiency, its economic performance and low instrumental requirements. It significantly reduces process time compared with other conventional mixing techniques [11]. Ultrasound technology has been used due to its capacity of increasing the interaction between phases by cavitation caused by the collapse of bubbles, and the ultrasonic jet, that disrupts the boundary phase and causes

emulsification [11,12]. Furthermore, when ultrasound is applied to an aqueous solution or suspension, increases in mixing, shearing, and mass transfer are observed [13,14]. Until recently, it was unusual the use of ultrasound in biological reactions, but some studies pointed that this technology could be used for biotechnological applications [13,15,16].

According to Kwiatkowska et al. [13] the influence of the low frequency ultrasound is not able to inactivate enzymes, but can affect these sensitive catalysts in some processes. However, it must be remarked that the influence of sonic radiation on the activity and stability of enzymes depends on the sonication parameters and the specific enzyme preparation. In this sense, some researchers reported the influence of ultrasound in the kinetic parameters and reduction of esterification reaction time [17,18]. Nevertheless, its application to enzymatic-catalyzed reactions is still scarcely explored, and it was not found in the literature any research on the application of ultrasound-assisted technology for lipase-catalyzed flavor esters synthesis.

The esterification between acetic acid and butanol catalyzed by immobilized lipase B from *Candida antarctica*, commercially available as Novozym 435 has been recently optimized using the standard mechanical mixing system. Results were positive, but reaction rates and reuse of the biocatalyst were low to justify its use at industrial level, demanding further optimization of the process to meet these requirements. Optimal results were obtained using hexane as solvent, and a concentration of 0.3 M acetic acid to avoid enzyme inactivation [19].

Considering these aspects, in this work it was evaluated the influence of ultrasound energy on the esterification reaction between acetic acid and butanol in hexane catalyzed by Novozym 435 starting from the conditions previously reported as optimal for this reaction under standard mixing system. The reaction parameters

temperature, substrate molar ratio, enzyme content, and added water were optimized using central composite design (CCD) and the response surface methodology (RSM). Finally, the enzyme stability on acetic acid and the recycling of the enzyme for multiple batches were analyzed and compared with the standard mechanical agitation technique.

2. MATERIALS AND METHODS

2.1. Materials

Lipase B from *Candida antarctica* immobilized in a macroporous resin (Novozym 435) was kindly donated by Novozymes (Spain). The substrates, solvents and other chemicals were purchased from Sigma-Aldrich (Sigma, St. Louis, USA) and were of analytical grade.

2.2. Esterification reaction

The substrates *n*-butanol and acetic acid (0.3 M based on a previous work [19]) were dissolved in *n*-hexane at different molar ratios into 50 mL Erlenmeyer flasks (working volume of 10 mL), followed by the addition of various amounts of water and enzyme. The reaction was carried out in an ultrasound bath (Unique Inc., model USC 2880A, 40 kHz, Brazil), for 2.5 h at various temperatures, according to the experimental design (Table 1).

Table 1: Process variables and their levels used in CCD

Variables	Name	Coded Levels				
		-2	-1	0	1	2
X_1	Temperature (^oC)	30	37.5	45	52.5	60

X_2	Substrate Molar Ratio[a]	1:1	2:1	3:1	4:1	5:1
X_3	Enzyme Content[b]	1	2.5	5	7.5	10
X_4	Added Water[b]	0	0.25	0.5	0.75	1

[a](butanol:acetic acid); [b](% by weight of substrate);

The progress of esterification was monitored determining the residual acid content by titration of 0.5 mL of sample with NaOH (0.005 N) until pH 7, using ethanol as quenching agent. The amount of ester was calculated as being equivalent to the consumed acid. A calibration curve was constructed to ensure the reliability of this acid determination using laboratory-made mixtures of acetic acid, *n*-butanol, and commercial butyl acetate.

2.3 Experimental design

In order to obtain the optimal conditions for the esterification a central composite design (CCD) with four variables was performed. The four variables, each with five levels, are presented in Table 1 with their coded and uncoded values. The CCD, with 28 experiments, was composed of 16 factorial points, 8 axial points (two axial points on the axis of design variable), and four replications at the central point. In each case, the percentage of yield of conversion for esterification was calculated. The second-order polynomial equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \qquad (1)$$

Where Y is the response variable, β_0 the constant, β_i, β_{ii}, β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j the coded level of variables x_i and x_j. The above quadratic equation was used to plot surfaces for all variables.

2.4 Enzyme reuse

After the esterification reaction, the immobilized enzyme was separated from the reaction medium by vacuum filtration using a sintered glass funnel. In some cases, in order to remove any water from the support, the recovered biocatalysts were washed with 10 volumes of *n*-hexane, dried for 20 h at 40 ºC and reused in a new fresh reaction. Hexane is highly volatile, providing a dry biocatalyst after 20 h. In other cases, the reuse was performed after vacuum filtration without further treatments.

2.5 Statistical analysis

The experimental design and analysis of results were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed as analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, p(t), were determined by Student's t-test; the second order model equation significance was determined by Fisher's F-test. The variance explained by model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

3. RESULTS AND DISCUSSION

3.1. Experimental design, model fitting and ANOVA

Response surface methodology is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and the observed results. In Table 2 are presented the results for the ultrasound-assisted butyl acetate synthesis using Novozym 435 as biocatalysts after 2.5 h of reaction. Highest yield of conversion (99.3 %) was obtained in treatment 22 (45 °C, 3:1 alcohol:acid, enzyme content 10 %, added water 0.5 %), while the less effective treatment was the 21, with 42.0 % yield of conversion (45 °C, 3:1 alcohol:acid, enzyme content 1 %, added water 0.5 %). Most treatments presented yields of conversion higher than 80 % within the 2.5 h of reaction. These results are in agreement with previous works [19,20], in which Novozym 435 has been described as a good catalyst for the butyl acetate production, in organic solvents. A similar yield was obtained under similar reaction conditions, except that the standard mechanical mixing was used [19], producing 96.08 % of conversion using Novozym 435 in 5 h of reaction. Romero et al. [21], has compared three different commercial immobilized lipases (from *C. antarctica*, from *Rhizomucor miehei*, and from *Thermomyces lanuginosus*) in the synthesis of isoamyl acetate. The lipase from *C. antarctica* (Novozym 435), presented the highest activity. The optimization process confirmed that sonication was similarly efficient when compared with the conventional method of mechanical mixing.

Table 2: Experimental design and results of CCD.

Treatment	X_1	X_2	X_3	X_4	Novozym 435 (%)
1	-1	-1	-1	-1	46.6
2	-1	-1	-1	1	48.3
3	-1	-1	1	-1	94.9
4	-1	-1	1	1	83.7
5	-1	1	-1	-1	60.7
6	-1	1	-1	1	64.4
7	-1	1	1	-1	91.7
8	-1	1	1	1	90.2
9	1	-1	-1	-1	83.4
10	1	-1	-1	1	85.8
11	1	-1	1	-1	98.0
12	1	-1	1	1	97.3
13	1	1	-1	-1	80.8
14	1	1	-1	1	77.1
15	1	1	1	-1	95.8
16	1	1	1	1	96.4
17	-2	0	0	0	77.9
18	2	0	0	0	81.6
19	0	-2	0	0	75.4
20	0	2	0	0	92.0
21	0	0	-2	0	42.0
22	0	0	2	0	99.3
23	0	0	0	-2	92.1
24	0	0	0	2	90.1
25	0	0	0	0	88.3
26	0	0	0	0	91.3
27	0	0	0	0	90.7
28	0	0	0	0	90.1

According to Fisher's statistical test for analysis of variance (ANOVA), the model was statistically significant and adequate to represent the actual relationship between the responses and the variables, as suggested the model *F*-value (11.99) and very low *p*-value ($p < 0.0001$). The values of the determination coefficient, R^2, and correlation coefficient, R, were, 0.92 and 0.96, respectively. This represents a highly satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation. The

coefficients of variables were determined for the second-order polynomial model and the statistical significant (5 %) are given below:

$$Y = 45.2 + 5.90X_1 + 2.18X_2 + 13.14X_3 - 2.70X_1^2 - 1.71X_2^2 - 4.97X_3^2 - 3.02X_1X_2 - 5.01X_1X_3 + 1.16X_2X_3 - 1.07X_3X_4 \quad (2)$$

Where Y is the percentage yield conversion, and X_1, X_2, X_3, and X_4 are the coded values of temperature, substrate molar ratio, enzyme content and added water, respectively.

3.2. Effect of process parameters

The effect of variables involved in the process could be evaluated based on their *p*-values described in Table 3. The enzyme content (26.28) and reaction temperature (11.80) presented the highest effects. Both effects are positive, meaning that the increase from level -1 to level 1, positively affects the response that, in this case, is the yield of conversion. The enzyme content was the most significant variable. Until a certain limit, the increase in the enzyme content will positively affect the reaction rate. However, at very high enzyme concentrations, the rate of reaction conversion will plateau, probably due to (1) the difficulty in maintaining uniform suspension of the biocatalysts, (2) the capture of the free water by the support, driving the enzyme to inactivation [22], or (3) the yields of reaction are so high that further increases in biocatalyst content do not provide a significant impact in the final yield of conversion. Results in this work suggest that around 5 to 7.5 % of enzyme is enough to obtain the highest possible yields of conversion.

Table 3 – Statistical analysis of central composite design

Variable	**Effect**	***p*-value**
Mean	90.111	<0.0001
Linear		
X_1*	11.805	0.0002
X_2*	4.359	0.0038
X_3*	26.287	<0.0001
X_4	-1.034	0,1474
Quadratic		
X_1X_1*	-5.307	0.0024
X_2X_2*	-3.328	0.0085
X_3X_3*	-9.851	0.0003
X_4X_4	0.384	0.5232
Interactions		
X_1X_2*	-60.295	0.0027
X_1X_3*	-10.017	0.0006
X_1X_4	0.721	0.3494
X_2X_3*	-2.320	0.0378
X_2X_4	0.870	0.2739
X_3X_4*	-2.131	0.0468

* Statistically significant at the 95% confidence level.

The reaction system may be strongly influenced by the temperature. Raising the temperature could positively affect the course of reaction increasing the free energy and enzyme activity. But if higher temperatures disrupt the enzyme tertiary structure, this will lead to a loss of activity [21]. In this work, reaction temperature presented a positive effect, inducing high yields of conversion along the tested temperature range. In the synthesis of ethyl butyrate using Novozym 435 [23], 37 ºC was found to be the best temperature. Kuo et al. [24], studying the synthesis of rose aromatic esters using the

same biocatalyst, found out that at any given reaction temperature from 50 to 70 °C, an increase in enzyme amount led to a higher conversion. Therefore, it can be suggested that Novozym 435 is active over a broad temperature range.

The relationship between enzyme content and reaction temperature with the respective response can be better understood by examining contour plot depicted in Fig. 1, where substrate molar ratio was fixed at level 0.6 and added water was fixed at level -1. It can be noted that simultaneously increasing the reaction temperature and the enzyme content, the yields of conversion are enhanced, confirmed by the linear effects estimated for the reaction parameters. Therefore, the variation in one of the variables affects the other in such a way that lower temperatures requires higher enzyme amount and vice versa.

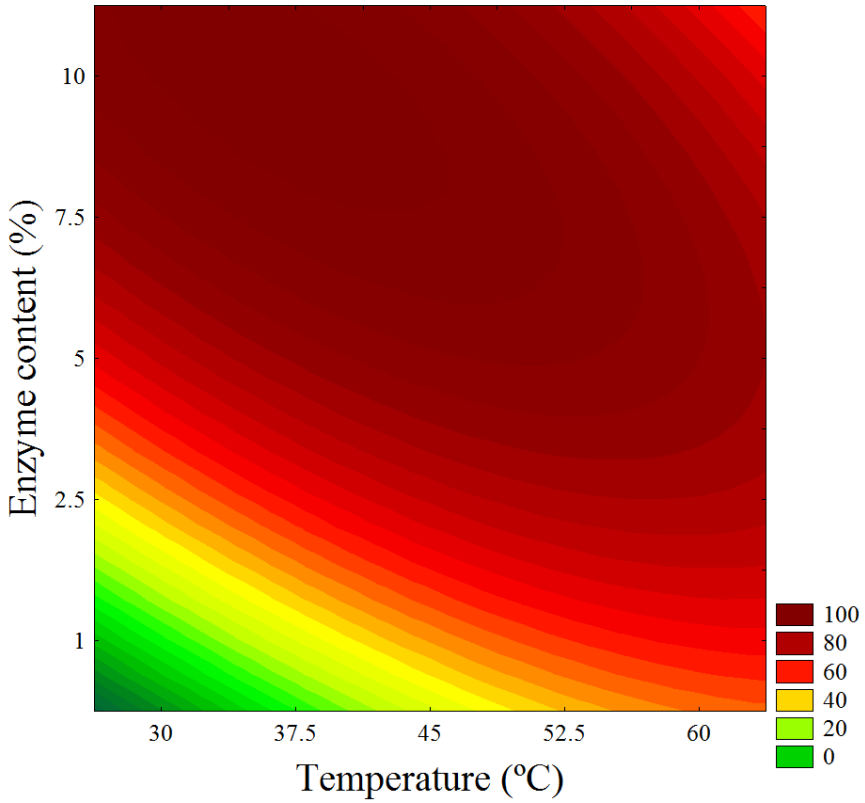

Fig. 1: Contour plots for the ultrasound-assisted synthesis of butyl acetate catalyzed by Novozym 435. Substrate molar ratio was fixed at level 0.6 and added water was fixed at level -1.

3.3. Optimal reaction conditions and model validation

The optimal conditions for ultrasound-assisted lipase-catalyzed butyl acetate synthesis were determined by the critical values using the Statistica 7.0 software, where the optimal values of each variable were obtained for the desired response that, in this work, was the maximal yield of conversion after 2.5 h of reaction. The optimal conditions were reaction temperature of 46 °C; substrate molar ratio of 3.6:1 butanol:acetic acid; enzyme content of 7 %; and added water of 0.25 %. At the optimal conditions, the predicted yield of conversion was 96 %. Using mechanical mixing the optimal conditions were slightly different: temperature 40 °C; substrate molar ratio of 3:1 butanol:acetic acid; enzyme content of 7.5 %; added water of 0.25 %, producing 91.5 % of ester yield after 2.5 h [19].

In order to validate the prediction model, experiments were carried out at the optimal conditions and the time-course of the ultrasound-assisted butyl acetate catalyzed by Novozym 435 is presented in Fig. 2. While the predicted molar conversion value was 96 %, the experimental result produced 95 % after 2.5 h of reaction. This result indicates that the observed value matches the predicted value. Therefore, equation (2) adequately predicts the yield of conversion. Moreover, it was observed at Fig. 2, that 2 h was enough time to reach a yield conversion of 94 %.

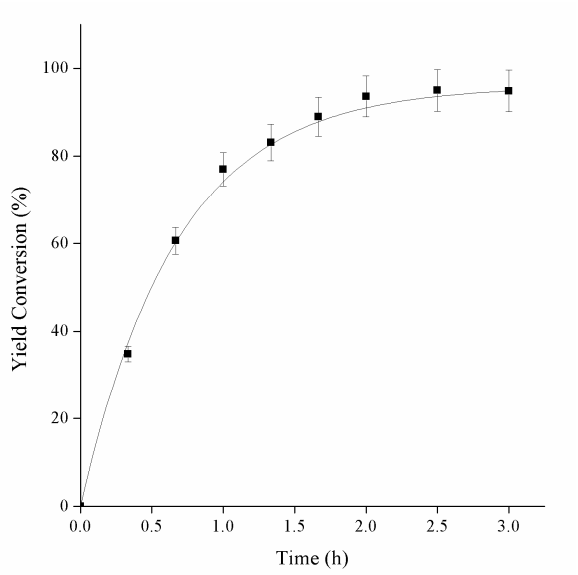

Fig. 2: Time-course of ultrasound-assisted butyl acetate synthesis under the optimal conditions at 0.3 M acetic acid.

3.4. Enzyme reuse

One of the most important properties of immobilized enzymes is the possibility of their recovery and reusability, which are important economic and environmental aspects and could define their future industrial applications. Recover and reuse were the main problems found using this biocatalyst when performing the esterification under standard mixing conditions [19]. Therefore, it was tested the reuse of the enzyme and the results of the repeated batches for ultrasound-assisted butyl acetate synthesis were presented in Fig. 3. The biocatalyst was washed with *n*-hexane and dried at 40 ºC in between batches [19], and control experiments were carried out without any treatment. Surprisingly, results indicated that there was no difference between treatment (washings with n-hexane) or the control, with reuse being possible either way, since the biocatalyst retained around 70 % of its initial activity after 14 reaction batches. In contrast, in the standard mixing system, the accumulation of acid and water in the biocatalyst after each cycle made necessary the washings with *n*-hexane to partially recover the enzyme

activity. Using the mechanical agitation, Novozym 435 was completely inactivated in the second direct reuse When compared with the best reuse treatment for the mechanical agitation, i.e., washing with n-hexane, the enhancement using ultrasound is still around 2.5-fold higher, even though the washings between the batches are not required.

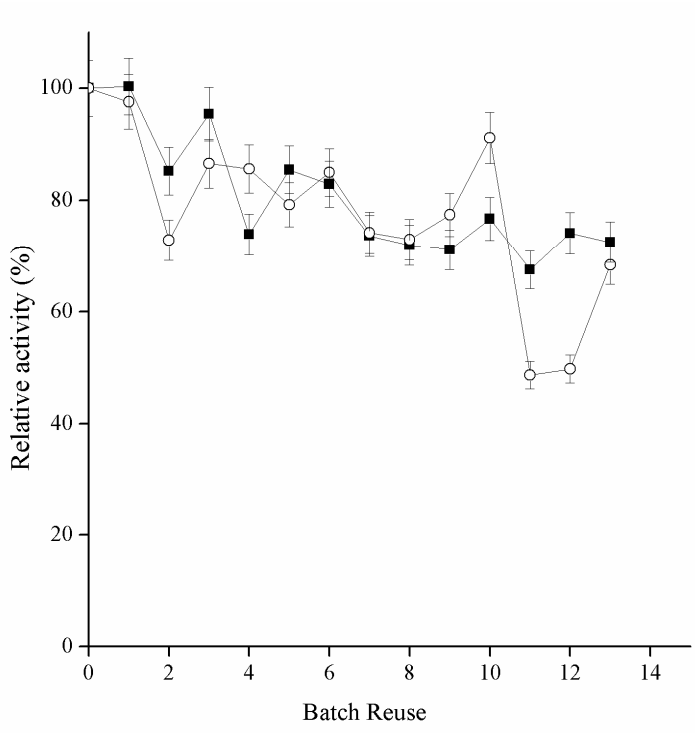

Fig. 3: Stability of Novozym 435 over repeated batches of ultrasound-assisted butyl acetate synthesis. (■) Control; (○) washing with n-hexane. All reactions were carried out at the optimal conditions.

The relative activity of the enzyme in the ultrasound mixing system was higher than that under mechanical mixing, showing that ultrasound technology improved operational stability. These results demonstrate the possible use of immobilized lipase combined with ultrasound technology for large-scale enzymatic syntheses of butyl acetate. According to Veljkovic et al. [25], ultrasound is not only efficient but also is

economically viable as it requires only one-third to a half the energy that is consumed by mechanical agitation.

3.4. Effect of acid concentration on enzyme activity

As acetic acid acidifies the reaction medium, it produces undesirable effects on enzyme activity and stability, thus its concentration is an important parameter for esterification. In a previous work [19], it was verified that 0.3 M was the maximum acetic acid concentration before Novozym 435 starts inactivating. The influence of ultrasound on the enzyme stability was tested in different concentrations of acetic acid and the results of the initial reaction rate are presented in Fig. 4. It was observed a linear increment in the initial reaction rate up to 2.0 M acetic, while above 2.5 M acid concentration, the initial reaction rate started decreasing and it was observed a reduction in the maximum yield conversion, suggesting enzyme inactivation. At 1.5 M acid concentration the maximum yield of conversion was 90 %; 84 % at 2.0 M, and 75 % at 2.5 M, after 2.5 h of reaction. If compared against the maximum acid concentration allowed in the standard mechanical mixing (0.3 M, maximum yield of conversion 94 %, after 2.5 h of reaction), it is clear that the increase of acetic acid concentration provides a much higher concentration of ester. Therefore, it could be suggested that both 2.0 M and 2.5 M are suitable concentrations of acetic acid for ultrasound-assisted butyl acetate synthesis. It is important to observe that the optimal acid concentration used in this work is much higher than most reported in the cited literature. This might be explained by the better mixing of the acetic acid in the organic solvent due to ultrasound, avoiding the acid to become accumulated in the microenvironment of the enzyme, and perhaps also avoiding the formation of a water phase in this microenvironment that could inactivate the enzyme [19].

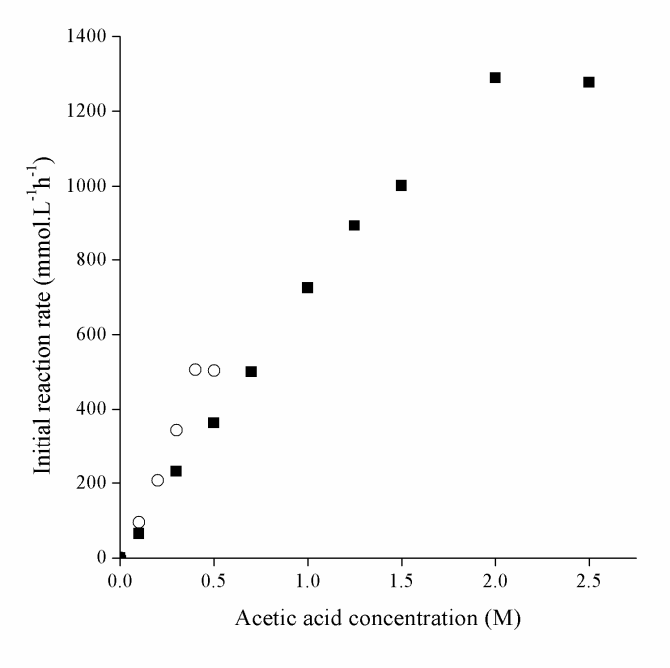

Fig. 4: Effect of acetic acid concentration on initial reaction rate in the butyl acetate synthesis using (■) ultrasound system and (○) mechanical stirring.

The time course of ultrasound-assisted esterification at 2.0 M acid concentration is presented in Fig. 5, showing that after 2 h 80 % of yield conversion was reached. The productivity for ultrasound-assisted butyl acetate synthesis was around 800 mmol.$L^{-1}h^{-1}$, while for mechanical agitation was 115 mmol.$L^{-1}h^{-1}$. It represents an improvement of 7-fold in the process productivity for each batch, which if combined with the enhancement in the operational stability, may produce a large increase in the overall process productivity.

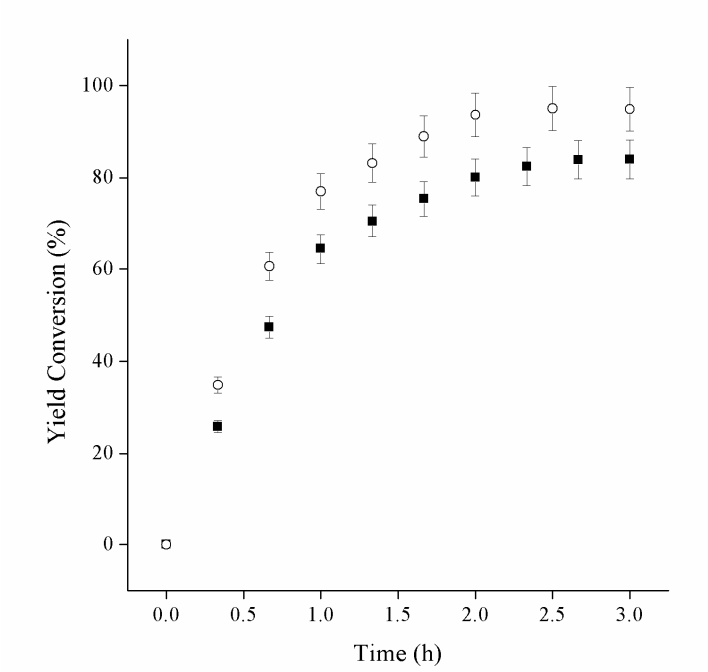

Fig. 5: Time course of ultrasound-assisted butyl acetate synthesis under optimal conditions at (■) 2.0 M and (○) 0.3M acetic acid.

4. CONCLUSION

The ultrasound energy has shown to be a good technology to improve the enzymatic esterification reaction catalyzed by Novozym 435 for flavor ester synthesis in three ways: (a) higher biocatalyst operational stability, (b) biocatalyst stabilization in the presence of high concentrations of acetic acid in *n*-hexane, and (c) increased productivity of ester, when compared to the traditional method of mechanical agitation. The reaction parameters were optimized and the optimal conditions were similar to mechanical agitation. In 2 h was possible to obtain around 95 % of yield conversion at 0.3 M acetic acid. Nevertheless, in spite of the similarity in the reaction conditions, the ultrasound-assisted technology allowed the direct reuse of the enzyme without the needed n-hexane washings. The enzyme could be reused, keeping around 70 % of its original activity after 14 reaction cycles, without any treatment. This represents a

reduction in the generated residues, and save one unit operation in the process, decreasing the cost of production. Under ultrasound irradiation, it was possible to use up to 2.5 M acetic acid, increasing the process productivity. Therefore, improving the acid concentration, the number of batches reuses, avoiding the unit operations of washing to recover the enzyme activity, and the fact that ultrasound requires only one-third to half the energy consumed by mechanical agitation, it can be stated that ultrasound-assisted butyl acetate synthesis catalyzed by Novozym 435 could be scaled up to industrial size in a near future.

Acknowledgments

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REFERENCES

[1] A. Rajendran, A. Palanisamy, V. Thangavelu, Lipase catalyzed ester synthesis for food processing industries, Braz. Arch. Biol. Technol., 52 (2009) 207-219.

[2] K.-E. Jaeger, T. Eggert, Lipases for biotechnology, Curr. Opin. Biotechnol., 13 (2002) 390-397.

[3] S. Torres, M.D. Baigori, S.L. Swathy, A. Pandey, G.R. Castro, Enzymatic synthesis of banana flavour (isoamyl acetate) by *Bacillus licheniformis* S-86 esterase, Food Res. Int., 42 (2009) 454-460.

[4] A.R.M. Yahya, W.A. Anderson, M. Moo-Young, Ester synthesis in lipase-catalyzed reactions, Enzyme Microb. Technol., 23 (1998) 438-450.

[5] S. Serra, C. Fuganti, E. Brenna, Biocatalytic preparation of natural flavours and fragrances, Trends Biotechnol., 23 (2005) 193-198.

[6] H. Abbas, L. Comeau, Aroma synthesis by immobilized lipase from *Mucor* sp, Enzyme Microb. Technol., 32 (2003) 589-595.

[7] R. Ben Salah, H. Ghamghui, N. Miled, H. Mejdoub, Y. Gargouri, Production of butyl acetate ester by lipase from novel strain of *Rhizopus oryzae*, J. Biosci. Bioeng., 103 (2007) 368-372.

[8] M. Karra-Chaabouni, H. Ghamgui, S. Bezzine, A. Rekik, Y. Gargouri, Production of flavour esters by immobilized *Staphylococcus simulans* lipase in a solvent-free system, Process Biochem., 41 (2006) 1692-1698.

[9] G.V. Kumar, M.N. Rao, Enzymatic synthesis of butyl butyrate using response surface methodology, J. Food Sci. Technol., 41 (2004) 560-562.

[10] G.D. Yadav, P.S. Lathi, Kinetics and mechanism of synthesis of butyl isobutyrate over immobilised lipases, Biochem. Eng. J., 16 (2003) 245-252.

[11] E.V. Rokhina, P. Lens, J. Virkutyte, Low-frequency ultrasound in biotechnology: state of the art, Trends Biotechnol., 27 (2009) 298-306.

[12] C.B. Hobuss, D. Venzke, B.S. Pacheco, A.O. Souza, M.A.Z. Santos, S. Moura, F.H. Quina, K.G. Fiametti, J. Vladimir Oliveira, C.M.P. Pereira, Ultrasound-assisted synthesis of aliphatic acid esters at room temperature, Ultrason. Sonochem., 19 (2012) 387-389.

[13] B. Kwiatkowska, J. Bennett, J. Akunna, G.M. Walker, D.H. Bremner, Stimulation of bioprocesses by ultrasound, Biotechnol. Adv., 29 (2011) 768-780.

[14] M.M. Zheng, L. Wang, F.H. Huang, L. Dong, P.M. Guo, Q.C. Deng, W.L. Li, C. Zheng, Ultrasonic pretreatment for lipase-catalyed synthesis of phytosterol esters with different acyl donors, Ultrason. Sonochem., 19 (2012) 1015-1020.

[15] J.V. Sinisterra, Application of ultrasound to biotechnology: an overview, Ultrasonics, 30 (1992) 180-185.

[16] L. Batistella, L.A. Lerin, P. Brugnerotto, A.J. Danielli, C.M. Trentin, A. Popiolski, H. Treichel, J.V. Oliveira, D. De Oliveira, Ultrasound-assisted lipase-catalyzed transesterification of soybean oil in organic solvent system, Ultrason. Sonochem., 19 (2012) 452-458.

[17] V.G. Deshmane, P.R. Gogate, A.B. Pandit, Process intensification of synthesis process for medium chain glycerides using cavitation, Chem. Eng. J., 145 (2008) 351-354.

[18] V.G. Deshmane, P.R. Gogate, A.B. Pandit, Ultrasound assisted synthesis of isopropyl esters from palm fatty acid distillate, Ultrason. Sonochem., 16 (2009) 345-350.

[19] A.B. Martins, N.G. Graebin, A.S.G. Lorenzoni, R. Fernandez-Lafuente, M.A.Z. Ayub, R.C. Rodrigues, Rapid and high yields of synthesis of butyl acetate catalyzed by Novozym 435: reaction optimization by response surface methodology, Process Biochem., 46 (2011) 2311-2316.

[20] N.G. Graebin, A.B. Martins, A.S.G. Lorenzoni, C. Garcia-Galan, R. Fernandez-Lafuente, M.A.Z. Ayub, R.C. Rodrigues, Immobilization of lipase B from *Candida antarctica* on porous styrene-divinylbenzene beads improves butyl acetate synthesis, Biotechnol. Prog., 28 (2012) 406-412.

[21] M.D. Romero, L. Calvo, C. Alba, A. Daneshfar, H.S. Ghaziaskar, Enzymatic synthesis of isoamyl acetate with immobilized *Candida antarctica* lipase in n-hexane, Enzyme Microb. Technol., 37 (2005) 42-48.

[22] D. Wei, C. Gu, Q. Song, W. Su, Enzymatic esterification for glycoside lactate synthesis in organic solvent, Enzyme Microb. Technol., 33 (2003) 508-512.

[23] J.L.R. Friedrich, F.P. Peña, C. Garcia-Galan, R. Fernandez-Lafuente, M.A.Z. Ayub, R.C. Rodrigues, Effect of immobilization protocol on optimal conditions of ethyl butyrate synthesis catalyzed by lipase B from *Candida antarctica*, J. Chem. Technol. Biotechnol., in press (2012) DOI: 10.1002/jctb.3945.

[24] C.-H. Kuo, S.-H. Chiang, H.-Y. Ju, Y.-M. Chen, M.-Y. Liao, Y.-C. Liu, C.-J. Shieh, Enzymatic synthesis of rose aromatic ester (2-phenylethyl acetate) by lipase, J. Sci. Food Agric., 92 (2012) 2141-2147.

[25] V.B. Veljković, J.M. Avramović, O.S. Stamenković, Biodiesel production by ultrasound-assisted transesterification: State of the art and the perspectives, Renew. Sustain. Energy Rev., 16 (2012) 1193-1209.

4. CONSIDERAÇÕES FINAIS

A enzima Novozym 435 se mostrou um excelente catalisador para a reação de esterificação entre ácido acético e butanol. Com o ultrassom, em 2 h, foi possível obter cerca de 95 % de conversão, já sem o uso do ultrassom, foram obtidos 90 % de conversão em 2,5 h. A concentração de ácido acético, usando agitação mecânica, foi 0,3 M sem que houvesse inativação enzimática. Esta concentração foi utilizada no planejamento experimental para a obtenção das condições ótimas em ambos os métodos de agitação. As condições ótimas encontradas foram muito semelhantes comparando os dois métodos. As condições ótimas da reação e os bons resultados nas taxas de conversão obtidos neste trabalho refletem a importância do uso de MSR, onde todas as variáveis foram estudadas simultaneamente, permitindo identificar suas interações e definir as suas melhores combinações.

No entanto, apesar da semelhança das condições reacionais, a tecnologia ultrassônica permite a reutilização direta da enzima sem a necessidade da lavagem com *n*-hexano, como tratamento. Além do mais, a enzima pôde ser reutilizada tendo cerca de 70% da sua atividade original após 14 ciclos de reação, sem qualquer tratamento, diminuindo assim o custo da produção do éster. Consequentemente, esse comportamento favorece a redução dos resíduos químicos gerados, e reduz uma operação unitária no processo. Ainda, sob irradiação ultrassônica, foi possível a utilização de até 2,5 M de ácido acético, aumentando a produtividade do processo.

Portanto, o uso do ultrassom traz inúmeras vantagens como: o aumento da concentração do ácido, o elevado número de reutilizações entre ciclos, a diminuição de uma operação (operação de lavagem para recuperar a atividade da enzima). Além disso, pelo ponto de vista econômico, o ultrassom necessita de apenas um terço da energia que

é requerida pela agitação mecânica. Assim pode-se afirmar que, por razões tecnológicas e econômicas, a síntese do acetato de butila catalisada pela Novozym 435, usando energia ultrassônica, pode ser ampliada para escalas industriais.

O ultrassom é capaz de ativar vários mecanismos que afetam positivamente as reações enzimáticas e os processos, mas estes não são muito conhecidos. Deste modo, os resultados apresentados neste estudo representam um progresso na sua utilização na síntese enzimática de ésteres de aroma.

Em resumo a energia ultrassônica tem mostrado ser uma boa tecnologia para melhorar a reação de esterificação enzimática de três formas:

(a) aumento da estabilidade operacional do biocatalisador,

(b) estabilização do biocatalisador na presença de concentrações elevadas de ácido acético em *n*-hexano,

(c) aumento da produtividade do éster, em comparação com o método tradicional de agitação mecânica.

Baseado nos resultados obtidos propõe-se o estudo dessa síntese em larga escala usando a tecnologia ultrassônica. Assim como o uso de reatores de fluxo contínuo, uma vez que os resultados foram positivos em relação à reutilização da enzima sem nenhuma etapa de lavagem. Da mesma forma, sugere-se o estudo de técnicas de purificação do éster no produto final e sua real aplicação em alimentos.

Por outro lado, é possível utilizar a mesma técnica utilizando diversas enzimas comerciais ou não, diferentes combinações de ácidos e álcoois, em outras concentrações, resultando em outros aromas. Encontra-se na literatura reações sem o

uso de solventes orgânicos, ou em líquidos iônicos e em gases no seu estado crítico, outro aspecto relevante a ser estudado.

Outra perspectiva interessante a ser estudada é o mecanismo cinético da reação. A reação de esterificação, por se tratar de uma reação com dois substratos e dois produtos, não segue o mecanismo cinético de Michaelis-Menten, e sim o mecanismo Ping-Pong Bi-Bi. O estudo do mecanismo cinético é importante, pois através dele se conhece diversos parâmetros, como a velocidade. Esta por sua vez, depende de fatores intrínsecos (relacionados com a energia de ativação), da concentração dos reagentes e do catalisador em si.

REFERÊNCIAS BIBLIOGRÁFICAS

ABIAM. Associação Brasileira da Indústria e Comércio de Ingredientes e Aditivos para Alimentos. Disponível em: < http://www.abiam.com.br/> Acesso em: 15 de nov. 2012.

AL-ZUHAIR, S. Production of biodiesel: possibilities and challenges. **Biofuels, Bioproducts and Biorefining,** v. 1, n. 1, p. 57-66, 2007.

ALVAREZ-MACARIE, E.; BARATTI, J. Short chain flavour ester synthesis by a new esterase from Bacillus licheniformis. **Journal of Molecular Catalysis - B Enzymatic,** v. 10, n. 4, p. 377-383, 2000.

BATISTELLA, L. et al. Ultrasound-assisted lipase-catalyzed transesterification of soybean oil in organic solvent system. **Ultrasonics Sonochemistry,** v. 19, n. 3, p. 452-458, 2012.

BERGER, R. G. Biotechnology of flavours-the next generation. **Biotechnology Letters,** v. 31, n. 11, p. 1651-1659, 2009.

BEZBRADICA, D. et al. The effect of substrate polarity on the lipase-catalyzed synthesis of aroma esters in solvent-free systems. **Journal of Molecular Catalysis B: Enzymatic,** v. 45, n. 3-4, p. 97-101, 2007.

BICAS, J. L. et al. Biotechnological production of bioflavors and functional sugars. **Ciência e Tecnologia de Alimentos,** v. 30, n. 1, p. 7-18, 2010.

BRASIL. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. **Portaria nº 540, de 27 de outubro de 1997: Regulamento Técnico: Aditivos Alimentares - definições, classificação e emprego.** Disponível em: <http://portal.anvisa.gov.br/wps/wcm/connect/d1b6da0047457b4d880fdc3fbc4c6735/PORTARIA_540_1997.pdf?MOD=AJPERES> Acesso em: 15 de nov. 2012.

BRASIL. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. **RDC nº 2, de 15 de janeiro de 2007.** Disponível em: < http://www.anvisa.gov.br/legis/resol/2007/rdc/02_170107rdc.htm> Acesso em: 15 de nov. 2012.

BUCHHOLZ, K.; KASCHE, V.; BORNSCHEUER, U. T. **Biocatalysts and Enzyme Technology**. Weinheim: Wiley-VHC, 2005.

CARDELLO, A. V. et al. Measuring emotional responses to foods and food names using questionnaires. **Food Quality and Preference,** v. 24, n. 2, p. 243-250, 2012.

CHANG, S. W. et al. Optimal formation of hexyl laurate by lipozyme IM-77 in solvent-free system. **Journal of Agricultural and Food Chemistry,** v. 54, n. 19, p. 7125-7129, 2006.

CHIANG, W.-D.; CHANG, S.-W.; SHIEH, C.-J. Studies on the optimized lipase-catalyzed biosynthesis of cis-3-hexen-1-yl acetate in n-hexane. **Process Biochemistry,** v. 38, n. 8, p. 1193-1199, 2003.

COUTO, R. et al. Geranyl acetate synthesis in a packed-bed reactor catalyzed by novozym in supercritical carbon dioxide and in supercritical ethane. **Industrial & Engineering Chemistry Research,** v. 50, n. 4, p. 1938-1946, 2011.

DAMONDARAM, S.; PARKIN, K. L.; FENNEMA, O. R. **Química de alimentos de Fennema**. 4 ed. Porto Alegre: Artmed, 2010.

DEBULIS, K.; KLIBANOV, A. M. Dramatic enhancement of enzymatic activity in organic solvents by lyoprotectants. **Biotechnology and Bioengineering,** v. 41, n. 5, p. 566-571, 1993.

DESHMANE, V. G.; GOGATE, P. R.; PANDIT, A. B. Process intensification of synthesis process for medium chain glycerides using cavitation. **Chemical Engineering Journal,** v. 145, n. 2, p. 351-354, 2008.

DESHMANE, V.G.;GOGATE, P.R.; PANDIT, A.B. Ultrasound assisted synthesis of isopropyl esters from palm fatty acid distillate. **Ultrasonics Sonochemistry,** v. 16, n. 3, p. 345-350, 2009.

DUBAL, S. A. et al. Biotechnological routes in flavour industries. **Advanced Biotech,** v. 20, 2008.

EISENMENGER, M. J.; REYES-DE-CORCUERA, J. I. Enhanced synthesis of isoamyl acetate using an ionic liquid-alcohol biphasic system at high hydrostatic pressure. **Journal of Molecular Catalysis B: Enzymatic,** v. 67, n. 1-2, p. 36-40, 2010.

FAN, X.; NIEHUS, X.; SANDOVAL, G. **Lipases as biocatalyst for biodiesel production**. 861**:** 471-483 p. 2012.

FRIEDRICH, J. L. R. et al. Effect of immobilization protocol on optimal conditions of ethyl butyrate synthesis catalyzed by lipase B from Candida antarctica. **Journal of Chemical Technology & Biotechnology**, p. n/a-n/a, 2012.

GARCIA-GALAN, C. et al. Potential of Different Enzyme Immobilization Strategies to Improve Enzyme Performance. **Advanced Synthesis & Catalysis,** v. 353, n. 16, p. 2885-2904, 2011.

GRAEBIN, N. G. et al. Immobilization of lipase B from Candida antarctica on porous styrene-divinylbenzene beads improves butyl acetate synthesis. **Biotechnology Progress,** v. 28, n. 2, p. 406-412, 2012.

GUBICZA, L. et al. Large-scale enzymatic production of natural flavour esters in organic solvent with continuous water removal. **Journal of Biotechnology,** v. 84, n. 2, p. 193-196, 2000.

GUNAWAN, E. R. et al. Study on response surface methodology (RSM) of lipase-catalyzed synthesis of palm-based wax ester. **Enzyme and Microbial Technology,** v. 37, n. 7, p. 739-744, 2005.

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

HOBUSS, C. B. et al. Ultrasound-assisted synthesis of aliphatic acid esters at room temperature. **Ultrasonics Sonochemistry,** v. 19, n. 3, p. 387-389, 2012.

HORCHANI, H. et al. Staphylococcal lipases: Biotechnological applications. **Journal of Molecular Catalysis B: Enzymatic,** v. 76, n. 0, p. 125-132, 2012.

JAEGER, K.-E.; EGGERT, T. Lipases for biotechnology. **Current Opinion in Biotechnology,** v. 13, n. 4, p. 390-397, 2002.

JONG, S. C.; BIRGMINGHAM, J. M. **Mushrooms as a source of natural flavour and aroma compounds**. Hong Kong: The Chinese University Press, 1993.

KARRA-CHAABOUNI, M. et al. Production of flavour esters by immobilized *Staphylococcus simulans* lipase in a solvent-free system. **Process Biochemistry,** v. 41, n. 7, p. 1692-1698, 2006.

KING, S. C.; MEISELMAN, H. L. Development of a method to measure consumer emotions associated with foods. **Food Quality and Preference,** v. 21, n. 2, p. 168-177, 2010.

KING, S. C.; MEISELMAN, H. L.; THOMAS CARR, B. Measuring emotions associated with foods: Important elements of questionnaire and test design. **Food Quality and Preference,** v. 28, n. 1, p. 8-16, 2013.

KUO, C.-H. et al. Enzymatic synthesis of rose aromatic ester (2-phenylethyl acetate) by lipase. **Journal of the Science of Food and Agriculture,** v. 92, n. 10, p. 2141-2147, 2012.

KWIATKOWSKA, B. et al. Stimulation of bioprocesses by ultrasound. **Biotechnology Advances,** v. 29, n. 6, p. 768-780, 2011.

LONGO, M. A.; SANROMÁN, M. A. Production of Food Aroma Compounds: Microbial and Enzymatic Methodologies. **Food Technology and Biotechnology,** v. 44, n. 335-353, 2006.

LORENZONI, A. S. G. et al. Optimization of pineapple flavour synthesis by esterification catalysed by immobilized lipase from Rhizomucor miehei. **Flavour and Fragrance Journal,** v. 27, n. 2, p. 196-200, 2012.

MARTINS, A. B. et al. Rapid and high yields of synthesis of butyl acetate catalyzed by Novozym 435: Reaction optimization by response surface methodology. **Process Biochemistry,** v. 46, n. 12, p. 2311-2316, 2011.

OGNJANOVIC, N.; BEZBRADICA, D.; KNEZEVIC-JUGOVIC, Z. Enzymatic conversion of sunflower oil to biodiesel in a solvent-free system: Process optimization and the immobilized system stability. **Bioresource Technology,** v. 100, n. 21, p. 5146-5154, 2009.

OZYILMAZ, G.; GEZER, E. Production of aroma esters by immobilized *Candida rugosa* and porcine pancreatic lipase into calcium alginate gel. **Journal of Molecular Catalysis B: Enzymatic,** v. 64, n. 3-4, p. 140-145, 2010.

PIRES-CABRAL, P.; DA FONSECA, M. M. R.; FERREIRA-DIAS, S. Modelling the production of ethyl butyrate catalysed by *Candida rugosa* lipase immobilised in polyurethane foams. **Biochemical Engineering Journal,** v. 33, n. 2, p. 148-158, 2007.

POLAINA, J.; MACCABE, A. P.; EDITORS. **Industrial Enzymes: Structure, Function and Applications**. Dordrecht: Springer, 2007.

POLÔNIO, M. L. T.; PERES, F. Consumo de aditivos alimentares e efeitos à saúde: desafios para a saúde pública brasileira. **Cadernos de Saúde Pública,** v. 25, p. 1653-1666, 2009.

RAJENDRAN, A.; PALANISAMY, A.; THANGAVELU, V. Lipase catalyzed ester synthesis for food processing industries. **Brazilian Archives of Biology and Technology,** v. 52, n. 1, p. 207-219, 2009.

ROKHINA, E. V.; LENS, P.; VIRKUTYTE, J. Low-frequency ultrasound in biotechnology: state of the art. **Trends in Biotechnology,** v. 27, n. 5, p. 298-306, 2009.

ROMERO, M. D. et al. Enzymatic synthesis of isoamyl acetate with immobilized Candida antarctica lipase in n-hexane. **Enzyme and Microbial Technology,** v. 37, n. 1, p. 42-48, 2005.

SCHRADER, J. et al. Applied biocatalysis for the synthesis of natural flavour compounds – current industrial processes and future prospects. **Biotechnology Letters,** v. 26, n. 6, p. 463-472, 2004.

SERRA, S.; FUGANTI, C.; BRENNA, E. Biocatalytic preparation of natural flavours and fragrances. **Trends in Biotechnology,** v. 23, n. 4, p. 193-198, 2005.

SHARMA, R.; CHISTI, Y.; BANERJEE, U. C. Production, purification, characterization, and applications of lipases. **Biotechnol Adv,** v. 19, n. 8, p. 627-62, 2001.

SINISTERRA, J. V. Application of ultrasound to biotechnology: an overview. **Ultrasonics,** v. 30, n. 3, p. 180-185, 1992.

TAMALAMPUDI, S. et al. Enzymatic production of biodiesel from Jatropha oil: A comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. **Biochemical Engineering Journal,** v. 39, n. 1, p. 185-189, 2008.

TORRES, S. et al. Enzymatic synthesis of banana flavour (isoamyl acetate) by Bacillus licheniformis S-86 esterase. **Food Research International,** v. 42, n. 4, p. 454-460, 2009.

VEGA, P. V.; FLORENTINO, B. L. **Toxicologia de Alimentos**. México, D.F.: 2000.

WEI, D. et al. Enzymatic esterification for glycoside lactate synthesis in organic solvent. **Enzyme and Microbial Technology,** v. 33, n. 4, p. 508-512, 2003.

YAHYA, A. R. M.; ANDERSON, W. A.; MOO-YOUNG, M. Ester synthesis in lipase-catalyzed reactions. **Enzyme and Microbial Technology,** v. 23, n. 7–8, p. 438-450, 1998.

YU, Y. et al. Lipase/esterase-catalyzed synthesis of aliphatic polyesters via polycondensation: A review. **Process Biochemistry,** v. 47, n. 7, p. 1027-1036, 2012.

ZHENG, M. M. et al. Ultrasonic pretreatment for lipase-catalyed synthesis of phytosterol esters with different acyl donors. **Ultrasonics Sonochemistry,** v. 19, n. 5, p. 1015-1020, 2012.