UNIVERSIDADE ESTADUAL DE CAMPINAS

Faculdade de Engenharia de Alimentos

CAMILO BARROSO TEIXEIRA

PRODUCTION, CHARACTERIZATION AND APPLICATION OF *PENICILLIUM* SP LIPASIC SOLID FERMENTED ON FATTY ACIDS OBTAINMENT BY COTTONSEED OIL HYDROLYSIS ASSISTED BY ULTRASOUND

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Campinas/SP

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Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do Título de Doutor em Ciência de Alimentos.

Thesis presented to Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Food Science.

Supervisor/Orientador: PROF. DRA. GABRIELA ALVES MACEDO

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO CAMILO BARROSO TEIXEIRA, E ORIENTADO PELA PROFA. DRA. GABRIELA ALVES MACEDO

Campinas

2015

Agência(s) de fomento e nº(s) de processo(s): CNPq, 141104/2011-2

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Claudia Aparecida Romano - CRB 8/5816

T235p

Teixeira, Camilo Barroso, 1984-
Production, characterization and application of *Penicillium* sp. lipasic solid fermented on fatty acids obtainment by cottonseed oil hydolysis assisted by ultrasound / Camilo Barroso Teixeira. – Campinas, SP : [s.n.], 2015.

Orientador: Gabriela Alves Macedo.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Lipase. 2. *Penicillium*. 3. Fermentação em estado sólido. 4. Hidrólise. 5. Ultrassom. I. Macedo, Gabriela Alves. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Produção, caracterização e aplicação de sólido fermentado lipásico de *Penicillium* sp. na obtenção de ácidos graxos pela hidrólise de óleo de algodão assistido por ultrassom
Palavras-chave em inglês:
Lipase
Penicillium
Solid-state fermentation
Hydrolysis
Ultrasound
Área de concentração: Ciência de Alimentos
Titulação: Doutor em Ciência de Alimentos
Banca examinadora:
Gabriela Alves Macedo [Orientador]
Eliana Setsuko Kamimura
Fabiano Jares Contesini
Luciana Francisco Fleuri
Marcus Bruno Soares Forte
Data de defesa: 15-12-2015
Programa de Pós-Graduação: Ciência de Alimentos

BANCA EXAMINADORA

Dra. Gabriela Alves Macedo

(DEPAN/FEA/UNICAMP - Orientadora)

Dra. Eliana Setsuko Kamimura

(FZEA/USP)

Dr. Fabiano Jares Contesini

(CTBE/CNPEM)

Dra. Luciana Francisco Fleuri

(DQB/IBB/UNESP- Botucatu)

Dr. Marcus Bruno Soares Forte

(DEA/FEA/UNICAMP)

Dr. Renato Grimaldi

(DTA/FEA/UNICAMP - Suplente)

Dra. Rosana Goldbeck

(DEA/FEA/UNICAMP - Suplente)

Dra. Tânia Forster Carneiro

(DEA/FEA/UNICAMP - Suplente)

A ata de defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

Dedico todo esforço e mérito desse trabalho aos meus pais pelo apoio integral e amor incondicional. Dedico também a todos seres humanos que acreditam e lutam para que a ciência (exata, biológica e humana) seja um fundamento essencial para a evolução da humanidade.

Simplificar é complexo.

RESUMO

Esse estudo objetivou o desenvolvimento de um biocatalisador com atividade lipolítica produzida por fermentação em estado sólido (FES) utilizando subprodutos agrícolas como substrato e micro-organismo *Penicillium* sp. para avaliação da aplicação na hidrólise de óleo de algodão assistido por ultrassom (US). A tese foi dividida em três capítulos, o primeiro é destinado a uma revisão bibliográfica enfatizando a importância do desenvolvimento de uma bioeconomia, na utilização de óleos e gorduras para produtos comerciais, reaproveitamento de biomassa agrícola, novas aplicações da FES, de lipases e a utilização de US na biocatálise. O segundo capítulo relata a produção de lipase por FES utilizando três subprodutos como substratos: farelo de trigo, de soja e de algodão. Planejamento de misturas simplex centroide (PMSC) foi utilizado para avaliar a melhor formulação usando atividade de lipase (U/g) e custo/unidade de atividade (US$/10³U) como respostas. A função desejabilidade otimizou simultaneamente as duas respostas, gerando uma formulação com maior atividade e menor custo para o meio de fermentação usando 100% farelo de algodão, depois utilizado para otimizar os parâmetros físicos na FES: temperatura, relação água/sólido e tamanho de partícula. O delineamento central composto rotacional (DCCR) foi empregado para avaliar os parâmetros atividade de lipase, proteína e atividade específica. As condições ótimas: 30ºC, 70% relação água/sólido e partícula de 2,4 mm de tamanho foram determinados pela função desejabilidade. Essa abordagem permitiu uma redução de 20% de água no meio de fermentação. Para determinar melhor tempo de fermentação os experimentos realizados nas condições ótimas revelaram o melhor tempo em 48 horas e uma redução de 50% do tempo ótimo inicial (96 horas). No terceiro capítulo, o objetivo foi caracterizar a enzima e avaliar a aplicação na hidrólise de óleo de algodão assistido por US e em banho com agitação. A caracterização da lipase compreendeu a determinação das condições ótimas e de estabilidade para temperatura e pH; especificidade (tamanho de cadeia) e estabilidade em solventes orgânicos. As condições ótimas foram: atividade em 40ºC e pH 7 e estabilidade em 30ºC e pH 3. Os resultados indicam maior afinidade por ácidos graxos de cadeia longa (paranitrofenol palmitato p-NPP) e alta estabilidade em solventes apolares (hexano) e baixa em polares (metanol e etanol). Na hidrólise do óleo de algodão, dois experimentos foram conduzidos: em banho com agitação e outro utilizando US através de sonda (19 kHz) em contato direto com meio reacional. Os dois estudos foram desenhados por DCCR e usados como variáveis independentes: concentração de fermentado sólido lipásico (FSL) e relação tampão/óleo para o sistema em banho (40ºC, 120 rpm) e concentração de FSL, relação tampão/óleo e densidade energética (W/mL) para o sistema reacional com US. Os resultados demonstraram que aumento de LSF e relação

tampão/óleo resultam em aumento da hidrólise do óleo de algodão. Por outro lado, a potência de US gera diminuição da hidrólise, possivelmente inibindo a reação. As condições ótimas foram as mesmas para os dois sistemas: 20% FSL, relação tampão/óleo de 4. O tempo ótimo de reação foi definido pela função desejabilidade obtendo 30 minutos e 54% de hidrólise para US e 1 hora e 28,5 % para banho com agitação.

Palavras-chave: lipase, *Penicillium*, fermentado sólido, hidrólise, ultrassom.

ABSTRACT

This study aimed to develop a biocatalyst with lipolytic activity through solid-state fermentation (SSF) using agricultural byproducts as substrate and a strain of *Penicillium* sp. to evaluate the application on cottonseed oil hydrolysis assisted by ultrasound (US). Divided into three chapters, the first intended for a literature review emphasizing the importance of developing a bioeconomy, the use of oils and fats for commercial products, agricultural biomass reuse, SSF new applications, lipase and the US in biocatalysis. The second chapter relates to lipase production by SSF using three by-products as substrates: wheat bran (WB), soybean meal (SM) and cottonseed meal (CSM). Simplex centroid mixture design (SCMD) evaluated the best formulation using lipase activity (U/g) and cost/unit (US$/10³U) as responses. The function desirability simultaneously optimized both responses, generating the best formulation with higher activity and lower cost using 100% CSM as fermentation medium to optimize the physical parameters of the SSF: temperature, the water/solid ratio and size particle. Central composite rotatable design (CCRD) evaluated the parameters lipase activity, protein and specific activity. The optimum conditions determined by desirability function were: 30°C, 70% water/solid and particle size of 2.4 mm. This approach allowed a reduction of 20% water in the fermentation medium. To determine the best fermentation time, experiments carried out in optimum conditions showed the best time in 48 hours, a 50% reduction from the initial optimum time (96 hours). In the third chapter, the goal was to characterize the enzyme and to evaluate the application in cottonseed oil hydrolysis assisted by US and other in shaking bath. The characterization included the determination of optimum and stability conditions of temperature and pH for lipase activity; specificity (chain size) and stability in organic solvents. The optimum conditions were: 40 °C and pH 7 for activity and 30°C and pH 3 for stability. Higher affinity for long-chain fatty acids (p-NPP) and higher stability in apolar solvents (hexane) and lower in polar (methanol and ethanol). In the hydrolysis of cottonseed oil, two experiments were conducted: in shaking bath and another using US probe (19 kHz) in direct contact with the reaction medium. Both studies were designed by CCRD and used as independent variables: concentration of lipasic solid fermented (LSF) and buffer/oil ratio for the system in bath (40 °C, 120 rpm) and concentration of LSF, buffer/oil ratio and power density (W/mL) to US system. The results showed that increasing LSF and buffer/oil ratio resulted on improvement of cottonseed oil hydrolysis. Nevertheless, the US power generates a decrease, possibly inhibiting the reaction. The optimum conditions were the same for both systems: 20% FSL, buffer/oil ratio = 4 The optimum reaction time defined by the desirability function was 30 minutes and 54% of hydrolysis yield to US and 1 hour and 28.5% for shaking bath.

Keywords : Lipase, *Penicillium*, solid fermented, hydrolysis, ultrasound.

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INTRODUÇÃO GERAL

O avanço da bioeconomia no mundo reflete uma mudança de mentalidade da sociedade com objetivo de reverter ou diminuir os danos causados ao planeta por uma economia movida apenas pela geração de lucro. O mundo corporativo continua incipiente e incapaz de promover uma economia ambientalmente responsável e economicamente favorável. Essas mudanças somente serão possíveis com o comprometimento integral da sociedade civil, acadêmica e empresarial.

O desenvolvimento da biotecnologia tem permitido a aplicação de avanços gerados pela biologia para a criação de novos processos produtivos e novos produtos capazes de abrandar parte dos danos ambientais como a poluição e o esgotamento de recursos naturais promovendo um estímulo ao uso desses de forma sustentável. Entre essas tecnologias, a biocatálise e a fermentação em estado sólido (FES) são consideradas peças fundamentais para a bioeconomia considerando a possibilidade de substituir processos produtivos químicos antigos que geram resíduos tóxicos ao meio ambiente.

A biocatálise e a FES estão interligadas, ambas são consideradas como tecnologias verdes que utilizam biomassa como matéria-prima para obtenção de diversos produtos e, por isso, capazes de integrar a cadeia produtiva de alimentos, químicos, fármacos e combustíveis com uma possível diminuição de geração de subprodutos tóxicos. As enzimas são os principais biocatalisadores que podem ser produzidas por FES e sua aplicação industrial vem aumentando com os anos de acordo com a evolução de tecnologias capazes de gerar enzimas e processos enzimáticos cada vez mais estáveis e rentáveis.

A lipase é uma enzima capaz de hidrolisar triacilgliceróis em meio aquoso e esterificar em meio orgânico. É conhecida por sua promiscuidade por substrato e por ser capaz de sintetizar diversos compostos, obtendo-se, portanto, diversos produtos de interesse comercial. Suas aplicações comerciais já abrangem a área de detergentes, cosméticos, alimentos, biocombustíveis e fármacos, além de novas pesquisas que demonstram seu potencial para desenvolver novos produtos e aplicações como biopolímeros para aplicações biomédicas (Sen and Puskas, 2015), intermediários de drogas antivirais (Moni *et al.*, 2015) e antioxidantes lipofílicos (Roby *et al.*, 2015).

Para aumentar a produtividade e rendimento de conversão das reações enzimáticas, estratégias como a intensificação de bioprocessos permite que tecnologias consideradas "verdes" sejam utilizadas na biocatálise. Entre elas, estudos recentes têm demonstrado a

capacidade do ultrassom (US) em acelerar as reações orgânicas catalisadas por lipases (Martins *et al.*, 2013; Michelin *et al.*, 2015; Remonatto *et al.*, 2015), demonstrando seu potencial para diminuir o tempo de reação, aumento do rendimento de conversão e redução do gasto energético (ver ANEXO 4 (Artigo Revisão Biodiesel). Os efeitos de transferência de massa e de cavitação permitiram a emulsificação de líquidos imiscíveis, aumentando sua eficiência com relação aos sistemas agitados mecanicamente (AbismaïL *et al.*, 1999).

Este estudo objetivou o estudo de seleção de micro-organismos produtores de lipase pertencentes à fauna brasileira e a produção de lipase por FES a partir de subprodutos da agroindústria como farelo de trigo, de soja e de algodão que possuem uma composição complexa rica em nutrientes adequada para o crescimento de micro-organismos; além da disponibilidades desses subprodutos no mercado nacional.

Neste contexto, o trabalho foi dividido em três capítulos. O primeiro é uma revisão bibliográfica a respeito fundamentos e avanços recentes para lipase, FES e reações catalisadas por lipases assistidas por US. O segundo capítulo visou selecionar uma linhagem fúngica isolada da fauna brasileira para ser o biocatalisador da produção de lipase por FES. Após a seleção da linhagem, foi feita a seleção de substrato para FES avaliando os farelos de trigo, soja e algodão para verificar qual mistura apresenta maior rendimento de atividade de lipase por menor custo de matéria-prima. Após a seleção, foi otimizado os parâmetros físicos da FES para maximizar a produção de lipase. O terceiro capítulo refere-se à caracterização da enzima obtida com relação a sua performance e afinidade de substrato. O terceiro capítulo se destina à avaliação do modo de aplicação de enzima como sólido fermentado lipásico (SFL) para a hidrólise do óleo vegetal em banho com agitação orbital ou assistido por sonicação.

CAPÍTULO 1. INTEGRATION OF FATS AND OILS; SOLID-STATE FERMENTATION (SSF) AND ULTRASOUND TECHNOLOGY: THE LIPASE ROLE ON BIOECONOMY DEVELOPMENT

Camilo Barroso Teixeira, Gabriela Alves Macedo

Biochemistry Laboratory, Food Science Department, School of Food Engineering, University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil

Submitted to *Chemical Society Reviews*

ABSTRACT

Vegetable and animal fats and oils represents the main renewable feedstock in oleochemical industry with essential importance on world's bioeconomy development. Several commercial products from food, chemical and pharmaceutical companies presents triacylglycerol, fatty acids and other lipid compounds in its formulation. Lipases represents an innovative biocatalyst to produce commercial fatty products due to its high specificity, high purity products, biodegradability, offering no chemical contamination in final products compared to chemical catalysts. Lipase can be obtained by solid-state fermentation (SSF), which is also a green technology capable of using byproducts from vegetable oils extraction as raw materials to produce lipases, generating a possibility of develop a sustainable production chain. Although several advantages of lipases, the process stills expensive and unproductivity with low enzyme stability, mass transfer limitation and low reaction rates. As a solution for these drawbacks, physical technologies like ultrasound (US) emerged as an alternative for biocatalysis process intensification contributing for improve efficiency and productivity in lipase catalyzed reaction. This review presents recent advances and some technical aspects of fatty acids products, SSF process and lipase catalyzed reactions assisted by US.

RESUMO

Óleos e gorduras vegetais e animais representam a principal matéria-prima renovável na indústria oleoquímica com importância essencial no desenvolvimento da bioeconomia mundial. Vários produtos comerciais a partir de alimentos, produtos químicos e indústria farmacêutica apresentam triacilgliceróis, ácidos gordos e outros compostos lipídicos na sua formulação. A lipase representa um biocatalisador inovador para obtenção de produtos graxos comerciais devido à sua alta especificidade, produtos de alta pureza, biodegradabilidade, não oferecendo nenhuma contaminação química aos produtos finais em comparação com catalisadores químicos. A lipase pode ser obtido por fermentação em estado sólido (FES), que é também uma tecnologia verde que utiliza subprodutos de extração de óleos vegetais, como matérias-prima, gerando uma possibilidade de desenvolver uma cadeia de produção sustentável. Embora várias vantagens das lipases, o processo continua caro e improdutivo devido à baixa estabilidade da enzima, limitação de transferência de massa e baixas taxas de reação. Como uma solução para estes inconvenientes, as tecnologias físicas, como ultrassom (US) surgiram como uma alternativa para a intensificação do processo de biocatálise contribuindo para melhorar a eficiência e produtividade na reação. Esta revisão apresenta os avanços recentes e alguns aspectos técnicos dos produtos de ácidos graxos, processo FES e reações catalisadas por lipase assistidos por US.

1. Introduction

Fats and oils represent one of most used renewable materials for chemical products manufacture, besides its importance on human feeding. Its use on biodiesel fuel creates a necessity on increase the agriculture production in all world to meet demand in transport fuel market. The diversity number of products with fatty acids in its composition increases demonstrating its importance on a bioeconomy development.

Agriculture covers almost a quarter of the territory of Brazil and is expected to expand due to growing demand for food, animal feed and raw materials for liquid biofuels (Fao, 2009; Lossau *et al.*, 2015). Consequently, a proportional increase is estimated to generate agricultural and forestry waste. The solid-state fermentation (SSF) process has the potential to develop new applications using the bioconversion of agro-industrial waste into biofuels and other value-added products (Farinas, 2015) as enzymes like lipases.

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) have gained recognition as biocatalysts since the nineties due to their high activity, no co-factors requirement, organic solvents synthetic activity and, mainly, for wide substrate specificity and mild operating conditions (Ravelo *et al.*, 2015). Lipases have potential for catalyze reactions to obtain fatty acids (triacylglycerol hydrolysis), esterification, transesterification and alcoholysis. This broad of reactions allow obtaining several compounds with high value for commercial application such as omega-3 rich monoacylglycerols (Solaesa *et al.*, 2016), flavor compounds (Wang *et al.*, 2016), propylene carbonate (Yadav, Hude and Talpade, 2015), poly-ϵ-caprolactone (Chew *et al.*, 2015) and ibuprofen monoester (Ravelo *et al.*, 2015) for example.

Lipase catalyzed reactions present some limitations as low stability and low rate conversion, high cost enzymes and mass transfer restrictions due to reactions systems with immiscible reactants. Besides the development of new enzymes with improved characteristics, another approach to solve these limitation is the process intensification. Physical technologies such as microwave and ultrasound (US) have successfully improved mass and heat transfer on lipase performance for different reaction systems and products (Costa *et al.*, 2014; Teixeira, Madeira Junior and Macedo, 2014; Tomke and Rathod, 2015; Yadav, Hude and Talpade, 2015). The US technology permits to overcome the mass transfer resistance with higher efficiency than conventional agitation system, besides the cavitation phenomena improves catalysis. The application of US for lipase reactions is a green alternative to develop new bioprocesses with eco-friendly characteristics (Lerin *et al.*, 2014).

2. Bioeconomy

One of the most important targets of the green chemistry and the sustainability, besides the residue generation decrease and toxic substances use, is the substitution of nonrenewable natural resources (e.g. petrol and mineral coal) through renewable biomass application as raw material for chemical commodities production. These concepts are part of bioeconomy essence, which is based on plants capability for solar energy utilization in chemical synthesis, using carbon dioxide and water as substrates through photosynthesis. The second generation bioeconomy is based on agricultural biomass utilization for food, animal feed, biofuels and chemicals (Sheldon and Sanders, 2015).

These biobased chemical products have specified markets for commercialization on specialty and fine chemistry areas, however, the applications for raw chemical products must grow. The citric acid and the ethanol, for example, are considered bulk biochemicals, while enzymes, monoclonal antibodies and pure enantiomer compounds as the L-amino acids are excellent or special biochemicals. Like in others sectors, the performance and the demand for consume are decisive factors. The consumer's preference could be an important factor for such markets as cosmetics which currently have a bio-based compostion (Kircher, 2014).

Historically, vegetable and animal fats and oil always were the renewable raw material most used by chemical industry (Biermann *et al.*, 2011), being for surfactants, cosmetics and lubricants. Moreover, vegetable oils have been used by decade in formulations of paints, as coating materials and resins (Meier, Metzger and Schubert, 2007). Vegetable fats as rapeseed, palm, coconut, palm and sunflower oils have different fatty acids, triacylglycerol profiles with different carbon chain distribution and the utilization of these resources for industrial chemicals production, like biodiesel, is still considered a political discussion with divided opinions. For increasing the large-scale biodiesel production, the industrial use of edible can be a problem due to its possible competition with food industry, generating an increase feedstock prices and cause a possible food shortage. As a solution, non-edible food emerged as an alternative. Another possible problem is related to the environmental impacts on land use for planting and its changes for emissions of greenhouse gases, biodiversity, water use and energy balance. Geraldes Castanheira *et al.* (2014) evaluated the sustainability of the biodiesel production chain in Brazil and concluded that environmental impacts can be reduced by increasing agricultural productivity, diversifying types of raw material, adopting appropriate policies, aiming at an integrated optimization of food production and bioenergy through the agro-economic-ecological zoning, allowing the appropriate use of land for each purpose.

The basic products, such as fatty acids, fatty alcohols and esters with different physical properties have various types of applications in various areas depending on the distribution of carbon chain. Alkyl chain on C12 band represents important raw material for detergents as sodium dodecyl sulfate, for C18 makes part on lubricants formulation (isoamyl oleate). Natural fatty alcohols produced by heterogeneous catalysis of high-pressure hydrogenation between methyl esters or fatty acids. Unsaturated fats and oils can be catalytically modified by functionalization, oligomerization, oxidation or metathesis. On this way, new functional groups are introduced on the oleochemical substrate. The carbon chain size could be modified and new products with different characteristics and different properties are available for different application areas due to catalytically variations of fats and oils (Behr, Westfechtel and Pérez Gomes, 2008).

These various numbers of commercial applications such as detergents, lubricants, cosmetics and biofuels show the current stage of fatty acids and triacylglycerols appreciation. The direct conversion of fatty acids or triglycerides mixtures allows an increased amount of possible products generating a vegetable oils appreciation as a raw material for new biopolymers production for example (Deuss, Barta and De Vries, 2014). Most of these applications have in common a first hydrolysis step for the use of free fatty acids. Triglycerides and fatty acids are used for production of various chemical compounds of commercial interest as hydroxylated fatty acids (Cao and Zhang, 2013), organic phase change material (He *et al.*, 2015), cosmetic components as emulsifiers, emollients , detergents , thickeners (Ansorge-Schumacher and Thum, 2013), antioxidants such as phenolic esters (Roby *et al.*, 2015), adhesives (Agirre *et al.*, 2010), polyester (Gobin *et al.*, 2015), antibacterial coatings (Kumar *et al.*, 2008), organogels (Sagiri *et al.*, 2015) and lubricants (Kamalakar *et al.*, 2014).

With the intense development in the last decade for the organic synthesis, catalysis and biotechnology using plant oils and oleochemicals, new applications emerged for fatty acids and plant triglycerides. Among them, the omega- functionalization of fatty acids containing unsaturation in the alkyl chain, application of olefins metathesis reaction and the "de novo" fatty acids synthesis using various renewable carbon sources. Among other technologies are used for processing the microorganisms or enzymes are divided into three application focus: biotransformation of oils and renewable fats; biotransformation of intermediaries such as alkanes in fatty acids; and "de novo " synthesis of fatty acids or triglycerides using carbon sources such as glucose (Biermann *et al.*, 2011).

Lipases are not the only enzymes that modify lipids. Phospholipase, P450-monooxygenases and other biocatalysts are studied with respect to their lipid modification capacity. They are used because of quimo- , regio-; and stereoselectivity; apart from mild operating conditions compared to chemical processes (Bornscheuer, 2014).

3. Lipases

Lipases are triacylglycerol ester hydrolases (EC 3.1.1.3) which have the ability to catalyze hydrolysis reactions as in the synthesis of triglycerides. However , its specificity is not restricted to the ester linkage of triglycerides but also various types of compounds having ester bonds with carboxylic acids different from glycerides (Jensen, Galluzzo and Bush, 1990; Malcata *et al.*, 1992). This dynamism enables its application in different biochemical reactions including esterification, transesterification, interesterification, acidolysis, aminolysis, alcoholysis, acylation and racemic resolution production (Gutarra *et al.*, 2009; Yang *et al.*, 2010; Salihu and Alam, 2015). Its commercial application expands to biofuels, synthetic organic compounds, detergents, perfumes, cosmetics, leather processing, pharmaceuticals, medical diagnostics, food and feed (Gandhi, 1997; Masomian *et al.*, 2013; Salihu and Alam, 2015).

These enzymes can be obtained from microbial, plant and animal sources, however, commercially, microbial are more advantageous because the possibility of obtaining it by fermentation with higher productivity and lower production cost. These microbial sources may be fungi, bacteria and algae that produce lipases with unique characteristics with the possibility of industrial applications (Hasan, Shah and Hameed, 2006)

The evolution of extraction and purification methods of proteins as well as genetic engineering and subsequent cloning is responsible for lipases development and optimization to economically viable commercial applications as a specific alternative to chemical processes. Environmental concern has also been made to develop various types of applications due to the fact that reactions with lipases have a closer reaction mechanism of existing in nature for the metabolism of human beings. Therefore, the associated mechanisms and processes are considered more environmentally sound than chemical synthesis. Other advantages include its specificity of lipases (stereospecificity, selectivity) is considered greater than the inorganic catalysts, generating less amount of by-products and catalytic efficiency, resulting in lower activation energies and, consequently, a need for milder process conditions reducing the cost of energy and biocompounds thermal degradation (Paiva, Balcao and Malcata, 2000). The

specificity and selectivity are considered major advantages of lipases compared to the chemical catalysts. They can be modeled using the molecular properties of the enzyme, the structure of the substrate and factors affecting the binding of the enzyme with the substrate. Due to this high substrate specificity, is necessary a deep knowledge of lipase's substrate characteristics to find the ideal for use in the reaction (Castro *et al.*, 2004; Poppe *et al.*, 2015). Considering that oils and fats used in reactions have different compositions, a new strategy is employed to increase the productivity of the reactions, like the combined use of different lipases, which exhibit different specificities for substrates. This approach purposed by Alves *et al.* (2014b) is based on the fact that biocatalytic mixture composition of different lipases could be more effective on heterogeneous substrates than using isolated specific lipases (Alves *et al.*, 2014b; Poppe *et al.*, 2015).

The solvent system determines the best type of lipase indicated for a given reaction. Tolerance, stability and compatibility with different reaction media are key features for each specific type. It is these properties that determine its effectiveness in the application for waste treatment, leather processing, flavor synthesis, biodiesel and detergent formulation. The lipases catalyze organic reactions which have different substrates polarities thus different solubilities. Esterification reactions between fatty acids and alcohols have a net problem of immiscibility, which hinders the diffusion of lipase and the substrates in the medium, thereby reducing the mass transfer and the reaction conversion yield. As a solution, many studies and processes are using organic solvents, such as hexane, to facilitate the solubilization and diffusion of reaction components (Salihu and Alam, 2015).

Organic solvents have high ability to dissolve hydrophobic compounds, change the thermodynamic equilibrium towards the direction of the synthesis rather hydrolysis; low contamination, lipase specificity modification, ease of extraction and reuse of enzymes compared to the aqueous medium (Doukyu and Ogino, 2010; Salihu and Alam, 2015).

Different applications use lipases with improved catalytic potential. The wild microbial strains offer good characteristics, however, for use in reactions to specific lipases it has low yield and high deactivation. Molecular cloning techniques and suitable expression of proteins have been widely used to improve the catalytic performance of the lipase (Zheng, Baumann and Reymond, 2004; Salihu and Alam, 2015). Other approaches are also known to enhance the catalytic effectiveness of lipases solvents such as engineering, directed evolution and mutagenesis (Jaeger *et al.*, 2001; Salihu *et al.*, 2011; Salihu and Alam, 2015).

Current lipase commercial applications aimed at the beverage, dairy and baking markets. The largest market region is North America, followed by Europe, Asia- Pacific and the rest of the world (Micromarketmonitor, 2015). Novozymes (owner of 48 % of the global market for industrial enzymes) launched two new products in 2014 with new lipase formulations: one for application in detergents (Lipex®) and one for biodiesel (Eversa®) (Novozymes, 2015).

The lipase activity is a function of the interfacial medium composition and simultaneous changes in the enzymatic conversion. Inhibition of lipase is attributed to hydrolysis of the substrate and not to the desorption of the enzyme as was previously thought (Reis *et al.*, 2009). To prove this theory, experimental and theoretical modeling related to the properties of triglycerides lipolysis products confirmed that Sn-2 monoglycerides quickly increased its concentration in the interface and cast out the free fatty acids, di- and triglycerides that were in low total concentration. Some results also demonstrated that Sn-2 monoglyceride also expelled regioespecífics Sn-1.3 lipases from oil-water interface. Sustained hypothesis is that the lipase forms a sub-layer in the aqueous phase located just below the interface covered by monoglyceride. Triacylglycerols form the apolar phase of the system and the Sn-1.3 regioespecífics lipases involved in lipolysis are removed from the reaction zone, which may be described as a self-regulatory mechanism. This hypothesis has been validated by an lipolysis experiment that was reduced by 90% when added Sn-2 monoglyceride in the system (Reis *et al.*, 2008; Reis *et al.*, 2009).

The binding energy of the enzyme substrate is the greater resultant force for enzyme activity. For this connection occurs, there must be a desolvation in the active site of the enzyme. Many enzymes have hydrophobic active sites facilitating the partition of the water of hydrophobic substrates to the active site. When this water is replaced by an organic solvent, the hydrophobic substrate is thermodynamically stable (Klibanov, 1997; Doukyu and Ogino, 2010).

3.1.Immobilized lipases

For all biocatalytic routes, the cost of the catalysts and the process must be compared to the chemical route. Moreover, one should consider the costs of raw materials required for organic synthesis and the preparation of the catalyst. There are three major factors to justify the immobilization of enzymes: increased stability; increase in the specific volume in the concentration of biocatalyst; recycle and separation simplification of the biocatalyst (Liese and Hilterhaus, 2013).

The interactive studies of molecular modeling, molecular biology and biochemistry can lead to a more rational approach to stabilize enzymes by immobilization. Depending on the structure and mechanism, most immobilizing control methods will be developed, resulting immobilized enzymes with higher activity, as has been shown recently by Novozymes (Svendsen *et al.*, 2007; Liese and Hilterhaus, 2013). **Erro! Fonte de referência não encontrada.** shows the various and most used methods of immobilization for lipases.

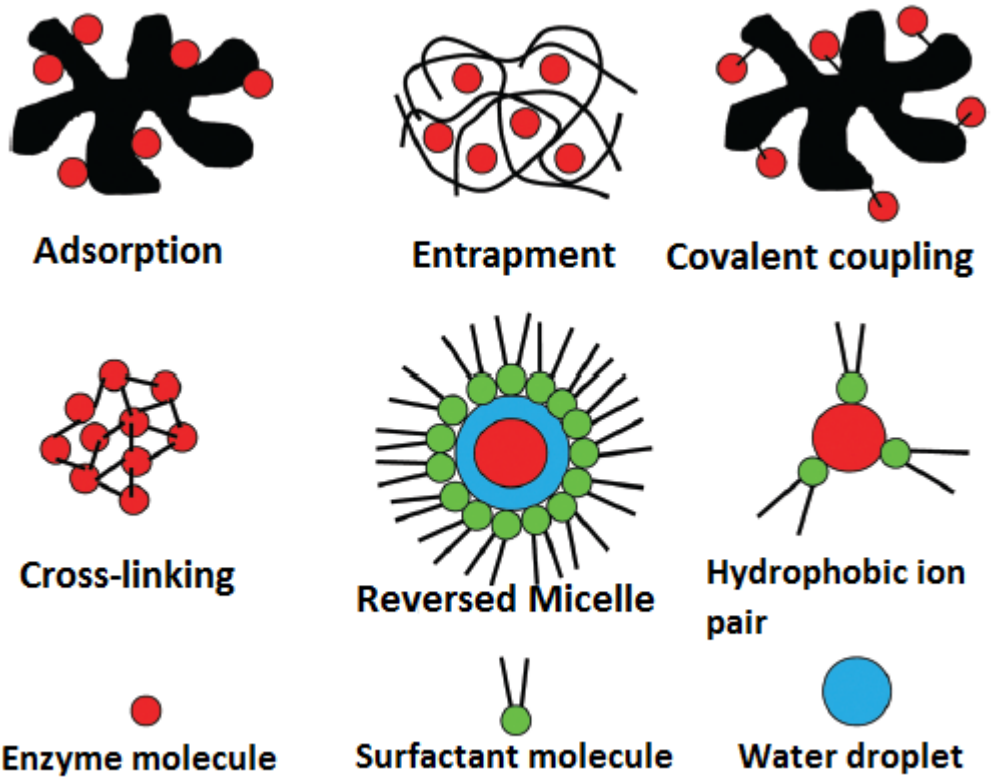

Figure 2 Schematic representation of immobilization methods [adapted from Adlercreutz (2013) with permission from The Royal Society of Chemistry].

From the standpoint of producers, to consider the application of the immobilized enzyme as feasible, the production cost should favor a new application or offer other advantages compared to the enzyme in a soluble form. The fact of the reusability of the immobilized enzymes not directly benefits producers, however, encourages consumers to buy the product in immobilized form. In the case of enzyme producer, the gains can not be restricted only to sales but also with the intellectual property generated in the development of new products and processes obtained from the use of immobilized enzymes. Otherwise , consumers who need the immobilized enzyme must purchase the enzyme in soluble form and immobilize it by themselves, or outsource for any specialized company (Thayer, 2012; Dicosimo *et al.*, 2013). Traditional chemical companies increased their production and immobilization of its own

enzymes in parallel to the development process . However, the demand for the product must be chosen carefully to ensure that the immobilized enzyme is really needed and provide reasonable return (Thayer, 2012; Dicosimo *et al.*, 2013).

Some parameters influence the structure of an immobilized enzyme in the biocatalytic process. Enzymes must suit the reaction conditions such as temperature, solvent, substrate concentration and reaction type. Moreover, it is necessary to know the breakdown of the enzyme attached to the surface of support for consolidation of the enzyme stabilization knowledge. Therefore, it is necessary to develop biology tools and molecular chemistry to optimize the attachment of the enzyme on the surface of support combined with modern analytical technologies strategies to increase the activity and stability of the enzyme. Also the patterns should be identified in the substrate transport phenomena through engineering processes to increase the yield of biocatalyst with high activity and stability (Liese and Hilterhaus, 2013).

Despite the current challenges, the development and commercial large-scale application of immobilized enzymes will continue to expand to chemical production as well as for applications to consumer. A more comprehensive understanding of the factors that justify the implementation of immobilized enzymes will be responsible for guiding the new generation of biocatalysts can build new positive results into the industry (Dicosimo *et al.*, 2013). One possibility is the combination of enzymes and inorganic catalysts for creating multi - catalytic systems for the synthesis in a stage of chemical and materials.

3.2.Recent applications

3.2.1. Structured lipids

New triglycerides can be obtained by replacing the fatty acids or by changing their position within the molecule. These modified fats are known as structured lipids and can be produced by chemical or enzymatic route and functional properties for medical applications, food, cosmetics and lubricants (Khodadadi *et al.*, 2013; Paula *et al.*, 2015).

The interest of enzymatic interesterification for synthesis of structured lipids with specific functional properties has grown due to the advantages of enzymatic route on the chemical, as milder process conditions, allowing less unit operations in the process, lower capital investment and reduced degradation of antioxidants (De Martini Soares *et al.*, 2013; Paula *et al.*, 2015).

Structured lipids are already commercially used and the example cocoa butter equivalent is the best known, where palmitic, stearic and oleic acids account for more than 95%

of the total fatty acids. The commercial production of this cocoa butter synthesized by lipase from palm oil and stearic acid was initially patented by Unilever (Coleman and Macrae, 1981) and Fuji Oil (Matsuo *et al.*, 1983) in the transesterification or acidolysis process of tristearin or stearic acid low cost oils (Biermann *et al.*, 2011).

Triglycerides structured with a standard definition of the fatty acids position are important compounds for various applications in human nutrition. Such lipids containing medium chain fatty acids at the sn -1 and sn -3 positions and a long chain preferably at the sn -2 position. They are used now to treat patients with pancreatic insufficiency as well as providing quick energy. Another example is Betapol® that is used for infant nutrition. It contains oleic acid at the sn -1 and sn -3 position and palmitic acid at the sn -2 position (OPO). The enzymatic production is beneficial with respect to chemical synthesis due to increased chain length and regiospecificity of the fatty acid by lipase position reached that can be used to generate pure products with desired nutritional properties. Betapol is manufactured through the transesterification of tripalmitin with oleic acid using *Rhizomucor miehei* lipase (Novozyme RMIM®). However, the product contains only 65% of palmitic acid at the sn -2 position (Biermann *et al.*, 2011).

3.2.2. Cosmetics

The lipids present in the bilayer of the present stratum corneum into the epidermis are composed of fatty acids, ceramides and cholesterol, contributing as a barrier to skin permeability. Normal skin cleansing treatments are associated with damage to the stratum due to cleaning surfactants can cause breaching of lipid layer, extracting it from the skin. These disrupted cells are associated with a variety of pathological skin conditions (Ananthapadmanabhan, Mukherjee and Chandar, 2013).

The application of lipases for cosmetic products and ingredients synthesis is already considered, especially when it comes from the demand for green chemistry and natural products. However, lack of specific enzymatic preparations with respect to the substrate and stability reducing process cost effectiveness hampers the commercialization of these processes. To facilitate research and development, academic and industrial areas have focused on resolving these limitations when many have already been resolved. Thus, it is expected to further implementation of catalysis with lipases in the production of cosmetics (Ansorge-Schumacher and Thum, 2013).

The Brazilian cosmetic company Natura S.A has developed and patented an enzymatic esterification process of fatty acids derived from Amazonian oils and butters for esters production used in cosmetics. Cupuaçu butter, sapucainha, ucuúba, murumuru, palm olein, pataua oil, tucuma oil, inaja , passion fruit, pequi were esterified with caprylic alcohol, isoamyl, lauric, myristic, stearic, sorbitol, glycerol and others polyols. The process consists in the addition of lipase in stirred reactor at 40-80 °C, water removal system and separation by vacuum filtration (Teixeira and Da Silva, 2011).

The organic reactions of alcoholysis, esterification and transfer of groups acis from esters to other nucleophiles like amines or thiols involving fatty acids are important characteristics of lipases to obtain compounds used in the cosmetic industry. Its compatibility with the lipophilic medium and non-aqueous emulsions is advantageous for the process as well as its high stereoselectivity and considered stability are factors that favor their inclusion in the cosmetics processment. Such application can be divided into two forms: as an ingredient in the formulation or as biocatalysts for synthesis of specific chemical (Ansorge-Schumacher and Thum, 2013).

4. Solid state fermentation

Biotechnology uses microorganisms for obtaining products generated from its primary and secondary metabolites. Its conversion capacity using solid substrates was essential to solid-state fermentation (SSF) development. In this case, molds and yeasts are microorganisms that have been most widely used because of its ability to grow at low water activity required for the system (Singhania *et al.*, 2009). Although SSF is defined as bioprocess conducted with low free water, the substrate must have sufficient moisture to allow the growth and metabolic activity of the microorganism. The solid matrix may be the carbon source and other nutrients or may be an inert material as a support for growth (Barrios-González, 2012). The SSF is defined as a three-phase heterogeneous process between solid, liquid and gas, which occurs in the microbial culture capable of converting substrates into many types of products. Due to its peculiarities, such as low energy demand, associated with high product yield and low waste water generation with less chance of bacterial contamination, SSF has been a major focus in the development of bioprocesses for the past two decades (Thomas, Larroche and Pandey, 2013).

The economic viability and environmental sustainability of the SSF is based on the use of agro-industrial byproducts as inexpensive substrates. So, it has gained credibility among various industrial corporations with several advantages in applications traditionally held by

submerged fermentation (SmF). Perhaps the biggest drawback of SSF in relation SmF is the insufficient knowledge of the physiology and growth of fungi and other microorganisms on solid nutrient substrates and complex matrices (Barrios-González, 2012).

Despite the increased attention be directed to the discernment of biological mechanisms, most of research in the SSF is directed to the search for substrates and optimization of different products processment and new applications. The survey is usually directed by the engineering and knowledge of fungal physiology, mainly from the viewpoint of the main fermentation parameters effects (temperature, humidity, aeration) on microbial growth. This has been the basis for mathematical models, which are useful for scale up, as well as process control and bioreactors design (Barrios-González, 2012). These parameters are considered important to the development of any bioprocess, and for SSF, which include the selection of substrate and microorganism, optimization of process parameters and purification of the final product is considered as a challenge. The development of predictive models that establish a relationship between microbial physiology with the interaction between environmental physicochemical factors is considered the ultimate goal for the development and control of bioprocesses. Factors, such as temperature, pH, aeration, water activity, moisture, bed properties and nature of the solid substrate, interfere with microbial metabolism. Among these factors, humidity and nature of the solid substrate used are the most important affecting the SSF processes. The selection of moisture depends on the nature of the substrate (absorption characteristics), and the type of microorganism employed. Fungi require low humidity, 40-60% may be sufficient, but the selection of the substrate depends on several factors mainly related to cost and availability and must involve the selection of various agro-industrial wastes (Singhania *et al.*, 2009).

Oil seed cakes obtained as residue from the vegetable oil extraction have been used to produce lipase by SSF. This residual oil in cake serves as an inducer (Singhania, Soccol and Pandey, 2008). Various agricultural by-products have been reported as effective substrates for lipase production including brans (wheat, rice, soybeans), oil cakes (soybean, olive , sesame, babassu) and bagasse (sugarcane) (Salihu *et al.*, 2012).

The scale up, the purification of the final products and estimation of biomass are major challenges for research. SSF scale up has been the limiting factor; however, recently, with the advent of biochemical engineering, various bioreactors have been designed to develop a process on an industrial scale, in addition to online monitoring for improve the process control of various parameters such as the system heat and mass transfers (Singhania *et al.*, 2009).

Moreover, the biomass determination is a major challenge because the existence of only indirect methods for determining biomass, like measurement of glucosamine, ergosterol, DNA, modification of dry weight and CO_2. Most of them have their limitations, but recently, digital image processing have been developed as a tool to quantify biomass in SSF (Singhania *et al.*, 2009).

The SSF has evolved in Brazil over the past 15 years with the development of research in processes and applications. The Brazilian economy, characterized as strongly with agro-industrial production of coffee, sugarcane, soy, consequently also features a high generation of agricultural byproducts. From these characteristics, brazilian groups surveyed headed by the biotechnological processes laboratory of the Federal University of Parana (UFPR) has been devoted to the SSF processes development using agricultural by-products as substrates for protein enrichment, biological detoxification , production of biomolecules such as enzymes , organic acids , aromatic compounds foods, biopesticides, edible mushrooms, pigments, xanthan gum and hormones (Soccol and Vandenberghe, 2003). With this investment in research in the field, Brazil currently has become one of the countries that most published in the Scopus database on SSF area.

4.1.Solid-State Fermentation applications

SSF is a promising technique for the industrial development of bioprocesses and bioproducts. Its recent advances include the production of enzymes, biopolymers, pigments, secondary metabolites with potential for large-scale production with technical and economic feasibility. The studies of production escalation on bioreactors with different configurations apparently have shown that the best results were achieved using tray reactors. Data generated in these studies with regard to heat and mass transfer effects are used for mathematical modeling in the design process. However, the perspective is focused on the use of agricultural byproducts as substrates and different microorganisms to obtain different products (Thomas, Larroche and Pandey, 2013).

Agricultural waste or byproducts constitute a major proportion of agricultural production worldwide (almost 30 %). They are comprised of lignocellulosic materials, fruit and vegetable waste, the sugar industry waste as well as the meat industry. These agricultural byproducts presents high concentrations composition of bioactive and nutraceutical compounds such as polyphenols, carotenoids and fiber. It is a high-value biomass and has the potential to solve the problem of animal nutrition and the global demand for protein and calories (Ajila *et al.*, 2012).

As an alternative to generate value to these agricultural byproducts, SSF is being studied for use in the generation of various compounds and products using these agricultural byproducts: microbial lipids for biodiesel production using lignocellulosic biomass (Cheirsilp and Kitcha, 2015), biohydrogen by anaerobic pathway (Han *et al.*, 2015b; Motte *et al.*, 2015), mushrooms for medicine and food usin sunflower sedds as substrates for fermentation (Postemsky and Curvetto, 2015), biopolymers (Tang *et al.*, 2015), hypocholesterolemic drugs (Dikshit and Tallapragada, 2015), organic acids (Mondala, 2015), biobleaching pulp in the paper industry (Sharma *et al.*, 2015), biofertilizers production (Lim and Matu, 2015), biosurfactants (Velioglu and Ozturk Urek, 2014), bioremediation (Waghmare *et al.*, 2014), methane production through food waste bioremediation (Uçkun Kiran, Trzcinski and Liu, 2015), use of food residue for production of proteins, lipids and aromas (Aggelopoulos *et al.*, 2014), conversion of lignocellulose material into lipids (Kitcha and Cheirsilp, 2014), production of β-glucosidase in soybean flour for deglucosilation of isoflavones to bioactive aglicone (Handa *et al.*, 2014), solid probiotic foods development (Rodríguez De Olmos, Bru and Garro, 2015), fermented coffee (Li, Li and Li, 2010), xantam gum (Bilanovic, Malloy and Remeta, 2010). Table 1 summarizes some of the recent studies for the different applications of the SSF.

Among the new studies for producing enzymes by SSF includes: phytase production (Gaind and Singh, 2015), alpha- amylase (Sahnoun *et al.*, 2015), use of mixtures of solid agricultural byproducts as substrates for phytase production (Suresh and Radha, 2015), simultaneous production of phytase and tanase in orange peel (Macedo and Madeira, 2013) and simultaneous detoxification of castor bean cake (Madeira Jr, Macedo and Macedo, 2011); Continuous production of enzymes using autotrophic fungus (Mueller, Wilkins and Prade, 2015); production of lignocellulolytic enzymes using grape skin and residue of olive (Nalini and Parthasarathi, 2014); laccases production and application in the synthesis of gold nanoparticles (El-Batal *et al.*, 2015).

Table 1 Recent studies on SSF applications.

Microrganism	Substrate	Product	Time (hours)	Temperature (ºC)	Reference
A. awamori e A. oryzae	Food Residues	Glucoamylase and protease	24	30	(Han *et al.*, 2015a)
Grifola sordulenta	Sunflower Seed	Mushrooms	1080	24	(Postemsky and Curvetto, 2015)
Purpureocillium lilacinum	Shrimp residue	Chitosanase	96	32	(Nidheesh, Pal and Suresh, 2015)
Amycolatopsis mediterranei	Ragi bran	Rifamycin	216	30	(Nagavalli *et al.*, 2015)
Paecilomyces variotii	Orange peel	Flavanones	48	32	(Madeira Jr *et al.*, 2014)
A. oryzae	Soybean bran	α-amylase	-	30	(Sahnoun *et al.*, 2015)
A. flavus	Mustard cake	Phytase	96	37	(Gaind and Singh, 2015)
L. paracasei e *B. longum*	Soybean bran	Solid probiotic	24	37	(Rodríguez De Olmos, Bru and Garro, 2015)

Recent advances focus on developing processes and bioreactors for intensification of SSF to increase production scale. Among these advances, we can mention: development of fixed-bed bioreactors for the production of amylase, cellulase, xylanase and proteases (Castro, Castilho and Freire, 2015). An integrated process for lignocellulolitic enzymes production by SSF and subsequent use for hydrolysis of biomass and SmF for ethanol production (Machado *et al.*, 2013). Novozymes patent for development of enzymes production process utilizing tray reactor with three layers of substrates (Ranganathan, 2014), large-scale fermenters (Luth and Eiben, 2009), novozymes automated fermenter (Andersen *et al.*, 2014).

4.2.Brazilian scenario for SSF and lipase production

Agricultural waste are quite available and considered as raw material at low cost. The use of this material has been studied to produce lipases. The cakes derived from the extraction of vegetable oils are commonly used as substrates for lipase production by the SSF. The composition of these substrates, as the microorganism, determine the production capability (Salihu *et al.*, 2012).

The production of lipases by SSF has been gaining prominence in research and the number of publications is increasing every year demonstrating the potential of this area (Figure 1).

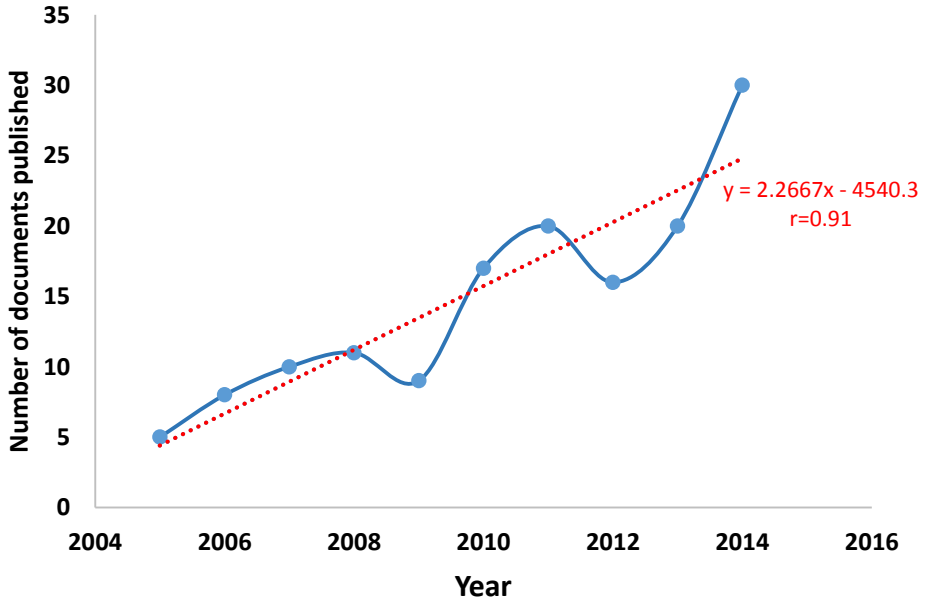

Figure 1 Number of papers published per year on solid state fermentation for lipase production (Scopus, 2015).

Brazil and India are the countries that had invested in the production of lipases by SSF and are the two largest publishers in the area (Figure 2). The two countries have fairly representative agrarian economies on the world stage and therefore great potential for application of technology.

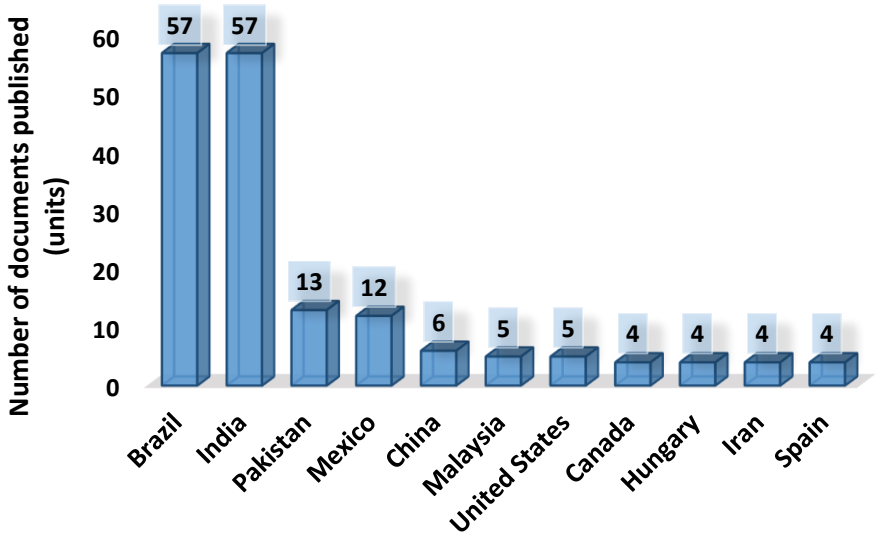

Figure 2 Number of documents published by country for SSF lipase production (Scopus, 2015).

Recent advances in research for the production of lipases by SSF points to different focuses and approaches. Among these, the use of agricultural byproducts such as mixing the substrate with different supply nutrients to increase productivity (Fleuri *et al.*, 2014; Salgado *et al.*, 2014) and production of thermostable enzymes (Ávila-Cisneros *et al.*, 2014), A study of olive meal supplementation was conducted to produce protease and lipase of *Candida utilis* by SSF with various sources of nitrogen and carbon as maltose, starch, yeast extract. Moftah *et al.* (2012) observed that the best results were obtained with maltose, while glucose and oleic acid obtained the lower yields of lipase and protease activity.

Biodiesel production using non-food sources of vegetable oils have stimulated the growth of research for detoxification of the byproducts generated as oil cakes and reused as animal feed. Among the existing sources in Brazil stand out from the castor bean (*Ricinus communis*) and *Jatropha curcas*.

Despite having high protein concentration, jatropha has toxic phorbol esters, anti - nutritive factors such as phytate, trypsin inhibitor, lecithin and saponin, therefore, can not be used by the food industry and feed. Comparison SSF and SmF study using *B. licheniformis* performed better in SmF to reduce phorbol esters (62 %), phytates (42%), trypsin inhibitor (75%) and only lecithin was not reduced (Phengnuam and Suntornsuk, 2013). A recent study of detoxification of *Ginkgo biloba* L. residue by SSF showed that in addition to detoxification capacity, *Candida tropicalis* and *Aspergillus oryzae* are microorganisms capable of producing cellulase and protease simultaneously, with potential use of the fermented material for animal feed (Zhou *et al.*, 2015). In addition to anti-nutritional or toxic factors, the discharge of this

material in the environment becomes a problem. Therefore, many studies focus on detoxification of this material and the possibility of use as a substrate for obtaining bioproducts. One such study aimed on detoxification and lipase and protease production by *Aspergillus versicolor* CJS -98 through *Jatropha* sp.cake in SSF process (Veerabhadrappa, Shivakumar and Devappa, 2014).

They used a new strain of *Streptomyces fimicarius* able to degrade 97% of phorbol esters in *Jatropha* cake, that was evaluated for feeding carp and has proved to be non-toxic. Joshi, Mathur and Khare (2011) also examined the SSF process of J*atropha* residue, however, using a strain of *Pseudomonas aeruginosa* and obtained the complete degradation of phorbol esters in nine days.

Another detoxification studies of castor bean , led by Brazilian groups Godoy *et al.* (2009) and Godoy *et al.* (2012) evaluated the SSF detoxification of castor beans using a *Penicillium simplicissimum* strain and they found that after 72 hours of fermentation the fermented material did not inhibit cell growth in vitro. Furthermore, they observed a relationship between the protease production and detoxification of materials, besides the ability to produce lipase.

4.3. Lipasic fermented solid (LFS)

In addition to the immobilization, a new application mode that has been recently studied for lipase is the lipasic fermented solid (LFS), which is used for biodiesel synthesis. The LFSs do not require purification processes and therefore can be considered as naturally low cost immobilized biocatalysts (Zago *et al.*, 2014).

The lipase production by SSF shows an interesting possibility of application due to the system in which the microorganism grows is composed by solid particles of organic material with a free water low concentration. This allows for LFS acting as a support for the enzyme without the need for prior extraction in esterification reactions (Fernandes *et al.*, 2007). This approach has the objective of obtaining lower costs for lipase applications that do not require highly purified enzymes. The LFS is obtained by SSF and after fermentation is generally standardized for particle size, frozen and lyophilized only, without purification.

The first work reporting the LFS application was related to the kinetic resolution of highly enantioseletive fungal lipases produced by SSF (Nagy *et al.*, 2006). From this, others works have been studying the application of LFS, mainly for biodiesel synthesis.

Research conducted by brazilian groups have explored the use of LFS, such as Fernandes *et al.* (2007) which studied the enzymatic reactions of esterification and transesterification by LFS in organic medium. After that, some more advanced studies have reported the biodiesel synthesis in fixed bed column bioreactor using LFS produced by *Burkholderia cepacia* LTEB11 (Salum *et al.*, 2010). A more recent study uses a LFS hybrid process for producing *Acrocomia aculeate* acid oil biodiesel through the hidroesterification process using a plant lipase for hydrolysis and a LFS for esterification as low cost biocatalysts (Aguieiras *et al.*, 2014). Another study evaluated the hybrid process for production of waste acid soybean oil biodiesel by subcritical water hydrolysis process, followed by enzymatic esterification using LFS in packed-bed reactor. The fermented solid substrate consists of some agricultural residues, as was done by Zago *et al.* (2014) that produced a SFL from *Rhizopus microsporus* CPQBA 312-07 DRM in sugarcane bagasse and sunflower seed cake with aim on catalyze the corn oil ethanolysis. The results obtained were 91% conversion in 48 hours, obtained at 44°C using n-heptane as solvent. Another study using bagasse and sunflower seed as solid medium for fermentation evaluated the LFS produced by *Rhizopus oryzae* and *Rhizopus microsporus* for the interesterification of palm stearin, palm kernel oil and concentrate triglycerides enriched with ω3 polyunsaturated fatty acids getting results solid fat content suitable for margarine and "shortenings" (Rasera *et al.*, 2012). Besides the interesterification, hydrolysis was tested for effluent pretreatment by Rosa, Cammarota and Freire (2006). LFS produced by *Penicillium restrictum* using industrial waste babassu oil used to pretreatment of dairy effluent getting results in accordance with environmental legislation for fatty waste.

Research groups from other countries have studied the use of LSF as well. Among the published works we can mention: Direct biodiesel synthesis catalyzed by LSFof *Burkholderia cenocepacia* (Liu *et al.*, 2013) and SFL production by *Burkholderia cenocepacia* and application in optimization and kinetics of biodiesel synthesis (Liu *et al.*, 2014).

4.4.Recent Advances in SSF

Kumar *et al.* (2011) used greasy residue, Czapek -Dox medium and wheat bran as substrate for lipase production by *Penicillium chrysogenum* SSF. The determined optimal conditions were 30 °C, pH 7 and 1:1:2 (w/ w/v) fatty residue:wheat bran: Czapek -Dox medium. Silva *et al.* (2011) optimized the lipase production of *Penicillium brevicompactum* and *Penicillium verrucosum* by SSF in terms of hydrolytic activity and esterification and found that there was no correlation between the activity of hydrolysis and esterification.

Rigo *et al.* (2010) evaluated the lipase production by *Penicillium* P58 and P74 by SSFF using soybean meal, urea and soybean oil as substrates. Other studies assessed the conditions of extraction and concentration of lipases produced by SSF(Menoncin *et al.*, 2010) and the production of lipase by a brazilian wild strain of *Penicillium simplicissimum* by SSF using babassu cake, obtaining an enzyme with thermostability with maximum activity 50 °C , unusual among lipases derived from mesophilic fungi (Gutarra *et al.*, 2009).

Two strategies for inoculation were studied by Gutarra *et al.* (2007) for the production of *Penicillium simplicissimum* lipase by SSF using babassu meal as a substrate. Conventional inoculation with fungal spore was compared with pellets grown by inoculating into liquid medium and fermented cake. The pellets delay lipase production while the fermented cake had equivalent performance to the conventional inoculation method. Fermented cake under optimal conditions increased by 1.5 times to produce lipase with 10 times less than conventional spores inoculum.

One study evaluated the lipase immobilization of *Aspergillus niger* ATCC 1015 in matrices of structural fibers from *Carica papaya* wood and vegetable sponge *Luffa aegyptiaca*. Ethanolic formaldehyde, formaldehyde and glutaraldehyde were used for covalent "cross-link" of enzymes retaining 90 % of enzyme activity with glutaraldehyde after 11 replicates. The use of *Carica papaya* wood resulted in decrease on enzyme activity of 45.3 % while vegetable sponge resulted in increased activity in 21.4 % (Osho, Popoola and Kareem, 2014).

Ferrarezi *et al.* (2014) tested 54 thermophilic fungi for lipase production by selecting *Thermomucor indicae seudaticae* with higher yield. The fungus was immobilized in sponge bushing , however, the maximum activity was obtained at 40 °C .

Zubiolo *et al.* (2014) studied the lipase production of *Aspergillus niger* by pumpkin seed flour SSF and encapsulating it by the sol-gel matrix as immobilization method achieving 71 % yield. The enzyme had a change in thermal stability from 45 °C (free form) to 60 °C for immobilized. However, with the immobilization, the enzyme had a 31.3 % lower substrate affinity than for the free form.

Singh *et al.* (2014) obtained a thermo-alkaline-tolerant lipase produced by *Schizophyllum commune* ISTL04 FES using *Leucaena leucocephala* seed substrate with maximum activity at 60ºC and pH 11.

Zubiolo *et al.* (2014) made a comparative study of SmF and SSF for producing *Fusarium* sp lipase. In SmF, were evaluated sources of carbon and nitrogen as crambe, corn, flaxseed, olive and palm oil, ammonium sulfate, sodium nitrate, Triton X-100 and yeast extract. For SSF, sugarcane bagasse, castor bean pies, corn, soybean and crambe. Analyzing the results, they realized that to SmF, the best results were obtained with crambe oil, Triton X-100, ammonium sulphate and yeast extract. For SSF, crambe cake humidified with phosphate buffer. Estimating the cost/enzymatic activity, SSF with crambe cake and distilled water was 87.2 % cheaper than SmF.

A study evaluated the lipase producing *Candida rugosa* NCIM 3462 by SSF using pies of sesame, peanut and coconut as substrates. The best results were obtained from sesame cake (Rajendran and Thangavelu, 2013). Another study evaluated the lipase production of *Trichoderma harzianum* using sugarcane bagasse, castor bean and 1% olive oil (Coradi *et al.*, 2013). Bagasse was also evaluated in the production of *Rhizopus oryzae* lipase in tray reactor at 45 °C, humidity 80 % and 0.5 cm thickness bed. The results showed differences in activity between the position of each tray. The top had greater activity, following by the middle and the base achieving 215.16 , 199.36 and 52 , 64 Ugds $^{-1}$ respectively (Vaseghi *et al.*, 2013).

Dayanandan *et al.* (2013) evaluated the effects of each oil cake for the production of lipase *Aspergillus tamarii* MTCC 5152 by SSF obtaining the best results for wheat bran with addition of 2.5 % of sesame cake. The enzyme was applied to tannery waste fatty for biodiesel production.

5. Ultrasound (US) enzymatic reactions

The process intensification is essential for effective sustainability of the chemical process industry (Stankiewicz and Moulijn, 2000). This scaling can be done using multifunctional devices (Dautzenberg and Mukherjee, 2001; Stankiewicz, 2003), increasing reaction rates by using sophisticated equipment or new energy sources. New approaches are being developed for bioprocesses intensification, including the use of thin-layer reactors (Pellis *et al.*, 2015). Among the new sources of energy, the ultrasound (US) or the energy associated with the liquid flow can be used to generate the cavitation phenomenon resulting in an effective process intensification (Gogate, 2008).

US is a sound frequency above the human perception used for different purposes in different areas. It can be classified by the level and frequency of energy: 1) high and low frequency power (2-10 MHz), US known as diagnostics used for medical imaging and chemical

analysis; 2)low frequency and high power (20-100 kHz), used for cleaning, welding and sonochemistry (Rokhina, Lens and Virkutyte, 2009). Classified as a green technology due to its high energy efficiency, simplified instrumentation, decreased process time compared to traditional techniques, as well as economically viable performance (Suslick, 1990; Mason and Lorimer, 2002) .

One of the biggest advances in science is related to the ease and efficiency in conversion and translation of knowledge in technology and commercial products. US technology follows this trend and its application in food technology for processing, preservation and bioactive compounds extraction as well. US can provide a network of advantages in terms of productivity, yield and selectivity, with less processing time, better quality, besides being environmentally safe (Chemat, Zill E and Khan, 2011). The influence of the ultrasound on biological reactions started when Wood and Loomis (1927) observed a rapid denaturation of frogs and fish myoglobins that survived for 20 minutes. Biological molecules, both *in vivo* and *in vitro* are sensitive to high power ultrasound (20-40 kHz) suffering degradation from the effects of cavitation bubbles generation, responsible for the detrimental physical effects to the cells, as well as the production of free radicals. However, recent research is directed to the use of ultrasound in pulses, as sublethal doses, has shown positive results for use in bioprocesses. The mechanism of action has not been fully described and probably varies with the substrates involved, frequency and power of the US applied (Kwiatkowska *et al.*, 2011).

Sonochemistry is the science that studies the chemical reactions in which the molecules are under application of strong ultrasonic radiation (20kHz - 10MHz) (Suslick *et al.*, 1991; Gedanken, 2004). These radiation effects can be used in various commercial applications and especially in enhancing physical and chemical processes for chemical synthesis (increased rate of reaction and conversion yield), wastewater treatment (chemical complexes degradation as p-nitrophenol, rhodamine B, dichloromethane phenol); textile processing (increasing the efficacy of staining technique), biotechnology (standardization, water sterilization, cell disruption, to release intracellular enzymes and foam control in bioreactors), crystallization, polymer chemistry, extraction (Luche and Bianchi, 1998; Thompson and Doraiswamy, 1999; Gogate, 2002; 2008; Sutkar and Gogate, 2009).

5.1.General aspects

Moderate cavitation can not be destructive, with the possibility of combined application of enzymes in bioprocesses to increase the enzymes transport to the substrate surface resulting in the opening of the substrate surface to the action of enzymes as a result from

the mechanical energy generated by the cavitation. Furthermore, cavitation is increased in heterogeneous systems (Kwiatkowska *et al.*, 2011).

Cavitation is also the physical phenomenon responsible for sonochemical process. A number of theories has been developed for describing how a 20 kHz low frequency is able to break chemical bonds. All agree that the main event in the phenomenon is the creation, growth and collapse of a bubble that is formed in the liquid. The stage, which leads to bubble growth, occurs through diffusion of solute vapor inside the bubble volume. The last stage is the collapse of bubble, which occurs when the size reaches this maximum value (Gedanken, 2004). From this, it is considered the mechanism of "hot spot" that is the theory which explains why the chemical bonds are broken with the bubble collapse. This theory says that the collapse generates high temperatures (5000-25000 K). Since this occurs in less than a nanosecond, high cooling rates in excess of 10^{11} K / s are also achieved (Gedanken, 2004). This high energy released in this collapse is responsible for the breakdown or synthesis of chemical bonds in molecules.

In most works, proposed mechanisms of the US effects in enzymatic and microbial reactions are mostly based on theory than on experimental data. The theories are based on the physical effects caused by US as increased emulsification, providing better agitation and consequent mass transfer and mechanical stress. Other accepted theory suggests that US increases the cell membrane permeability and changes the surface potential, resulting in the calcium channels activation, weakening the cell wall, increasing the permeability and selectivity, accelerating the molecular transport or increasing the dissolution rate of insoluble compounds, increasing the mass transfer inside and outside the cell. For bi-phase reactions, it is suggested that US reduces the adsorption of organic compounds on enzyme surface, providing better processing and recycling of immobilized enzyme (Kwiatkowska *et al.*, 2011).

Vapor pressure, viscosity and surface tension are liquid phase properties that affects the cavitation phenomenon. With respect to vapor pressure, a low pressure is preferable due to gas formation in cavitation causes a greater energy dissipation out of the system, reducing the cavitation effect. With respect to viscosity, it is necessary that cavitation overcome the cohesive forces present in the liquid so it can experience the cavitation phenomenon. These forces increase the resistance to mass transfer and therefore require a larger energy to occur the phenomenon. Therefore, high viscosity liquids have a reduction in the cavitation intensity. Liquids with high surface tension, like water, have higher intense cavitation. Some theoretical studies suggest that hydrophilic and even organic solvents such as glycerol results in higher

intensity than acetone, ethanol or formamide. Furthermore, the cavitational activity can be increased by adding surfactants (Gogate, Sutkar and Pandit, 2011).

The cavitation is also responsible for the free radicals generation that contributes to catalytic reaction efficiency. Juretic *et al.* (2015) evaluated the formation of free radicals in water with the application of ultrasound probe through a batch and continuous flow. They observed that the formation of hydroxyl radicals in batch systems was directly proportional to the sonication time and the applied energy density. However, for continuous systems were inversely proportional. Comparing the system in batch and continuous flow was observed that sonication time and residence time have a greater effect on the formation of free radicals in terms of energy density.

The ultrasonic bath and the probe are the two most used application modes to generate ultrasound. In the reactions, it is classified direct sonication when using the probe or indirectly using the ultrasonic bath. In the bath, the transducer is placed at the base of the tank and the US is transmitted through the liquid. In this case, part of the energy is dissipated and is not absorbed by the reaction. With the probe, the transducer is in direct contact with the reaction medium, with less power dissipation by being more efficient in energy transfer (Barboza and Serra, 1992; Lerin *et al.*, 2014).

Another physical effect caused by the US broadcast by a liquid is known as "acoustic streaming", which is defined as a tendency of a stable fluid to flow near the surface of obstacles and vibrating elements; near the limiting walls in a high intensity ultrasonic field. The acoustic streaming is induced due to space attenuation of a sound wave in free space and the transfer momentum to the medium by a vibrating object (Kumar *et al.*, 2006).

The propagation of a high intensity sound wave in a fluid environment can cause a strong decrease in pressure, sufficient to produce a stable flow of fluid. It is reported that acoustic transmission is effective on improving the transport processes rates occurring within a fluid solid interface, including heat transfer, changes in morphology of biological cells and removing weakly bonded surface layers (Kumar *et al.*, 2006).

The viscosity, as well as other parameters, is also considered very important for acoustic cavitation effect (Briggs, Johnson and Mason, 1947; Behrend, Ax and Schubert, 2000). Viscosity is the molecular interaction of a liquid measured qualitatively indicating a direct relationship between the intensity of these attraction forces and interactions between molecules, and thus, the greater the interaction, the greater the viscosity, the greater the resistance to mass

transfer and hence, the energy required for cavitation onset (Behrend, Ax and Schubert, 2000). An increased viscosity in the continuous phase can improve the sonication emulsification, however, an increase in viscosity of the dispersed phase decreases the ability of ultrasonic emulsification. Nevertheless, the sonication can be compared to high pressure homogenization in efficiency, relatively more effective for high viscosity solutions emulsification (Maa and Hsu, 1999). When the interfacial tension is low, the shear force required to destabilization of the interface and for emulsification is also low, thus facilitating an increase in the number of droplets in the emulsion (Gaikwad and Pandit, 2008).

The development of ultrasonic processing systems are not well adapted for the pharmaceutical emulsion production to delivery therapeutic agents, grease emulsions for parenteral nutrition or liposomes. These products requires easy cleaning and sterilization equipment, as well as having an aseptic operation. Furthermore, as ultrasonic emulsification is mainly governed by cavitation, ions or particles are issued to the product by abrasion of the ultrasonic probe which , in most cases , is made of metal and may be a critical point for product contamination (Freitas *et al.*, 2006).

Behrend, Ax and Schubert (2000) evaluated the cavitation spread of probe in oil phase, using high-speed video techniques, concluding that the cavitation area is very small and close to the surface of US probe, which makes the emulsification process require an improvement in the approach for an efficient process design.

The height of the probe in the liquid medium interferes with the fluid dynamics of the system. The position at which the probe is placed in the reactor has a direct effect on the flow profile and agitation of the heterogeneous reaction system (Figure 3). Kojima, Imazu and Nishida (2014) evaluated the time effect when the ultrasonic probe is placed in the reaction medium of emulsion diesel oil and water. They observed that the emulsion stability varies according to where the probe position. The greater the distance from probe to base of the reactor, the greater the emulsion stability. The micro agitation generated by the probe in liquid surface formed convective currents in the mixture solution by the stirring required for stable emulsion formation.

In most methods, the emulsion is prepared by shearing processes. The final size of the resulting emulsion is homogenized balance between two opposite forces: breaking the droplet and the droplet re-coalescence. In an ultrasound system, the acoustic cavitation provides the shear force required to emulsion occurs. The collapse of cavitation bubbles produces high levels

of located turbulence (an explosion in micro-scale) that is responsible for emulsion mechanism (Leong *et al.*, 2009).

One way of producing sonication emulsion can be done by immersing an ultrasonic probe in the mixture with components or by adding the dispersed phase slowly in the continuous phase during sonication. This process works well for small batches, however its scale up becomes difficult due to the US intensity decreases rapidly with increasing distance from the probe, reducing the homogenization efficiency (Freitas *et al.*, 2006).

Much discussion is made about the US application mode. Through ultrasonic baths, the energy is dispersed uniformly into the liquid medium and some is lost without being absorbed by the reaction system. In contrast, through the probe, the energy is fully dispersed into the reaction medium directly, with a low energy loss, promoting high emulsification (Hielscher, 2007), however denaturing the enzyme present (Özbek and Ülgen, 2000). The US probe generates high cavitation, raising the temperature to over 100 °C in the region close to the probe (higher cavitation zone). Thus, the temperature control in this system is not very effective (Awadallak *et al.*, 2013).

The enzymes US effect is not yet fully elucidated, taking into account the publication of negative and positive results for the activation and inactivation of enzymes in the scientific literature. The molecular weight of the enzyme as well as the physiological localization of enzymes in the cell are the most important factors for tolerance of enzymes to US (Ceni *et al.*, 2011; Awadallak *et al.*, 2013).

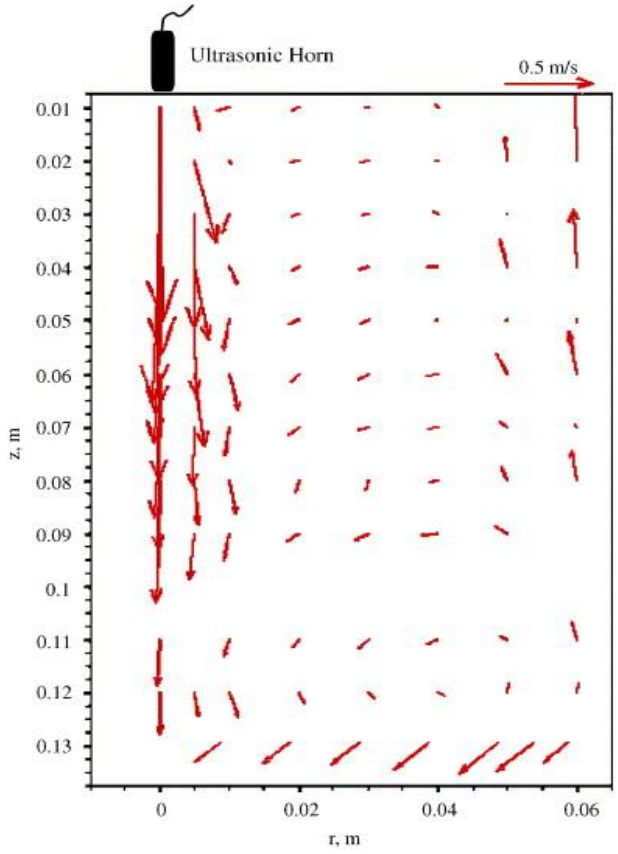

Figure 3 Bi-dimensional graphic (r, z) to the average velocity vectors at 35 kW/m³ (adapted from Kumar *et al.* (2006) with permission of Elsevier).

5.2.Lipase US-catalyzed reactions

US technology has been reported in the literature as a promising technology for the chemical and enzymatic synthesis, focusing on the interest compounds production for the pharmaceutical, chemical and food industries (Klíma, Bernard and Degrand, 1994; Tinkov *et al.*, 2009; Lerin *et al.*, 2014). This technology assists in substrate dissolution, improves mass transfer and provides enough energy to catalyze reactions and thus may induce conformational changes in the protein and disturbing weak interactions between molecules (Braginskaya *et al.*, 1990; Gebicka and Gebicki, 1997; Zhu *et al.*, 2010; Lerin *et al.*, 2014). These effects help to enhance the enzymatic reaction (Ishimori, Karube and Suzuki, 1981; Sinisterra, 1992) or may contribute to enzyme denaturation (Özbek and Ülgen, 2000). In addition, US uses only a third to half of the energy consumed by mechanical agitation (Lifka and Ondruschka, 2004; Ji *et al.*, 2006; Chand *et al.*, 2010). The US major advantages for enzymatic reactions are: decreased reaction time, decreased concentration of reagents needed and increased conversion efficiency (Lerin *et al.*, 2014).

There are several reports in literature for lipase US catalysis. China and Brazil are the countries that have published most documents in the Scopus database (Figure 4).

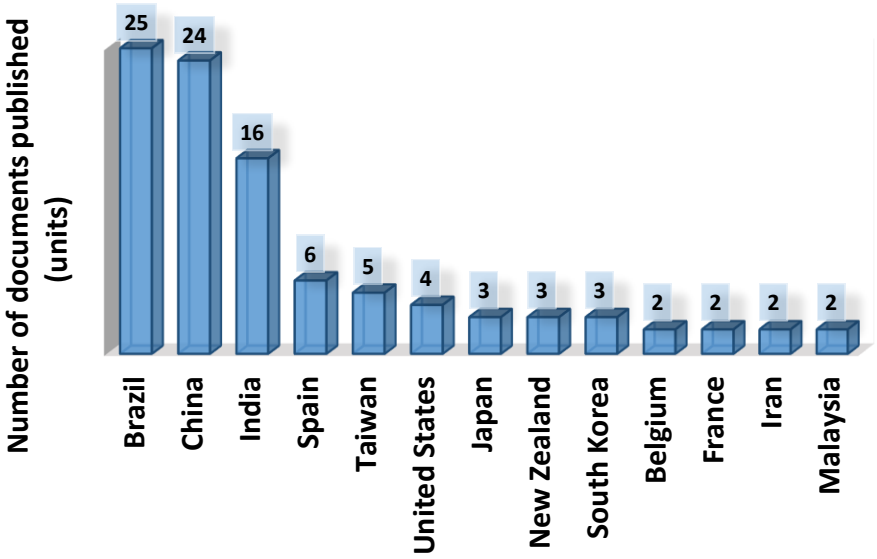

Figure 4 Number of documents published by country for lipase US catalysis (Scopus, 2015).

Results from experiments have shown that consumption decreases US enzymes, decrease reaction time and does not affect the secondary structure of the enzyme through microenvironments while tertiary structure of the enzyme might be affected. In the case of filamentous fungi, US alters the morphology and rheology of the medium without affecting the growth. It also improves the reaction rate by enzymatic synthesis in organic solvents, by adding small amounts of water in the system (Kwiatkowska *et al.*, 2011).

Most papers reporting the US application to improve enzymatic reactions using an ultrasonic bath system, so with indirect application of US. Some recent studies are summarized in Table 2.

Table 2 Recent studies with lipase US reactions.

Lipase	Substrate	Product	Time (hours)/ Temperature (ºC)	Reference
Novozym 435	Canola and Soybean oils	Mono and diacylglycerol	2/70	(Remonatto *et al.*, 2015)
Novozym 435	Macauba oil	Biodiesel	*0,5/65*	(Michelin *et al.*, 2015)
Lipozyme TL IM	Palm oil	Diacilglicerol	1,5/30	(Gonçalves *et al.*, 2012)
CAL B	Ascorbiac acid and Palmitic acid	Ascorbil-palmitate	2/70	(Lerin *et al.*, 2011)
Lipozyme RM IM	Tripalmitin and Oleic acid	1,3-dioleoyl-2-palmitoyl-glycerol	4/50	(Liu *et al.*, 2015)
Novozym 435	Dimethyl carbonate and glycerol	Glycerol carbonate	4/60	(Waghmare, Vetal and Rathod, 2015)

The reported lipase applications relates different reactions to obtain various products. Among these, a recent one demonstrates an application to rapeseed oil and soybean oil glycerolysis using Novozym 435**.** Solvent-free medium using ultrasonic bath and mechanical agitation for producing monoglycerides and diglycerides. The results showed that the highest yield (75% conversion) for canola oil used optimal conditions: 0.8/1 glycerol/oil molar ratio, 70 °C, 900 rpm, 120 min reaction, 10% enzyme and 52.8 W.cm² of US power density (Remonatto *et al.*, 2015). Another recent study had evaluated the glycerolysis using Novozym 435 in mechanical stirring reactor under US irradiation (Fiametti *et al.*, 2012). Another possibility of diacylglycerol production was made by partial hydrolysis of soybean oil catalyzed by US and lipase, generating better conversion yields and shorter reaction times compared with the thermal process (Babicz *et al.*, 2010).

Applying US with lipases for biodiesel production with different vegetable oil sources. Among these, some reported the use of coconut oil, Macaúba (*Acrocomia aculeata*) in solvent-

free medium using Novozym 435 under ultrasound. The best results of 70% conversion were obtained with 132 W, 65 °C in 30 minutes of reaction allowing re-use of the enzyme to 5 cycles without losing activity (Michelin *et al.*, 2015). As the Macaúba, soybean oil is also used for biodiesel production using Novozym 435 in packed-bed reactor under US irradiation. In this study, the best results were obtained with 61.6 W ultrasonic power 65 °C resulting in almost 100% conversion (Trentin *et al.*, 2014). Another study uses coconut oil with ethanol using 1% Novozym 435 at 50 °C. The application of US (43 kHz) in bath has allowed an increase of 20 times in conversion rate (Tupufia *et al.*, 2013). As can be seen, various types of vegetable oil sources are possible, even waste frying oil and dimethyl carbonate, conducted in solvent-free reaction under irradiation in bath using also US and Novozym 435. In addition to applications from different sources oils, some studies have focused on comparison between mechanical stirring and US. Gharat and Rathod (2013) studied the combination of the two methods which resulted in 86.6% conversion. In addition, other studies have focused on comparing different lipases. Batistella *et al.* (2012) evaluated the of soybean oil ethanolysis using two commercial lipases: Lipozyme RM IM and Novozym 435, the best results were obtained with Lipozyme RM IM. with 90% conversion in 4 hours at 60 °C and 100W of US power.

Flavors and fragrances synthesis for food industry is another application studied. Paludo *et al.* (2015) studied a combination of molecular sieve with US for the ethyl butyrate synthesis (mango and banana flavor) catalyzed by *Thermomyces lanuginosus* lipase (Lipozyme TL-IM). They obtained the best results using 35% enzyme, 0.7m butyric acid, 1: 1 (acid: alcohol), 30 °C with 61% conversion after 6 hours. With the addition of molecular sieve to remove water system the conversion yield increased by 1.5 times. Bansode and Rathod (2014) evaluated the esterification of isoamyl butyrate using Novozym 435 under US in solvent-free medium. For maximum conversion of 96% in 3 hours, the optimum parameters were 60 °C, 70W and 25kHz combining US with mechanical agitation of 80 rpm, 2% of enzyme and 2 g of molecular sieve. Another flavor synthesis study evaluated the esterification of butyl acetate in US using *Candida antarctica* lipase B immobilized on styrene-divinylbenzene beads obtaining 90% conversion in 1.5 hours under optimal conditions 48.8ºC, 7.7% enzyme 12:28% of water. The enzyme was used was 7 cycles by maintaining 70% of initial activity (Alves *et al.*, 2014a). Considering the addition of a solvent, another study evaluated the influence of low frequency US (40 kHz)in the esterification of acetic acid and butanol catalysed by Novozym 435, using n-hexane as a solvent. Optimum conditions were temperature 46°C, 7% enzyme, 0.25% water obtaining 94% conversion in 2.5 hour. US increased at 7.5 times the enzyme productivity when

compared to mechanical agitation. Furthermore, the presence of acetic acid increased recycle and enzyme stability (Martins *et al.*, 2013).

6. Conclusion

SSF, lipases and US represents an integration of different areas for bioeconomy development. The capacity of mixing complexes areas in research and development can establish a novel model of production capable of substituting the old chemical ones and generate new innovative products. Mostly, the combination of fermentation, biocatalysis and physical processes

Considering the SSF importance for adding value to agricultural by-products, as well as the lipase's ability to obtain new products with better efficiency under US irradiation, this review aims to research a process using SSF lipase production using agricultural by-products to produce a LFS capable of hydrolyzing vegetable oils in the US assisted process.

REFERENCES

ABISMAïL, B.*et al.* Emulsification by ultrasound: drop size distribution and stability. **Ultrasonics Sonochemistry,** v. 6, n. 1–2, p. 75-83, 3// 1999. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417798000273 >.

ADLERCREUTZ, P. Immobilisation and application of lipases in organic media. **Chemical Society Reviews,** v. 42, n. 15, p. 6406-6436, 2013. ISSN 0306-0012. Disponível em: < http://dx.doi.org/10.1039/C3CS35446F >.

AGGELOPOULOS, T.*et al.* Solid state fermentation of food waste mixtures for single cell protein, aroma volatiles and fat production. **Food Chemistry,** v. 145, n. 0, p. 710-716, 2/15/ 2014. ISSN 0308-8146. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0308814613010340 >.

AGIRRE, A.*et al.* Improving adhesion of acrylic waterborne PSAs to low surface energy materials: Introduction of stearyl acrylate. **Journal of Polymer Science Part A: Polymer Chemistry,** v. 48, n. 22, p. 5030-5039, 2010. ISSN 1099-0518. Disponível em: < http://dx.doi.org/10.1002/pola.24300 >.

AGUIEIRAS, E. C. G.*et al.* Biodiesel production from Acrocomia aculeata acid oil by (enzyme/enzyme) hydroesterification process: Use of vegetable lipase and fermented solid as low-cost biocatalysts. **Fuel,** v. 135, n. 0, p. 315-321, 11/1/ 2014. ISSN 0016-2361. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0016236114006358 >.

AJILA, C. M.*et al.* Bio-processing of agro-byproducts to animal feed. **Critical Reviews in Biotechnology,** v. 32, n. 4, p. 382-400, 2012. Disponível em: < http://informahealthcare.com/doi/abs/10.3109/07388551.2012.659172 >.

ALVES, J.*et al.* Combined Effects of Ultrasound and Immobilization Protocol on Butyl Acetate Synthesis Catalyzed by CALB. **Molecules,** v. 19, n. 7, p. 9562-9576, 2014a. ISSN 1420-3049. Disponível em: < http://www.mdpi.com/1420-3049/19/7/9562 >.

ALVES, J. S.*et al.* Combi-lipase for heterogeneous substrates: a new approach for hydrolysis of soybean oil using mixtures of biocatalysts. **RSC Advances,** v. 4, n. 14, p. 6863-6868, 2014b. Disponível em: < http://dx.doi.org/10.1039/C3RA45969A >.

ANANTHAPADMANABHAN, K. P.; MUKHERJEE, S.; CHANDAR, P. Stratum corneum fatty acids: their critical role in preserving barrier integrity during cleansing. **International Journal of Cosmetic Science,** v. 35, n. 4, p. 337-345, 2013. ISSN 1468-2494. Disponível em: < http://dx.doi.org/10.1111/ics.12042 >.

ANDERSEN, C.*et al.* **Solid state bioreactor adapted for automation**: Google Patents 2014.

ANSORGE-SCHUMACHER, M. B.; THUM, O. Immobilised lipases in the cosmetics industry. **Chemical Society Reviews,** v. 42, n. 15, p. 6475-6490, 2013. ISSN 0306-0012. Disponível em: < http://dx.doi.org/10.1039/C3CS35484A >.

ÁVILA-CISNEROS, N.*et al.* Production of Thermostable Lipase by *Thermomyces lanuginosus* on Solid-State Fermentation: Selective Hydrolysis of Sardine Oil. **Applied Biochemistry and Biotechnology,** v. 174, n. 5, p. 1859-1872, 2014/11/01 2014. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1007/s12010-014-1159-9 >.

AWADALLAK, J. A.*et al.* Enzymatic catalyzed palm oil hydrolysis under ultrasound irradiation: Diacylglycerol synthesis. **Ultrasonics Sonochemistry,** v. 20, n. 4, p. 1002-1007, 7// 2013. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417712002738 >.

BABICZ, I.*et al.* Lipase-catalyzed diacylglycerol production under sonochemical irradiation. **Ultrasonics Sonochemistry,** v. 17, n. 1, p. 4-6, 1// 2010. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417709001205 >.

BANSODE, S. R.; RATHOD, V. K. Ultrasound assisted lipase catalysed synthesis of isoamyl butyrate. **Process Biochemistry,** v. 49, n. 8, p. 1297-1303, 8// 2014. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1359511314002669 >.

BARBOZA, J.; SERRA, A. Ultra-som (I): influência do ultra-som na química. **Quim Nova,** v. 15, p. 302-315, 1992.

BARRIOS-GONZÁLEZ, J. Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. **Process Biochemistry,** v. 47, n. 2, p. 175-185, 2// 2012. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1359511311004132 >.

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, 5// 2012. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417711002409 >.

BEHR, A.; WESTFECHTEL, A.; PÉREZ GOMES, J. Catalytic Processes for the Technical Use of Natural Fats and Oils. **Chemical Engineering & Technology,** v. 31, n. 5, p. 700-714, 2008. ISSN 1521-4125. Disponível em: < http://dx.doi.org/10.1002/ceat.200800035 >.

BEHREND, O.; AX, K.; SCHUBERT, H. Influence of continuous phase viscosity on emulsification by ultrasound. **Ultrasonics Sonochemistry,** v. 7, n. 2, p. 77-85, 4// 2000. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417799000292 >.

BIERMANN, U.*et al.* Oils and Fats as Renewable Raw Materials in Chemistry. **Angewandte Chemie International Edition,** v. 50, n. 17, p. 3854-3871, 2011. ISSN 1521-3773. Disponível em: < http://dx.doi.org/10.1002/anie.201002767 >.

BILANOVIC, D. D.; MALLOY, S. H.; REMETA, P. **Solid or semi-solid state fermentation of xanthan on potato or potato waste**: Google Patents 2010.

BORNSCHEUER, U. T. Enzymes in lipid modification: Past achievements and current trends. **European Journal of Lipid Science and Technology,** v. 116, n. 10, p. 1322-1331, 2014. ISSN 1438-9312. Disponível em: < http://dx.doi.org/10.1002/ejlt.201400020 >.

BRAGINSKAYA, F.*et al.* Low intensity ultrasonic effects on yeast hexokinase. **Radiation and environmental biophysics,** v. 29, n. 1, p. 47-56, 1990. ISSN 0301-634X.

BRIGGS, H.; JOHNSON, J.; MASON, W. Properties of liquids at high sound pressure. **The Journal of the Acoustical Society of America,** v. 19, n. 4, p. 664-677, 1947. ISSN 0001-4966.

CAO, Y.; ZHANG, X. Production of long-chain hydroxy fatty acids by microbial conversion. **Applied Microbiology and Biotechnology,** v. 97, n. 8, p. 3323-3331, 2013/04/01 2013. ISSN 0175-7598. Disponível em: < http://dx.doi.org/10.1007/s00253-013-4815-z >.

CASTRO, A. M.; CASTILHO, L. R.; FREIRE, D. M. G. Performance of a fixed-bed solid-state fermentation bioreactor with forced aeration for the production of hydrolases by *Aspergillus awamori*. **Biochemical Engineering Journal,** v. 93, n. 0, p. 303-308, 1/15/ 2015. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X14003003 >.

CASTRO, H. F.*et al.* Modificação de óleos e gorduras por biotransformação. **Química Nova,** v. 27, n. 1, p. 146-156, 2004. ISSN 0100-4042.

CENI, G.*et al.* Ultrasound-assisted enzymatic transesterification of methyl benzoate and glycerol to 1-glyceryl benzoate in organic solvent. **Enzyme and Microbial Technology,** v. 48, n. 2, p. 169-174, 2/8/ 2011. ISSN 0141-0229. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0141022910002255 >.

CHAND, P.*et al.* Enhancing biodiesel production from soybean oil using ultrasonics. **Energy & Fuels,** v. 24, n. 3, p. 2010-2015, 2010. ISSN 0887-0624.

CHEIRSILP, B.; KITCHA, S. Solid state fermentation by cellulolytic oleaginous fungi for direct conversion of lignocellulosic biomass into lipids: Fed-batch and repeated-batch fermentations. **Industrial Crops and Products,** v. 66, n. 0, p. 73-80, 4// 2015. ISSN 0926-6690. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0926669014008000 >.

CHEMAT, F.; ZILL E, H.; KHAN, M. K. Applications of ultrasound in food technology: Processing, preservation and extraction. **Ultrasonics Sonochemistry,** v. 18, n. 4, p. 813-835, 7// 2011. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417710002385 >.

CHEW, P. L.*et al.* Extractive bioconversion of poly-ϵ-caprolactone by *Burkholderia cepacia* lipase in an aqueous two-phase system. **Biochemical Engineering Journal,** v. 101, p. 9-17, 9/15/ 2015. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X15001400 >.

COLEMAN, M. H.; MACRAE, A. R. **Fat process and composition**: Google Patents 1981.

CORADI, G.*et al.* Comparing submerged and solid-state fermentation of agro-industrial residues for the production and characterization of lipase by *Trichoderma harzianum*. **Annals of Microbiology,** v. 63, n. 2, p. 533-540, 2013/06/01 2013. ISSN 1590-4261. Disponível em: < http://dx.doi.org/10.1007/s13213-012-0500-1 >.

COSTA, I. C. R.*et al.* Lipase catalyzed ascorbyl palmitate synthesis under microwave irradiation. **Journal of Molecular Catalysis B: Enzymatic,** v. 102, p. 127-131, 4// 2014. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117714000460 >.

DAUTZENBERG, F. M.; MUKHERJEE, M. Process intensification using multifunctional reactors. **Chemical Engineering Science,** v. 56, n. 2, p. 251-267, 1// 2001. ISSN 0009-2509. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0009250900002281 >.

DAYANANDAN, A.*et al.* Enhanced production of Aspergillus tamarii lipase for recovery of fat from tannery fleshings. **Brazilian Journal of Microbiology,** v. 44, p. 1089-1095, 2013. ISSN 1517-8382. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1517-83822013000400010&nrm=iso >.

DE MARTINI SOARES, F. A. S.*et al.* Batch and continuous lipase-catalyzed interesterification of blends containing olive oil for trans-free margarines. **European journal of lipid science and technology,** v. 115, n. 4, p. 413-428, 2013. ISSN 1438-9312.

DEUSS, P. J.; BARTA, K.; DE VRIES, J. G. Homogeneous catalysis for the conversion of biomass and biomass-derived platform chemicals. **Catalysis Science & Technology,** v. 4, n. 5, p. 1174-1196, 2014. ISSN 2044-4753. Disponível em: < http://dx.doi.org/10.1039/C3CY01058A >.

DICOSIMO, R.*et al.* Industrial use of immobilized enzymes. **Chemical Society Reviews,** v. 42, n. 15, p. 6437-6474, 2013.

DIKSHIT, R.; TALLAPRAGADA, P. Bio-synthesis and screening of nutrients for lovastatin by *Monascus* sp. under solid-state fermentation. **Journal of Food Science and Technology**, p. 1-8, 2015/02/11 2015. ISSN 0022-1155. Disponível em: < http://dx.doi.org/10.1007/s13197-014-1678-y >.

DOUKYU, N.; OGINO, H. Organic solvent-tolerant enzymes. **Biochemical Engineering Journal,** v. 48, n. 3, p. 270-282, 2/15/ 2010. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X09002964 >.

EL-BATAL, A. I.*et al.* Laccase production by *Pleurotus ostreatus* and its application in synthesis of gold nanoparticles. **Biotechnology Reports,** v. 5, n. 0, p. 31-39, 3// 2015. ISSN 2215-017X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S2215017X14000538 >.

FAO. **Organization for Economic Co-Operation and Development (OECD), Food and agriculture organization of the United Nations (FAO) - Agricultural Outlook 2009-2018** 2009

FARINAS, C. S. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector. **Renewable and Sustainable Energy Reviews,** v. 52, p. 179-188, 12// 2015. ISSN 1364-0321. Disponível em: < http://www.sciencedirect.com/science/article/pii/S136403211500739X >.

FERNANDES, M. L. M.*et al.* Esterification and transesterification reactions catalysed by addition of fermented solids to organic reaction media. **Journal of Molecular Catalysis B: Enzymatic,** v. 44, n. 1, p. 8-13, 1/2/ 2007. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117706002505 >.

FERRAREZI, A. L.*et al.* Production and characterization of lipases and immobilization of whole cell of the *thermophilic Thermomucor* indicae seudaticae N31 for transesterification reaction. **Journal of Molecular Catalysis B: Enzymatic,** v. 107, n. 0, p. 106-113, 9// 2014. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117714001623 >.

FIAMETTI, K. G.*et al.* Kinetics of ultrasound-assisted lipase-catalyzed glycerolysis of olive oil in solvent-free system. **Ultrasonics Sonochemistry,** v. 19, n. 3, p. 440-451, 5// 2012. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417711001994 >.

FLEURI, L.*et al.* Production of fungal lipases using wheat bran and soybean bran and incorporation of sugarcane bagasse as a co-substrate in solid-state fermentation. **Food Science and Biotechnology,** v. 23, n. 4, p. 1199-1205, 2014/08/01 2014. ISSN 1226-7708. Disponível em: < http://dx.doi.org/10.1007/s10068-014-0164-7 >.

FREITAS, S.*et al.* Continuous contact- and contamination-free ultrasonic emulsification—a useful tool for pharmaceutical development and production. **Ultrasonics Sonochemistry,** v. 13, n. 1, p. 76-85, 1// 2006. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S135041770400183X >.

GAIKWAD, S. G.; PANDIT, A. B. Ultrasound emulsification: Effect of ultrasonic and physicochemical properties on dispersed phase volume and droplet size. **Ultrasonics Sonochemistry,** v. 15, n. 4, p. 554-563, 4// 2008. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417707001034 >.

GAIND, S.; SINGH, S. Production, purification and characterization of neutral phytase from thermotolerant *Aspergillus flavus* ITCC 6720. **International Biodeterioration & Biodegradation,** v. 99, n. 0, p. 15-22, 4// 2015. ISSN 0964-8305. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0964830514003825 >.

GANDHI, N. Applications of lipase. **Journal of the American Oil Chemists' Society,** v. 74, n. 6, p. 621-634, 1997/06/01 1997. ISSN 0003-021X. Disponível em: < http://dx.doi.org/10.1007/s11746-997-0194-x >.

GEBICKA, L.; GEBICKI, J. L. The effect of ultrasound on heme enzymes in aqueous solution. **Journal of Enzyme Inhibition and Medicinal Chemistry,** v. 12, n. 2, p. 133-141, 1997. ISSN 1475-6366.

GEDANKEN, A. Using sonochemistry for the fabrication of nanomaterials. **Ultrasonics Sonochemistry,** v. 11, n. 2, p. 47-55, 4// 2004. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417704000446 >.

GERALDES CASTANHEIRA, É.*et al.* Environmental sustainability of biodiesel in Brazil. **Energy Policy,** v. 65, n. 0, p. 680-691, 2// 2014. ISSN 0301-4215. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0301421513009865 >.

GHARAT, N.; RATHOD, V. K. Ultrasound assisted enzyme catalyzed transesterification of waste cooking oil with dimethyl carbonate. **Ultrasonics Sonochemistry,** v. 20, n. 3, p. 900-905, 5// 2013. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417712002362 >.

GOBIN, M.*et al.* Synthesis and characterisation of bio-based polyester materials from vegetable oil and short to long chain dicarboxylic acids. **Industrial Crops and Products,** v. 70, n. 0, p. 213-220, 8// 2015. ISSN 0926-6690. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0926669015002307 >.

GODOY, M. G.*et al.* Use of Vero cell line to verify the biodetoxification efficiency of castor bean waste. **Process Biochemistry,** v. 47, n. 4, p. 578-584, 4// 2012. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1359511311004478 >.

GODOY, M. G.*et al.* Use of a low-cost methodology for biodetoxification of castor bean waste and lipase production. **Enzyme and Microbial Technology,** v. 44, n. 5, p. 317-322, 5/6/ 2009. ISSN 0141-0229. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0141022909000179 >.

GOGATE, P. R. Cavitation: an auxiliary technique in wastewater treatment schemes. **Advances in Environmental Research,** v. 6, n. 3, p. 335-358, 9// 2002. ISSN 1093-0191. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1093019101000673 >.

GOGATE, P. R. Cavitational reactors for process intensification of chemical processing applications: A critical review. **Chemical Engineering and Processing: Process Intensification,** v. 47, n. 4, p. 515-527, 4// 2008. ISSN 0255-2701. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0255270107003042 >.

GOGATE, P. R.; SUTKAR, V. S.; PANDIT, A. B. Sonochemical reactors: Important design and scale up considerations with a special emphasis on heterogeneous systems. **Chemical Engineering Journal,** v. 166, n. 3, p. 1066-1082, 2/1/ 2011. ISSN 1385-8947. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1385894710011654 >.

GONÇALVES, K. M.*et al.* Palm oil hydrolysis catalyzed by lipases under ultrasound irradiation – The use of experimental design as a tool for variables evaluation. **Ultrasonics Sonochemistry,** v. 19, n. 2, p. 232-236, 3// 2012. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417711001398 >.

GUTARRA, M. L. E.*et al.* Inoculum strategies for *Penicillium simplicissimum* lipase production by solid-state fermentation using a residue from the babassu oil industry. **Journal of Chemical Technology & Biotechnology,** v. 82, n. 3, p. 313-318, 2007. ISSN 1097-4660. Disponível em: < http://dx.doi.org/10.1002/jctb.1674 >.

GUTARRA, M. L. E.*et al.* Production of an acidic and thermostable lipase of the mesophilic fungus *Penicillium simplicissimum* by solid-state fermentation. **Bioresource Technology,** v.

100, n. 21, p. 5249-5254, 11// 2009. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852409005872 >.

HAN, W.*et al.* Utilization of wheat for biohydrogen production by a combination of solid-state fermentation and batch fermentation. **International Journal of Hydrogen Energy,** v. 40, n. 17, p. 5849-5855, 5/11/ 2015a. ISSN 0360-3199. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0360319915006060 >.

HAN, W.*et al.* Biohydrogen production from food waste hydrolysate using continuous mixed immobilized sludge reactors. **Bioresource Technology,** v. 180, n. 0, p. 54-58, 3// 2015b. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852414018136 >.

HANDA, C. L.*et al.* Optimisation of soy flour fermentation parameters to produce β-glucosidase for bioconversion into aglycones. **Food Chemistry,** v. 152, n. 0, p. 56-65, 6/1/ 2014. ISSN 0308-8146. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0308814613017895 >.

HASAN, F.; SHAH, A. A.; HAMEED, A. Industrial applications of microbial lipases. **Enzyme and Microbial Technology,** v. 39, n. 2, p. 235-251, 6/26/ 2006. ISSN 0141-0229. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0141022905004606 >.

HE, H.*et al.* A novel polynary fatty acid/sludge ceramsite composite phase change materials and its applications in building energy conservation. **Renewable Energy,** v. 76, n. 0, p. 45-52, 4// 2015. ISSN 0960-1481. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960148114007150 >.

HIELSCHER, T. Ultrasonic production of nano-size dispersions and emulsions. **arXiv preprint arXiv:0708.1831**, 2007.

ISHIMORI, Y.; KARUBE, I.; SUZUKI, S. Acceleration of immobilized α-chymotrypsin activity with ultrasonic irradiation. **Journal of Molecular Catalysis,** v. 12, n. 2, p. 253-259, 1981. ISSN 0304-5102.

JAEGER, K. E.*et al.* Directed evolution and the creation of enantioselective biocatalysts. **Applied Microbiology and Biotechnology,** v. 55, n. 5, p. 519-530, 2001/05/01 2001. ISSN 0175-7598. Disponível em: < http://dx.doi.org/10.1007/s002530100643 >.

JENSEN, R. G.; GALLUZZO, D. R.; BUSH, V. J. Selectivity is an important characteristic of lipases (acylglycerol hydrolases). **Biocatalysis and Biotransformation,** v. 3, n. 4, p. 307-316, 1990. ISSN 1024-2422.

JI, J.*et al.* Preparation of biodiesel with the help of ultrasonic and hydrodynamic cavitation. **Ultrasonics,** v. 44, p. e411-e414, 2006. ISSN 0041-624X.

JOSHI, C.; MATHUR, P.; KHARE, S. K. Degradation of phorbol esters by *Pseudomonas aeruginosa* PseA during solid-state fermentation of deoiled Jatropha curcas seed cake. **Bioresource Technology,** v. 102, n. 7, p. 4815-4819, 4// 2011. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852411001076 >.

JURETIC, H.*et al.* Hydroxyl radical formation in batch and continuous flow ultrasonic systems. **Ultrasonics Sonochemistry,** v. 22, n. 0, p. 600-606, 1// 2015. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417714002302 >.

KAMALAKAR, K.*et al.* Novel Acyloxy Derivatives of Branched Mono- and Polyol Esters of Sal Fat: Multiviscosity Grade Lubricant Base Stocks. **Journal of Agricultural and Food Chemistry,** v. 62, n. 49, p. 11980-11987, 2014/12/10 2014. ISSN 0021-8561. Disponível em: < http://dx.doi.org/10.1021/jf504700m >.

KHODADADI, M.*et al.* Lipase-catalyzed synthesis and characterization of flaxseed oil-based structured lipids. **Journal of Functional Foods,** v. 5, n. 1, p. 424-433, 1// 2013. ISSN 1756-4646. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1756464612001818 >.

KIRCHER, M. The Emerging Bioeconomy: Industrial Drivers, Global Impact, and International Strategies. **Industrial Biotechnology,** v. 10, n. 1, p. 11-18, 2014/02/01 2014. ISSN 1550-9087. Disponível em: < http://dx.doi.org/10.1089/ind.2014.1500 >. Acesso em: 2015/04/08.

KITCHA, S.; CHEIRSILP, B. Bioconversion of lignocellulosic palm byproducts into enzymes and lipid by newly isolated oleaginous fungi. **Biochemical Engineering Journal,** v. 88, n. 0, p. 95-100, 7/15/ 2014. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X14000977 >.

KLIBANOV, A. M. Why are enzymes less active in organic solvents than in water? **Trends in Biotechnology,** v. 15, n. 3, p. 97-101, 3// 1997. ISSN 0167-7799. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0167779997010135 >.

KLÍMA, J.; BERNARD, C.; DEGRAND, C. Sonoelectrochemistry: effects of ultrasound on voltammetric measurements at a solid electrode. **Journal of Electroanalytical Chemistry,** v. 367, n. 1, p. 297-300, 1994. ISSN 1572-6657.

KOJIMA, Y.; IMAZU, H.; NISHIDA, K. Physical and chemical characteristics of ultrasonically-prepared water-in-diesel fuel: Effects of ultrasonic horn position and water content. **Ultrasonics Sonochemistry,** v. 21, n. 2, p. 722-728, 3// 2014. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417713002307 >.

KUMAR, A.*et al.* Characterization of flow phenomena induced by ultrasonic horn. **Chemical Engineering Science,** v. 61, n. 22, p. 7410-7420, 11/20/ 2006. ISSN 0009-2509. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0009250906005276 >.

KUMAR, A.*et al.* Silver-nanoparticle-embedded antimicrobial paints based on vegetable oil. **Nat Mater,** v. 7, n. 3, p. 236-241, 03//print 2008. ISSN 1476-1122. Disponível em: < http://dx.doi.org/10.1038/nmat2099 >.

KUMAR, S.*et al.* Use of evolutionary operation (EVOP) factorial design technique to develop a bioprocess using grease waste as a substrate for lipase production. **Bioresource Technology,** v. 102, n. 7, p. 4909-4912, 4// 2011. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852411000071 >.

KWIATKOWSKA, B.*et al.* Stimulation of bioprocesses by ultrasound. **Biotechnology Advances,** v. 29, n. 6, p. 768-780, 11// 2011. ISSN 0734-9750. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0734975011000814 >. Acesso em: 2011/12//.

LEONG, T. S. H.*et al.* Minimising oil droplet size using ultrasonic emulsification. **Ultrasonics Sonochemistry,** v. 16, n. 6, p. 721-727, 8// 2009. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417709000303 >.

LERIN, L.*et al.* A review on lipase-catalyzed reactions in ultrasound-assisted systems. **Bioprocess and Biosystems Engineering,** v. 37, n. 12, p. 2381-2394, 2014/12/01 2014. ISSN 1615-7591. Disponível em: < http://dx.doi.org/10.1007/s00449-014-1222-5 >.

LERIN, L. A.*et al.* Enzymatic synthesis of ascorbyl palmitate in ultrasound-assisted system: Process optimization and kinetic evaluation. **Ultrasonics Sonochemistry,** v. 18, n. 5, p. 988-996, 9// 2011. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417710002646 >.

LI, P. J.; LI, C. C.; LI, C. H. **Method for manufacturing coffee by solid state fermentation**: Google Patents 2010.

LIESE, A.; HILTERHAUS, L. Evaluation of immobilized enzymes for industrial applications. **Chemical Society Reviews,** v. 42, n. 15, p. 6236-6249, 2013. ISSN 0306-0012. Disponível em: < http://dx.doi.org/10.1039/C3CS35511J >.

LIFKA, J.; ONDRUSCHKA, B. Influence of Mass Transfer on the Production of Biodiesel. **Chemical Engineering & Technology,** v. 27, n. 11, p. 1156-1159, 2004. ISSN 1521-4125. Disponível em: < http://dx.doi.org/10.1002/ceat.200407033 >.

LIM, S.-F.; MATU, S. Utilization of agro-wastes to produce biofertilizer. **International Journal of Energy and Environmental Engineering,** v. 6, n. 1, p. 31-35, 2015/03/01 2015. ISSN 2008-9163. Disponível em: < http://dx.doi.org/10.1007/s40095-014-0147-8 >.

LIU, S.-L.*et al.* Ultrasonic pretreatment in lipase-catalyzed synthesis of structured lipids with high 1,3-dioleoyl-2-palmitoylglycerol content. **Ultrasonics Sonochemistry,** v. 23, n. 0, p. 100-108, 3// 2015. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417714003216 >.

LIU, Y.*et al.* Biodiesel synthesis directly catalyzed by the fermented solid of *Burkholderia cenocepacia* via solid state fermentation. **Fuel Processing Technology,** v. 106, n. 0, p. 303-309, 2// 2013. ISSN 0378-3820. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0378382012003062 >.

LIU, Y.*et al.* Solid-supported microorganism of *Burkholderia cenocepacia* cultured via solid state fermentation for biodiesel production: Optimization and kinetics. **Applied Energy,** v. 113, n. 0, p. 713-721, 1// 2014. ISSN 0306-2619. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0306261913006442 >.

LOSSAU, S.*et al.* Brazil's current and future land balances: Is there residual land for bioenergy production? **Biomass and Bioenergy,** v. 81, p. 452-461, 10// 2015. ISSN 0961-9534. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0961953415300635 >.

LUCHE, J.-L.; BIANCHI, C. **Synthetic organic sonochemistry**. Springer Science & Business Media, 1998. ISBN 0306459167.

LUTH, P.; EIBEN, U. **Solid state fermenter and procedure for solid state fermentation**: Google Patents 2009.

MAA, Y.-F.; HSU, C. C. Performance of Sonication and Microfluidization for Liquid–Liquid Emulsification. **Pharmaceutical Development and Technology,** v. 4, n. 2, p. 233-240, 1999. Disponível em: < http://informahealthcare.com/doi/abs/10.1081/PDT-100101357 >.

MACEDO, G. A.; MADEIRA, J. J. V. **Media for the culture of orange-bagasse waste for simultaneous production of the phytase and thanase enzymes by the microorganism *Paecilomyces variotii* by means of solid fermentation, the enzymes obtained and the uses thereof**: Google Patents 2013.

MACHADO, D. C. A.*et al.* **Integrated process for producing enzyme formulations from agro-industrial waste and biofuel production**: Google Patents 2013.

MADEIRA JR, J. V.; MACEDO, J. A.; MACEDO, G. A. Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using *Paecilomyces variotii*. **Bioresource Technology,** v. 102, n. 15, p. 7343-7348, 8// 2011. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852411006389 >.

MADEIRA JR, J. V.*et al.* Rich bioactive phenolic extract production by microbial biotransformation of Brazilian Citrus residues. **Chemical Engineering Research and Design,** v. 92, n. 10, p. 1802-1810, 10// 2014. ISSN 0263-8762. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0263876214003335 >.

MALCATA, F. X.*et al.* Kinetics and mechanisms of reactions catalysed by immobilized lipases. **Enzyme and Microbial Technology,** v. 14, n. 6, p. 426-446, 6// 1992. ISSN 0141-0229. Disponível em: < http://www.sciencedirect.com/science/article/pii/014102299290135B >.

MARTINS, A. B.*et al.* Ultrasound-assisted butyl acetate synthesis catalyzed by Novozym 435: Enhanced activity and operational stability. **Ultrasonics Sonochemistry,** v. 20, n. 5, p. 1155-1160, 9// 2013. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S135041771300045X >.

MASOMIAN, M.*et al.* A new thermostable and organic solvent-tolerant lipase from *Aneurinibacillus thermoaerophilus* strain HZ. **Process Biochemistry,** v. 48, n. 1, p. 169-175, 1// 2013. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S135951131200387X >.

MASON, T. J.; LORIMER, J. P. Applied sonochemistry. **The uses of power ultrasound in chemistry and processing**, p. 1-48, 2002.

MATSUO, T.*et al.* **Method for enzymatic transesterification of lipid and enzyme used therein**: Google Patents 1983.

MEIER, M. A. R.; METZGER, J. O.; SCHUBERT, U. S. Plant oil renewable resources as green alternatives in polymer science. **Chemical Society Reviews,** v. 36, n. 11, p. 1788-1802, 2007. ISSN 0306-0012. Disponível em: < http://dx.doi.org/10.1039/B703294C >.

MENONCIN, S.*et al.* Study of the Extraction, Concentration, and Partial Characterization of Lipases Obtained from *Penicillium verrucosum* using Solid-State Fermentation of Soybean Bran. **Food and Bioprocess Technology,** v. 3, n. 4, p. 537-544, 2010/08/01 2010. ISSN 1935-5130. Disponível em: < http://dx.doi.org/10.1007/s11947-008-0104-8 >.

MICHELIN, S.*et al.* Kinetics of ultrasound-assisted enzymatic biodiesel production from Macauba coconut oil. **Renewable Energy,** v. 76, n. 0, p. 388-393, 4// 2015. ISSN 0960-1481. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960148114007976 >.

MICROMARKETMONITOR. **Global Lipase Market Research Report**. 2015

MOFTAH, O.*et al.* Adding Value to the Oil Cake as a Waste from Oil Processing Industry: Production of Lipase and Protease by *Candida utilis* in Solid State Fermentation. **Applied Biochemistry and Biotechnology,** v. 166, n. 2, p. 348-364, 2012/01/01 2012. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1007/s12010-011-9429-2 >.

MONDALA, A. Direct fungal fermentation of lignocellulosic biomass into itaconic, fumaric, and malic acids: current and future prospects. **Journal of Industrial Microbiology & Biotechnology,** v. 42, n. 4, p. 487-506, 2015/04/01 2015. ISSN 1367-5435. Disponível em: < http://dx.doi.org/10.1007/s10295-014-1575-4 >.

MONI, L.*et al.* Ugi and Passerini Reactions of Biocatalytically Derived Chiral Aldehydes: Application to the Synthesis of Bicyclic Pyrrolidines and of Antiviral Agent Telaprevir. **The Journal of Organic Chemistry,** v. 80, n. 7, p. 3411-3428, 2015/04/03 2015. ISSN 0022-3263. Disponível em: < http://dx.doi.org/10.1021/jo502829j >.

MOTTE, J.-C.*et al.* Combination of dry dark fermentation and mechanical pretreatment for lignocellulosic deconstruction: An innovative strategy for biofuels and volatile fatty acids recovery. **Applied Energy,** v. 147, n. 0, p. 67-73, 6/1/ 2015. ISSN 0306-2619. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0306261915002196 >.

MUELLER, M.; WILKINS, M.; PRADE, R. A. **Methods for continuous enzyme production using a filamentous fungus with inhibited growth**: Google Patents 2015.

NAGAVALLI, M.*et al.* Solid State Fermentation and production of Rifamycin SV using *Amycolatopsis mediterranei*. **Letters in Applied Microbiology,** v. 60, n. 1, p. 44-51, 2015. ISSN 1472-765X. Disponível em: < http://dx.doi.org/10.1111/lam.12332 >.

NAGY, V.*et al.* Kinetic resolutions with novel, highly enantioselective fungal lipases produced by solid state fermentation. **Journal of Molecular Catalysis B: Enzymatic,** v. 39, n. 1–4, p. 141-148, 5/2/ 2006. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117706000294 >.

NALINI, S.; PARTHASARATHI, R. Production and characterization of rhamnolipids produced by *Serratia rubidaea* SNAU02 under solid-state fermentation and its application as biocontrol agent. **Bioresource Technology,** v. 173, n. 0, p. 231-238, 12// 2014. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852414013029 >.

NIDHEESH, T.; PAL, G. K.; SURESH, P. V. Chitooligomers preparation by chitosanase produced under solid state fermentation using shrimp by-products as substrate. **Carbohydrate Polymers,** v. 121, n. 0, p. 1-9, 5/5/ 2015. ISSN 0144-8617. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0144861714012004 >.

NOVOZYMES. **The Novozymes Report 2014**. 2015

OSHO, M. B.; POPOOLA, T.; KAREEM, S. O. Immobilization of *Aspergillus niger* ATCC 1015 on bionatural structures for lipase production. **Engineering in Life Sciences,** v. 14, n. 4, p. 449-454, 2014. ISSN 1618-2863. Disponível em: < http://dx.doi.org/10.1002/elsc.201300129 >.

ÖZBEK, B.; ÜLGEN, K. Ö. The stability of enzymes after sonication. **Process Biochemistry,** v. 35, n. 9, p. 1037-1043, 5// 2000. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0032959200001412 >.

PAIVA, A. L.; BALCAO, V. M.; MALCATA, F. X. Kinetics and mechanisms of reactions catalyzed by immobilized lipases☆. **Enzyme and microbial technology,** v. 27, n. 3, p. 187-204, 2000. ISSN 0141-0229.

PALUDO, N.*et al.* The combined use of ultrasound and molecular sieves improves the synthesis of ethyl butyrate catalyzed by immobilized *Thermomyces lanuginosus* lipase. **Ultrasonics Sonochemistry,** v. 22, n. 0, p. 89-94, 1// 2015. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417714001503 >.

PAULA, A. V.*et al.* Synthesis of Structured Lipids by Enzymatic Interesterification of Milkfat and Soybean Oil in a Basket-Type Stirred Tank Reactor. **Industrial & Engineering Chemistry Research,** v. 54, n. 6, p. 1731-1737, 2015/02/18 2015. ISSN 0888-5885. Disponível em: < http://dx.doi.org/10.1021/ie503189e >.

PELLIS, A.*et al.* Towards feasible and scalable solvent-free enzymatic polycondensations: integrating robust biocatalysts with thin film reactions. **Green Chemistry,** v. 17, n. 3, p. 1756-1766, 2015. ISSN 1463-9262. Disponível em: < http://dx.doi.org/10.1039/C4GC02289K >.

PHENGNUAM, T.; SUNTORNSUK, W. Detoxification and anti-nutrients reduction of *Jatropha curcas* seed cake by *Bacillus* fermentation. **Journal of Bioscience and Bioengineering,** v. 115, n. 2, p. 168-172, 2// 2013. ISSN 1389-1723. Disponível em: < http://www.sciencedirect.com/science/article/pii/S138917231200360X >.

POPPE, J. K.*et al.* Enzymatic reactors for biodiesel synthesis: present status and future prospects. **Biotechnology Advances**, 2015. ISSN 0734-9750.

POSTEMSKY, P. D.; CURVETTO, N. R. Solid-state fermentation of cereal grains and sunflower seed hulls by *Grifola gargal* and *Grifola sordulenta*. **International Biodeterioration & Biodegradation,** v. 100, n. 0, p. 52-61, 5// 2015. ISSN 0964-8305. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0964830515000487 >.

RAJENDRAN, A.; THANGAVELU, V. Utilizing Agricultural Wastes as Substrates for Lipase Production by *Candida rugosa* NCIM 3462 in Solid-State Fermentation: Response Surface Optimization of Fermentation Parameters. **Waste and Biomass Valorization,** v. 4, n. 2, p. 347-357, 2013/06/01 2013. ISSN 1877-2641. Disponível em: < http://dx.doi.org/10.1007/s12649-012-9140-8 >.

RANGANATHAN, L. **A solid state fermentation method**: Google Patents 2014.

RASERA, K.*et al.* Interesterification of fat blends using a fermented solid with lipolytic activity. **Journal of Molecular Catalysis B: Enzymatic,** v. 76, n. 0, p. 75-81, 4// 2012. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117711003274 >.

RAVELO, M.*et al.* Esterification of glycerol and ibuprofen in solventless media catalyzed by free CALB: Kinetic modelling. **Biochemical Engineering Journal,** v. 101, p. 228-236, 9/15/ 2015. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X15001953 >.

REIS, P.*et al.* Lipase reaction at interfaces as self-limiting processes. **Comptes Rendus Chimie,** v. 12, n. 1–2, p. 163-170, 1// 2009. ISSN 1631-0748. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1631074808001768 >. Acesso em: 2009/2//.

REIS, P. M.*et al.* Influence of Surfactants on Lipase Fat Digestion in a Model Gastro-intestinal System. **Food Biophysics**, Boston**,** v. 3, n. 4, p. 370-381, 06/26
01/15/received
05/21/accepted 2008. ISSN 1557-1858
1557-1866. Disponível em: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2854607/ >.

REMONATTO, D.*et al.* Lipase-Catalyzed Glycerolysis of Soybean and Canola Oils in a Free Organic Solvent System Assisted by Ultrasound. **Applied Biochemistry and Biotechnology**, p. 1-13, 2015/04/15 2015. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1007/s12010-015-1615-1 >.

ROBY, M. H.*et al.* Enzymatic production of bioactive docosahexaenoic acid phenolic ester. **Food Chemistry,** v. 171, n. 0, p. 397-404, 3/15/ 2015. ISSN 0308-8146. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0308814614013958 >.

RODRÍGUEZ DE OLMOS, A.; BRU, E.; GARRO, M. S. Optimization of fermentation parameters to study the behavior of selected lactic cultures on soy solid state fermentation. **International Journal of Food Microbiology,** v. 196, n. 0, p. 16-23, 3/2/ 2015. ISSN 0168-1605. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0168160514005819 >.

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, 5// 2009. ISSN 0167-7799. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0167779909000493 >.

ROSA, D. R.; CAMMAROTA, M. C.; FREIRE, D. M. G. Production and utilization of a novel solid enzymatic preparation produced by *Penicillium restrictum* in activated sludge systems treating wastewater with high levels of oil and grease. **Environmental engineering science,** v. 23, n. 5, p. 814-823, 2006. ISSN 1092-8758.

SAGIRI, S. S.*et al.* Stearic acid based oleogels: A study on the molecular, thermal and mechanical properties. **Materials Science and Engineering: C,** v. 48, n. 0, p. 688-699, 3/1/ 2015. ISSN 0928-4931. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0928493114008169 >.

SAHNOUN, M.*et al.* *Aspergillus oryzae* S2 alpha-amylase production under solid state fermentation: Optimization of culture conditions. **International Journal of Biological Macromolecules,** v. 75, n. 0, p. 73-80, 4// 2015. ISSN 0141-8130. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0141813015000379 >.

SALGADO, J.*et al.* Integrated Use of Residues from Olive Mill and Winery for Lipase Production by Solid State Fermentation with *Aspergillus* sp. **Applied Biochemistry and Biotechnology,** v. 172, n. 4, p. 1832-1845, 2014/02/01 2014. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1007/s12010-013-0613-4 >.

SALIHU, A.; ALAM, M. Z. Solvent tolerant lipases: A review. **Process Biochemistry,** v. 50, n. 1, p. 86-96, 2015. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1359511314005418 >.

SALIHU, A.*et al.* Lipase production: An insight in the utilization of renewable agricultural residues. **Resources, Conservation and Recycling,** v. 58, n. 0, p. 36-44, 1// 2012. ISSN 0921-3449. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0921344911002187 >.

SALIHU, A.*et al.* Effect of process parameters on lipase production by *Candida cylindracea* in stirred tank bioreactor using renewable palm oil mill effluent based medium. **Journal of Molecular Catalysis B: Enzymatic,** v. 72, n. 3–4, p. 187-192, 11// 2011. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117711001640 >.

SALUM, T. F. C.*et al.* Synthesis of biodiesel in column fixed-bed bioreactor using the fermented solid produced by *Burkholderia cepacia* LTEB11. **Process Biochemistry,** v. 45, n. 8, p. 1348-1354, 8// 2010. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1359511310001698 >.

SCOPUS. Analyze search results 2015. Disponível em: < www.scopus.com >. Acesso em: 05/04/2015.

SEN, S.; PUSKAS, J. Green Polymer Chemistry: Enzyme Catalysis for Polymer Functionalization. **Molecules,** v. 20, n. 5, p. 9358, 2015. ISSN 1420-3049. Disponível em: < http://www.mdpi.com/1420-3049/20/5/9358 >.

SHARMA, P.*et al.* An eco-friendly process for biobleaching of eucalyptus kraft pulp with xylanase producing *Bacillus halodurans*. **Journal of Cleaner Production,** v. 87, n. 0, p. 966-970, 1/15/ 2015. ISSN 0959-6526. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0959652614010221 >.

SHELDON, R. A.; SANDERS, J. P. M. Toward concise metrics for the production of chemicals from renewable biomass. **Catalysis Today,** v. 239, n. 0, p. 3-6, 1/1/ 2015. ISSN 0920-5861. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0920586114002600 >.

SILVA, M.*et al.* Production of multifunctional lipases by *Penicillium verrucosum* and *Penicillium brevicompactum* under solid state fermentation of babassu cake and castor meal. **Bioprocess and Biosystems Engineering,** v. 34, n. 2, p. 145-152, 2011/02/01 2011. ISSN 1615-7591. Disponível em: < http://dx.doi.org/10.1007/s00449-010-0455-1 >.

SINGH, M. K.*et al.* Novel lipase from basidiomycetes *Schizophyllum* commune ISTL04, produced by solid state fermentation of *Leucaena leucocephala* seeds. **Journal of Molecular Catalysis B: Enzymatic,** v. 110, n. 0, p. 92-99, 12// 2014. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117714002859 >.

SINGHANIA, R.; SOCCOL, C.; PANDEY, A. Application of Tropical Agro-industrial Residues as Substrate for Solid-state Fermentation Processes. In: PANDEY, A.;SOCCOL, C., *et al* (Ed.). **Current Developments in Solid-state Fermentation**: Springer New York, 2008. cap. 18, p.412-442. ISBN 978-0-387-75212-9.

SINGHANIA, R. R.*et al.* Recent advances in solid-state fermentation. **Biochemical Engineering Journal,** v. 44, n. 1, p. 13-18, 4/15/ 2009. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X08003331 >.

SINISTERRA, J. Application of ultrasound to biotechnology: an overview. **Ultrasonics,** v. 30, n. 3, p. 180-185, 1992. ISSN 0041-624X.

SOCCOL, C. R.; VANDENBERGHE, L. P. S. Overview of applied solid-state fermentation in Brazil. **Biochemical Engineering Journal,** v. 13, n. 2–3, p. 205-218, 3// 2003. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X0200133X >.

SOLAESA, Á. G.*et al.* Production and concentration of monoacylglycerols rich in omega-3 polyunsaturated fatty acids by enzymatic glycerolysis and molecular distillation. **Food Chemistry,** v. 190, p. 960-967, 1/1/ 2016. ISSN 0308-8146. Disponível em: < http://www.sciencedirect.com/science/article/pii/S030881461500953X >.

STANKIEWICZ, A. Reactive separations for process intensification: an industrial perspective. **Chemical Engineering and Processing: Process Intensification,** v. 42, n. 3, p. 137-144, 3// 2003. ISSN 0255-2701. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0255270102000843 >.

STANKIEWICZ, A. I.; MOULIJN, J. A. Process intensification: transforming chemical engineering. **Chemical Engineering Progress,** v. 96, n. 1, p. 22-34, 2000. ISSN 0360-7275.

SURESH, S.; RADHA, K. Effect of a mixed substrate on phytase production by *Rhizopus oligosporus* MTCC 556 using solid state fermentation and determination of dephytinization activities in food grains. **Food Science and Biotechnology,** v. 24, n. 2, p. 551-559, 2015/04/01 2015. ISSN 1226-7708. Disponível em: < http://dx.doi.org/10.1007/s10068-015-0072-5 >.

SUSLICK, K. S. Sonochemistry. **Science,** v. 247, n. 4949, p. 1439-1445, 1990. ISSN 0036-8075.

SUSLICK, K. S.*et al.* Sonochemical synthesis of amorphous iron. **Nature,** v. 353, n. 6343, p. 414-416, 10/03/print 1991. Disponível em: < http://dx.doi.org/10.1038/353414a0 >.

SUTKAR, V. S.; GOGATE, P. R. Design aspects of sonochemical reactors: Techniques for understanding cavitational activity distribution and effect of operating parameters. **Chemical Engineering Journal,** v. 155, n. 1–2, p. 26-36, 12/1/ 2009. ISSN 1385-8947. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1385894709005154 >.

SVENDSEN, A.*et al.* **Immobilised enzymes**: Google Patents 2007.

TANG, B.*et al.* Conversion of agroindustrial residues for high poly(γ-glutamic acid) production by *Bacillus subtilis* NX-2 via solid-state fermentation. **Bioresource Technology,** v. 181, n. 0, p. 351-354, 4// 2015. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852415000267 >.

TEIXEIRA, C. B.; MADEIRA JUNIOR, J. V.; MACEDO, G. A. Biocatalysis combined with physical technologies for development of a green biodiesel process. **Renewable and Sustainable Energy Reviews,** v. 33, p. 333-343, 5// 2014. ISSN 1364-0321. Disponível em: < http://www.sciencedirect.com/science/article/pii/S136403211400094X >.

TEIXEIRA, T. B. R. M.; DA SILVA, N. A. L. **Enzymatic process for obtaining a fatty ester**: Google Patents 2011.

THAYER, A. M. Biocatalysis. **Chemical & Engineering News,** v. 90, n. 22, p. 13-18, 2012.

THOMAS, L.; LARROCHE, C.; PANDEY, A. Current developments in solid-state fermentation. **Biochemical Engineering Journal,** v. 81, n. 0, p. 146-161, 12/15/ 2013. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X13002830 >.

THOMPSON, L.; DORAISWAMY, L. Sonochemistry: science and engineering. **Industrial & Engineering Chemistry Research,** v. 38, n. 4, p. 1215-1249, 1999. ISSN 0888-5885.

TINKOV, S.*et al.* Microbubbles as ultrasound triggered drug carriers. **Journal of pharmaceutical sciences,** v. 98, n. 6, p. 1935-1961, 2009. ISSN 1520-6017.

TOMKE, P. D.; RATHOD, V. K. Ultrasound assisted lipase catalyzed synthesis of cinnamyl acetate via transesterification reaction in a solvent free medium. **Ultrasonics Sonochemistry,** v. 27, p. 241-246, 11// 2015. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417715001157 >.

TRENTIN, C.*et al.* Continuous lipase-catalyzed esterification of soybean fatty acids under ultrasound irradiation. **Bioprocess and Biosystems Engineering,** v. 37, n. 5, p. 841-847, 2014/05/01 2014. ISSN 1615-7591. Disponível em: < http://dx.doi.org/10.1007/s00449-013-1052-x >.

TUPUFIA, S. C.*et al.* Enzymatic conversion of coconut oil for biodiesel production. **Fuel Processing Technology,** v. 106, n. 0, p. 721-726, 2// 2013. ISSN 0378-3820. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0378382012003967 >.

UÇKUN KIRAN, E.; TRZCINSKI, A. P.; LIU, Y. Enhancing the hydrolysis and methane production potential of mixed food waste by an effective enzymatic pretreatment. **Bioresource Technology,** v. 183, n. 0, p. 47-52, 5// 2015. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852415002023 >.

VASEGHI, Z.*et al.* Production of active lipase by *Rhizopus oryzae* from sugarcane bagasse: solid state fermentation in a tray bioreactor. **International Journal of Food Science & Technology,** v. 48, n. 2, p. 283-289, 2013. ISSN 1365-2621. Disponível em: < http://dx.doi.org/10.1111/j.1365-2621.2012.03185.x >.

VEERABHADRAPPA, M. B.; SHIVAKUMAR, S. B.; DEVAPPA, S. Solid-state fermentation of Jatropha seed cake for optimization of lipase, protease and detoxification of anti-nutrients in Jatropha seed cake using *Aspergillus versicolor* CJS-98. **Journal of Bioscience and Bioengineering,** v. 117, n. 2, p. 208-214, 2// 2014. ISSN 1389-1723. Disponível em: < http://www.sciencedirect.com/science/article/pii/S138917231300265X >.

VELIOGLU, Z.; OZTURK UREK, R. Concurrent Biosurfactant and Ligninolytic Enzyme Production by *Pleurotus* spp. in Solid-State Fermentation. **Applied Biochemistry and Biotechnology,** v. 174, n. 4, p. 1354-1364, 2014/10/01 2014. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1007/s12010-014-1136-3 >.

WAGHMARE, G. V.; VETAL, M. D.; RATHOD, V. K. Ultrasound assisted enzyme catalyzed synthesis of glycerol carbonate from glycerol and dimethyl carbonate. **Ultrasonics Sonochemistry,** v. 22, n. 0, p. 311-316, 1// 2015. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417714002041 >.

WAGHMARE, P. R.*et al.* Enzymatic hydrolysis and characterization of waste lignocellulosic biomass produced after dye bioremediation under solid state fermentation. **Bioresource Technology,** v. 168, n. 0, p. 136-141, 9// 2014. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852414002703 >.

WANG, Y.*et al.* High-yield synthesis of bioactive ethyl cinnamate by enzymatic esterification of cinnamic acid. **Food Chemistry,** v. 190, p. 629-633, 1/1/ 2016. ISSN 0308-8146. Disponível em: < http://www.sciencedirect.com/science/article/pii/S030881461500895X >.

WOOD, R. W.; LOOMIS, A. L. XXXVIII. The physical and biological effects of high-frequency sound-waves of great intensity. **The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science,** v. 4, n. 22, p. 417-436, 1927/09/01 1927. ISSN 1941-5982. Disponível em: < http://dx.doi.org/10.1080/14786440908564348 >. Acesso em: 2015/03/01.

YADAV, G. D.; HUDE, M. P.; TALPADE, A. D. Microwave assisted process intensification of lipase catalyzed transesterification of 1,2 propanediol with dimethyl carbonate for the green synthesis of propylene carbonate: Novelties of kinetics and mechanism of consecutive reactions. **Chemical Engineering Journal,** v. 281, p. 199-208, 12/1/ 2015. ISSN 1385-8947. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1385894715008761 >.

YANG, Z.*et al.* Both hydrolytic and transesterification activities of *Penicillium expansum* lipase are significantly enhanced in ionic liquid [BMIm][PF6]. **Journal of Molecular Catalysis B: Enzymatic,** v. 63, n. 1–2, p. 23-30, 4// 2010. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117709003026 >.

ZAGO, E.*et al.* Synthesis of Ethylic Esters for Biodiesel Purposes Using Lipases Naturally Immobilized in a Fermented Solid Produced Using Rhizopus microsporus. **Energy & Fuels,** v. 28, n. 8, p. 5197-5203, 2014/08/21 2014. ISSN 0887-0624. Disponível em: < http://dx.doi.org/10.1021/ef501081d >.

ZHENG, L.; BAUMANN, U.; REYMOND, J.-L. An efficient one-step site-directed and site-saturation mutagenesis protocol. **Nucleic acids research,** v. 32, n. 14, p. e115-e115, 2004. ISSN 0305-1048.

ZHOU, H.*et al.* Solid-state fermentation of *Ginkgo biloba* L. residue for optimal production of cellulase, protease and the simultaneous detoxification of *Ginkgo biloba* L. residue using *Candida tropicalis* and *Aspergillus oryzae*. **European Food Research and Technology,** v. 240, n. 2, p. 379-388, 2015/02/01 2015. ISSN 1438-2377. Disponível em: < http://dx.doi.org/10.1007/s00217-014-2337-2 >.

ZHU, K.*et al.* Study of ultrasound-promoted, lipase-catalyzed synthesis of fructose ester. **Frontiers of Chemical Engineering in China,** v. 4, n. 3, p. 367-371, 2010. ISSN 1673-7369.

ZUBIOLO, C.*et al.* Encapsulation in a sol–gel matrix of lipase from *Aspergillus niger* obtained by bioconversion of a novel agricultural residue. **Bioprocess and Biosystems Engineering,** v. 37, n. 9, p. 1781-1788, 2014/09/01 2014. ISSN 1615-7591. Disponível em: < http://dx.doi.org/10.1007/s00449-014-1151-3 >.

CAPÍTULO 2. DESIRABILITY FUNCTION APPLIED TO MIXTURE DESIGN COMBINED WITH CENTRAL COMPOSITE ROTATABLE DESIGN (CCRD) TO MAXIMIZE LIPASE PRODUCTION AND MINIMIZE COST IN *PENICILLIUM* SP. SOLID FERMENTATION USING AGROINDUSTRIAL RESIDUES

Camilo B. Teixeira[1]*, Luciana F. Fleuri[2]; Gabriela A. Macedo[1]

[1]Food Biochemistry Laboratory, Food Science Department, Food Engineering College (FEA/ UNICAMP), Campinas, São Paulo, Brazil.

[2] Chemistry and Biochemistry Department, Institute of Biosciences, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil

Submitted to *Bioresource Technology*

ABSTRACT

This study evaluated the solid-state fermentation for lipase production by a *Penicillium* sp. strain using wheat bran (WB), soybean meal (SM) and cottonseed meal (CSM) in a mixture substrates through a simplex centroid mixture design (SCMD) combined with central composite rotatable design (CCRD) to evaluate the best formulation for lipase activity (U/g), cost/unit (US$/10³U), protein extract (mg/mL). Desirability function was used to optimize simultaneously the responses for SCMD and CCRD. The best formulation was optimized by a CCRD study which evaluated the parameters temperature, size particle and water volume. The desirability function showed that CSM is the most suitable substrate in SCMD, although the second highest cost, obtaining the best results (89.2 U/g of lipase activity and 0.015 US$/10³U). The CCD optimization achieved a 20% reduction in water consumption in fermentation medium and a 50% reduction in fermentation time.

Keywords: Solid-state fermentation, desirability function, Mixture design, *Penicillium* sp., Lipase

RESUMO

Este estudo avaliou a fermentação em estado sólido (FES) para a produção de lipases por *Penicillium* sp . usando como substrato misturas de farelo de trigo (FT), farelo de soja (FS) e farelo de algodão (FA) através de planejamento de misturas simplex mistura centróide (PMSC) combinado com um design composto central rotacional (CCRD) para avaliar a melhor formulação para a atividade lipase (U/g) , custo/unidade (US$/$10^3$U) , concentração de proteína (mg/mL). A função desejabilidade foi usada para otimizar simultaneamente as respostas para PMSC e CCRD. A melhor formulação foi otimizada por um estudo CCRD que avaliou os parâmetros temperatura, tamanho de partículas e volume de água. A função desejabilidade mostrou que FA é o substrato mais adequado em PMSC, embora o segundo mais caro, obtendo-se os melhores resultados (89,2 U/g de atividade de lipase e de 0,015 US$/$10^3$U). A otimização do DCCR obteve uma redução de 20% no consumo de água no meio de fermentação e uma redução de 50 % no tempo de fermentação.

1. Introduction

The lipases's capacity of synthesizing many commercial chemical compounds has been shown the importance of this enzyme for oleochemichal, pharmaceutical, chemical and food industries. The production of lipase by solid-state fermentation (SSF) has been widely recognized as an alternative for submerge fermentation with possibility to use agriculture byproducts as raw material for fermentation medium composition. Considering this potential, Brazil plays an important role in world's foods and agriculture commodities production, being one of the biggest producers of soybean, sugarcane, waxes vegetable, meat, alcohol and others (Fao, 2015). The country's capacity to develop a bioeconomy has stimulated researches in SSF and biocatalysis with aim on reuse of byproducts generated by agriculture industry.

Oil cakes used for feed applications to poultry, fish and swine industry are rich in protein. Nevertheless, there is an increase necessity for cost reduction in industrial processes and value addition to agro-industrial residues. Therefore, oil cakes is an ideal source of proteinaceous nutrients for several biotechnological processes. Its applications includes fermentative production of enzymes, antibiotics, mushrooms, antioxidants, vitamins, etc. (Ramachandran *et al.*, 2007).

The SSF production of lipases has the opportunity to integrate the agro-industrial byproducts reuse with biocatalysis, two of most important tools for bioeconomy development, considering the application of bioresource's sustainable management using green technologies to generate biodegradable and non-toxic products. For its commercial establishment, design and development of competitive industrial processes should be the main objective for scientific researches considering the green engineering aspects (Anastas and Zimmerman, 2003).

The concept on sustainable development not only comprises the environmental safety but also the economical merit, which is capable of shifting this theory to commercial reality. Most of researches relies on environmental harmless and safety aspects of the process and do not consider the cost evaluation as a principle for economic sustainability.

The main objective of this study is to evaluate the application of desirability function to optimize simultaneously the responses on simplex centroid mixture design (SCMD) to evaluate enzyme activity and the mixture cost and use a central composite design (CCD) to optimize the physical parameters of the process to develop a two-step SSF. This approach relies on lipase production using a wild strain of *Penicillium* sp. and a mix of three different agro-industrial byproducts as substrates.

2. Methodology

2.1. Materials

Soybean meal (SM – 0.39 US$/kg) and Cottonseed meal (CSM – 0.27 US$/kg) were donated by Bunge (Rondonópolis, Mato Grosso, Brazil) and wheat bran Natu's®(WB - 0.19 US$/kg) was purchased in local market (Campinas, São Paulo, Brazil). A strain of *Penicillium* sp. isolated by Chemistry and Biochemistry Department of State University of São Paulo (UNESP - Botucatu, São Paulo, Brazil).

2.2.Protein Determination

The protein concentration in enzymatic extract was determined by Bradford method (Bradford, 1976) using bovine sore albumin as standard for calibration line.

2.3. Lipase Hydrolytic Activity

The lipase activity was determined using a system composed by 5 mL emulsion of extra virgin olive oil and 7% of arabic gum in a proportion of 1:3 (v/v) and 2 mL of 0.1M phosphate buffer pH 7.0 and 1 mL of enzyme extract. The system was incubated in a thermostatic shaker bath at 37°C, 30 minutes and agitation of 130 oscillations per minute. The reaction was stopped by addition of 10 mL acetone: ethanol mixture (1:1, v/v) and the released fatty acids titrated against NaOH 0.05 mol/L, using phenolphthalein as indicator. One unit of lipase activity was defined as one µmol of fatty acids released per minute under the assay conditions (Macedo, Park and Pastore, 1997; Lopes *et al.*, 2011).

2.4.Design of Experiments

2.4.1. Substrate Selection by Simplex Centroid Mixture Design

The simplex-centroid mixture design method evaluated the best formulation for fermentation medium composition for *Penicillium* sp. lipase SSF. The mixture components used were SM, CSM and WB. Each mixture was composed by standard mass fractions of the components from 0 to 1. Lipase activity (U/g) and cost/unit (US$/1000U) were the response variables. The operational conditions were: 500mL erlenmeyers flasks with cotton plug containing 10g of raw material, 10 g of distilled water, inoculated with 1 mL of 10^7 spores inoculum and incubated in a climatic chamber (Nova Ética, SP, Brasil) with 30 ºC, 90% humidity for 96 hours. The polynomial models generated by data regression were evaluated with the ANOVA analysis and validated experimentally. The Table 3 presents the experimental matrix with the mass fractions of the standard mixtures evaluated and its results.

2.4.2. Lipase SSF Optimization by Central Composite Rotatable Design (CCRD)

After selection of the best substrate formulation for fermentation medium, it was applied a CCRD to evaluate some physical operational parameters as size particle (1.4 – 3.3 mm), temperature (29.3 – 32.7 ºC) and water/solid ratio (19.9 -120.4 %). The responses variables were lipase activity (U/mL) and protein (mg/mL) of the enzyme extract. A 2^3 full factorial experimental design with 6 axial points and 3 replicates at central point was used to determine the best conditions for SSF lipase production. The Table 5 presents the experimental matrix with results**.** The ANOVA evaluated the polynomial models generated by least squares regression and the significant regression coefficients (95% of confidence) used for process optimization by the desirability function to obtain higher lipase activity and protein concentration.

2.4.3. Time-course Experiment

After optimization of fermentation medium and the physical parameters of the process, we evaluated the SSF kinetics in optimum conditions to determine the best time. The fermentation was performed in 24, 48, 72, 96, 120 hours and the response evaluated were lipase activity (U/mL), protein (mg/mL) and specific activity (U/mg protein). The desirability function was applied to optimize the responses simultaneously.

2.4.4. Multi-response Optimization by Desirability Function

The desirability function first created by Harrington (1965) and used for multiple responses by Derringer and Suich (1980) was applied for multiple response optimization of the regressions models obtained in the mixture design, central composite rotatable design and the time-course experiment.

2.4.5. Statistical Analysis

The desirability function optimization, regressions of simplex centroid mixture design and central composite rotatable design data and the analysis of variance (ANOVA) with a 10% significance level ($p < 0.1$) were performed on the statistical software STATISTICA 8.0 (Stat Soft Inc., Tulsa, Oklahoma, USA).

3. Results and Discussions

The simplex centroid mixture design evaluated the best formulation for the mixture of SM, CSM and WB for obtaining the best yield of lipase per mass of substrate, intending to be more efficient on byproducts mass utilization, and for the productivity which is related to capacity of generation of lipase activity per raw material cost, showing which mixture has minor

cost and the best enzyme activity. The three substrates were chosen due to its high availability in Brazilian market and its nutritional complexity. Its costs were direct correlated with protein concentration as SB>CS>WB. The lipase activity observed in the mixture design showed a direct correlation with lipid concentration of the mixture (r=0.84 data no shown) within CS>SB>WB. This indicates that lipase biosynthesis depends directly upon lipid concentration in the medium.

3.1.Mixture Design Analysis and Substrate Selection

The mixture design matrix **(Table 3)** shows the experimental values generated for the response variables lipase activity (U/g) and cost/unit (US$/10³U) from the independent variables standard mixture values (WB, SM and CSM).

Table 3 Mixture design experimental matrix with independent variables and response variables values.

Independent Variables			**Response Variables**	
Wheat Bran (WB)	Soybean Meal (SM)	Cottonseed Meal (CS)	**Lipase Activity (U/g of substrate)**	**Cost/unit (US$/1000U)**
1.00	0.00	0.00	**33.12**	**0.0299**
0.00	1.00	0.00	**42.74**	**0.0443**
0.00	0.00	1.00	**67.84**	**0.0198**
0.50	0.50	0.00	**48.61**	**0.0296**
0.50	0.00	0.50	**57.16**	**0.0204**
0.00	0.50	0.50	**62.50**	**0.0259**
0.67	0.17	0.17	**46.47**	**0.0258**
0.17	0.67	0.17	**54.49**	**0.0303**
0.17	0.17	0.67	**66.77**	**0.0206**
0.33	0.33	0.33	**68.91**	**0.0204**
0.33	0.33	0.33	**60.90**	**0.0231**
0.33	0.33	0.33	**70.51**	**0.0200**

Each value of independent variable corresponds to the mass fraction of component of the mixture. The sum of the mass fractions in the same row it is equal 1.

This relation between independent and response variable was translated into a polynomial model function by partial least squares regression method. The model was evaluated for its ability to fit the experimental behavior and prediction. The statistical analysis evaluated the fitness of two common polynomial models used for describe the mixture behavior: linear

and quadratic models were compared by the ANOVA analysis (**Table 4**) which indicated that all models were significant at 95% of confidence for both responses. The quadratic model was the most suitable and robust for describe the experimental data from the two responses observed due to its p-value be the lowest for both responses and the determination coefficient (R^2) higher. Therefore, we choose it for optimization and prediction, with the possibility to predict synergistic effects through interaction variables.

Table 4 Variance Analysis (ANOVA) for the response variables models.

Lipase activity (L)	**SS**	**df**	**MS**	**F**	**Ftab**	**p**	**R^2**
Model	936.066	2	468.0328	6.94	4.25	0.015007	0.6
Total Error	606.835	9	67.4261				
Lack of Fit	553.759	7	79.1084	2.98	19.35	0.274104	
Pure Error	53.076	2	26.5382				
Total Adjusted	1542.901	11	140.2637				
Lipase activity (Q)	**SS**	**df**	**MS**	**F**	**Ftab**	**p**	**R^2**
Model	1288.262	4	322.0656	8.85	4.12	0.007163	0.83
Total Error	254.638	7	36.3769				
Lack of Fit	201.562	5	40.3124	1.51	19.29	0.442542	
Pure Error	53.076	2	26.5382				
Total Adjusted	1542.901	11	140.2637				
Cost/unit (L)	**SS**	**df**	**MS**	**F**	**Ftab**	**p**	**R^2**
Model	0.000387	2	0.000193	10.33	4.25	0.004660	0.69
Total Error	0.000168	9	0.000019				
Lack of Fit	0.000163	7	0.000023	8.01	19.35	0.115318	
Pure Error	0.000006	2	0.000003				
Total Adjusted	0.000555	11	0.000050				
Cost/unit (Q)	**SS**	**df**	**MS**	**F**	**Ftab**	**p**	**R^2**
Model	0.000540	5	0.000108	40.99	4.38	0.000146	0.97
Total Error	0.000016	6	0.000003				
Lack of Fit	0.000010	4	0.000002	0.86	19.24	0.599154	
Pure Error	0.000006	2	0.000003				
Total Adjusted	0.000555	11	0.000050				

(L) – Linear model; (Q) – Quadratic model; SS – Sum of squares, df – degrees of freedom; MS – Mean Squares, F – Fischer test value, Ftab - Fischer table value p – p value; R^2 - Determination coefficient;

The quadratic model obtained and validated by ANOVA is expressed on **equation 1** for lipase activity (U/g) and on **equation 2** for cost/unit (US$/1000U):

Lipase Activity (U/g) *= 35.33*WB + 40.78*SB + 71.17*CSM + 60.35*WB*SB +40.70*SM*CSM* **(Equation 1)**

Cost/unit (US$/1000U) *= 0.03*WB + 0.044*SM + 0.02*CSM -0.034*WB*SM - 0.021*WB*CSM - 0.029*SM*CSM* (**Equation 2)**

Evaluating the effects of the components for response lipase activity, we observed that the highest regression coefficients were CSM > WB*SB > WB*SB > SB > WB. It was possible to observe that CSM showed the highest term for lipase activity due to its highest lipid concentration compared to SB and WB, respectively, which agrees with the correlation of lipid concentration in raw materials, being more meaningful than protein concentration due to SB (higher protein concentration) was the second highest term. The interaction coefficient SB*WB was the second highest term, higher than SB, showing a possible interaction between the matrices for enhance lipase activity through *Penicillium* sp. metabolism. WB is generally recognized as a substrate for lipase biosynthesis on SSF been synergistically affected when applied with other substrates as reported by Mahadik *et al.* (2002) and Edwinoliver *et al.* (2010).

For cost/unit response, the highest effects were SM > WB*SM > WB > CSM*SM > CS > WB*CSM. It is possible to observe that SM had the highest regression coefficient, considering that it has the highest cost and the second highest lipase activity, so the unitary cost for SM is the most expensive. CSM has the lowest unitary cost, although having the second highest material cost. This difference may be attributed for its higher differential in lipase activity compared to WB and SB. It also possible to observe that WB had a minor effect compared to SM. This difference reflects the SM's higher cost compared to WB. The interaction term WB*SM had the second highest effect with negative signal, indicating an inverse relation with unit/cost relation, which means that the interaction results in a decrease for cost/unit response. This fact indicates the synergistic effect between WB and SM due to WB's lower price and SM lipase activity.

Comparing the behavior of the three raw materials and its mixtures in the two responses measured, we can observe that CSM had the best results for both responses with major effect in lipase activity response and the second lowest for product cost, mainly due to its highest lipid concentration. SM and WB had different effects for the two responses as SM being higher in lipase activity and WB was lower in product cost. Despite higher lipase activity

than WB; SM is the most expensive raw material and therefore it has the weakest productivity. The three raw materials presented synergistic effects for lipase activity. For productivity, only CS*SM did not presented it. In lipase activity, CS*SM was the highest interaction effect and CS*WB was the highest for productivity response, mainly due to cost differences between WB and SM. This difference can be observed in triangular surfaces in Figure 5a and b**,** which represents cost/unit and lipase activity responses, respectively. The highest values for lipase activity are situated on CSM and SM region and for cost/unit, in WB and SM region (red color).

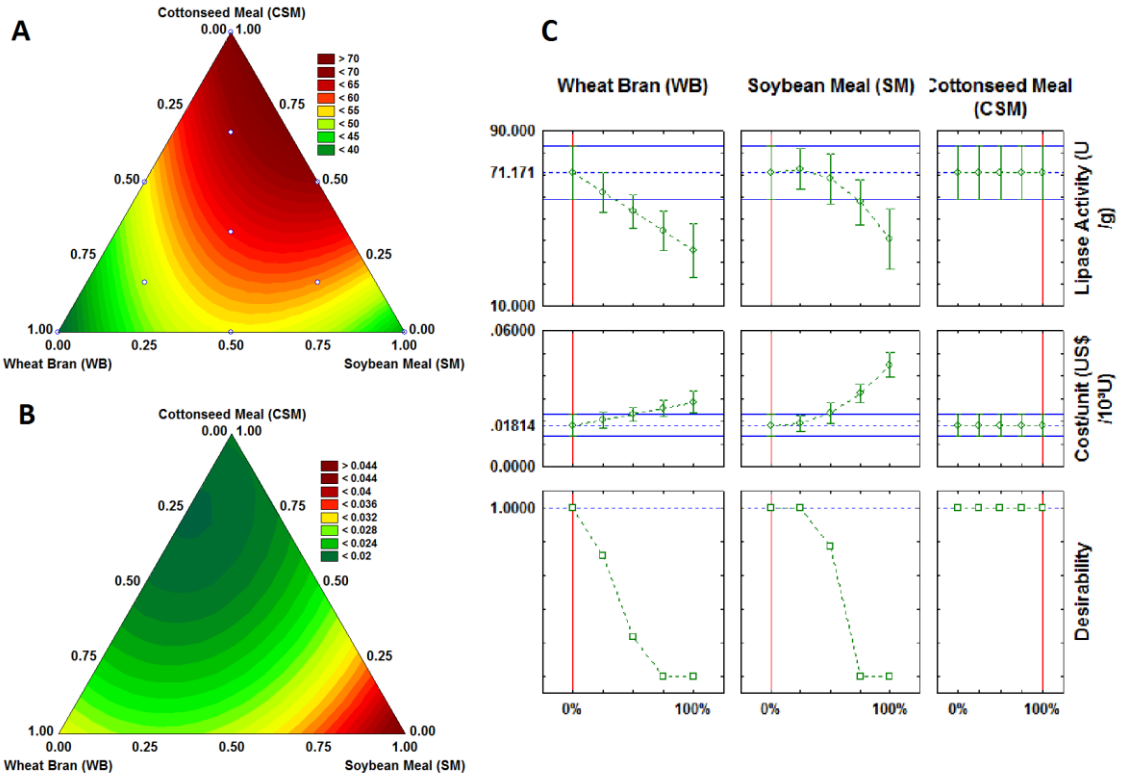

Figure 5 a) Triangular surface for lipase activity (U/g); b) Triangular surface for cost/unit (US$/1000U) c) Profiles for predicted values and desirability.

The double-response simultaneous optimization using desirability function **(**Figure 5c**)** showed that best mixture which gives the highest lipase activity and lowest cost/unit is composed by 100% cottonseed meal (CSM) obtaining a predict result of 71.17 U/g for lipase activity and 0.018 US$/10³U for cost/unit. For experimental validation, we evaluated the formulation with 100% CSM which results showed 89.2 ± 9.3 U/g of lipase activity and 0.015 US$/10³U for product cost. The simultaneous optimization of lipase activity and product cost permits the high efficiency use of raw materials and reduce the product cost. This approach allows a better evaluation of new technologies and sustainable processes.

Terrestrial animals and fish feeds uses CSM as a protein source due to its high protein content, relatively low price and sufficient availability (Li and Robinson, 2006; Sun *et al.*, 2015). However, recent papers present new applications for CSM. Sun *et al.* (2014) recently reported the SSF production of cottonseed peptides (CPs) prepared from CSM fermented by *Bacillus subtilis* BJ-1. They showed that the CP have mainly glutamic acid (211.7g.kg^{-1}), aspartic acid (81.4g.kg^{-1}) and arginine (97.5g.kg^{-1}). The CP presented a concentration-dependent effect for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, hydroxyl radical activity, metal-chelating ability, and reducing power. Emtiazi, Habibi and Taheri (2003) evaluated the production of extracellular lipase by *Pseudomonas* strain X isolated from castor oil. Maximum lipase activity obtained using CSM (400 U.mL^{-1}) in 50 h.

SM is widely used as substrate for SSF. Several authors reported its application: Rigo *et al.* (2010) evaluated the production of extracellular lipases by SSF in soybean meal with different supplements. New Brazilian microorganism *Penicillium* P58 and P74 produced different enzymes with alkaline and acidic characteristics. When urea and soybean oil supplemented the soybean meal, the product yield was 139.2 and 140.7U lipase/g of dry substrate in 48h of fermentation, in alkaline and acidic conditions, respectively. In neutral conditions, the enzyme extract yielded 180.0U lipase/g in 72h. They achieved high lipase activity (up to 200 U/g) using supplementation with 1g/100g of soybean oil and 3g/100g of urea, keeping C/N ratio at 6.11.

Vargas *et al.* (2008) studied the lipase production by *Penicillium simplicissimum* using soybean meal through a factorial design technique, evaluating the effects of incubation temperature, initial moisture of the meal and substrate supplementation with low cost supplements for lipase activity. They used soybean oil and oil rich wastewater from a slaughterhouse, corn steep liquor and yeast hydrolysate as supplementary carbon and nitrogen sources. They obtained that soybean meal without supplements is the best medium for lipase production by *P. simplicissimum.* Temperature and moisture had the highest effects for lipase production using soybean meal. The optimum growth conditions are 27.5 °C using substrate with 550 g.kg^{-1} of initial moisture generating lipase activity of 30 U gds^{-1} dry substrate.

Salgado *et al.* (2014) evaluated a two-phase olive mill waste (TPOMW) as substrate for SSF production of lipase, despite of its high phenolic compounds concentration and low nitrogen content. The best results produced by *A. ibericus* on a mixture of TPOMW, urea, and exhausted grape mark (EGM). Urea had the highest effect for lipase production. The

optimization of *A. ibericus* lipase production using a full factorial design (3^2) obtained a substrate composition (0.073g urea/g and 25% of EGM) reaching 18.67U/g of lipase activity.

Several recent works reported the application of oil cakes and different residues for lipase production by SSF: Santis-Navarro *et al.* (2011) used a microbial consortium from a wastewater sludges mixture in a medium containing s rich fat solid industrial wastes under thermophilic conditions (45ºC); Zubiolo *et al.* (2015) produced a lipase from *Aspergillus niger* by pumpkin seed flour SSF. Veerabhadrappa, Shivakumar and Devappa (2014) studied the SSF of *Jatropha* seed cake (JSC) by *Aspergillus versicolor* CJS-98. Fleuri *et al.* (2014) used *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. for lipase production by SSF and obtained the highest activities using WB and SM.

Many raw materials have been exploited as fermentable sources for extracellular lipase production from fungi as Olive oil cake, Babassu oil cake, Egg yolk, Almond meal, Sunflower oil, Soybean bran, Olive oil, Wheat bran and Soy cake (Singh and Mukhopadhyay, 2012; Singh *et al.*, 2014).

3.2.Central Composite Rotatable Design Analysis

After substrate selection for fermentation medium composition it was carried out a CCRD study to optimize the SSF process for lipase production using *Penicillium* sp., cottonseed meal (CS) and water as the reaction medium. The fermentation was carried for 96 hours at 30ºC with 70% relative humidity in climatic chamber with air circulation. The process was optimized using the most meaningful variables for SSF: Temperature, water/solid ratio and particle size as input variables. It is possible to observe in Table 5 that run 16 obtained the higher lipase activity with 16.03 U/mL of enzyme extract. For protein concentration, run 9 obtained 0.98 mg/mL of enzyme extract. The run 11 had the lowest lipase activity (4.01 U/mL) and run 7 had the lowest protein concentration (0.51 mg/mL). The results obtained in the CCRD study **(**Table 5**)** were submitted to a non-linear regression using the least squares method for estimate a polynomial model which fits best the experimental data in CCRD matrix. The analyze of the second order polynomial model was based on the most significant terms ($p<0.05$ for T-student) and reparametrized model was evaluated by ANOVA (Table 6) for its fitness for its suitability and reliability in prediction.

Table 5 CCRD experimental matrix with real and codified values of the independent variables and the results of response variables for cottonseed SSF.

	Independent Variables			**Response Variables**	
Runs	Temperature (ºC)	Water/solid (%)	Size Particle (mm)	**Lipase Activity (U/mL)**	**Protein (mg/mL)**
1	30.00(-1)	40.00(-1)	1.80(-1)	**9.94**	**0.97**
2	30.00(-1)	100.00(+1)	3.00(+1)	**12.82**	**0.82**
3	32.00(+1)	40.00(-1)	3.00(+1)	**10.90**	**0.53**
4	32.00(+1)	100.00(+1)	1.80(-1)	**9.62**	**0.57**
5	30.00(-1)	40.00(-1)	3.00(+1)	**10.90**	**0.95**
6	30.00(-1)	100.00(+1)	1.80(-1)	**13.62**	**0.85**
7	32.00(+1)	40.00(-1)	1.80(-1)	**9.13**	**0.51**
8	32.00(+1)	100.00(+1)	3.00(+1)	**13.94**	**0.69**
9	29.3(-1.68)	70.00(0)	2.40(0)	**15.95**	**0.98**
10	32.70(+1.68)	70.00(0)	2.40(0)	**10.58**	**0.67**
11	31.00(0)	19.90(-1.68)	2.40(0)	**4.01**	**0.88**
12	31.00(0)	120.4(+1.68)	2.40(0)	**12.82**	**0.72**
13	31.00(0)	70.00(0)	1.40(-1.68)	**8.49**	**0.60**
14	31.00(0)	70.00(0)	3.30(+1.68)	**14.10**	**0.75**
15	31.00(0)	70.00(0)	2.40(0)	**15.06**	**0.87**
16	31.00(0)	70.00(0)	2.40(0)	**16.03**	**0.86**
17	31.00(0)	70.00(0)	2.40(0)	**15.71**	**0.81**

Table 6 Analysis of variance (ANOVA) for the three response variables of the CCRD study: Lipase activity, protein, specific activity.

Lipase Activity	**SS**	**df**	**MS**	**Fcal**	**Ftab**	**p**
Regression*	149.556	7	21.365	11.803	3.29	0.0006
Residues	16.291	9	1.81			
Lack of Fit	15.803	7	2.258	9.241	19.35	0.101
Pure Error	0.489	2	0.244			
Total						
R^2	0.88					
Protein	**SS**	**df**	**MS**	**Fcal**	**Ftab**	**p**
Regression*	0.3221	4	0.10738	28.945	3.17	0.000005
Resídues	0.0482	13	0.0037			
Lack of Fit	0.0461	11	0.0041	4.061	19.40	0.214
Pure Error	0.029356	2	0.01			
Total	0.374889	16				
R^2	0.84					

SS – Sum of Squares, DG – Degrees of freedom; MS - Mean Squares, Fcal- Fischer's calculated value; Ftab – Fischer's table value.

*Statistically significant at 95% of confidence.

The ANOVA analysis showed that lipase activity and protein responses were statistically significant at 5 % of reliability, evaluating the Fischer's value (F) and p-value for regression and of each polynomial model. The lack of fit was not significant for these two responses and indicates that experimental repeatability (pure error) does not affect the total variation obtained by the polynomial model, which is considered adequate to explain the variations in experimental data. Therefore, we can only use for prediction the models obtained for lipase activity and protein responses (**Equations 3 and 4** respectively**)**.

Lipase Activity (U/mL) *= -2.62*(water/solid)² + 1.56*water/solid -1.41*particle size + 1.08*(particle size)² - 0.87*temperature + 0.74*temperature*particle size +15.6*

(Equation 3)

Protein (mg/mL) *= - 0.13*temperature – 0.07*(particle size)² + 0.05*temperature*water/solid + 0.82* **(Equation 4)**

It was possible to observe in lipase activity response that water/solid ratio was the higher regression coefficient with major influence followed by particle size and temperature, respectively. Only temperature did not present a quadratic term statistically significant for lipase activity. The interaction of temperature and water/solid ratio was the only one considered statistically significant with a positive correlation with lipase activity.

Analyzing the Figure 6a, b and c, we observed the representation of input variables for lipase activity. In 2a we can see that lipase activity decreases with increasing in temperature and increases when used with the mean particle size, achieving the bests results for lipase activity. In 2b, water/solids ratio had a great influence when used above 50%. In **2c**, we observed that size particle and water/solids ratio obtained the higher lipase activity when settled in middle value.

In Figure 6d, we observed the linear term of temperature with inversely correlation with protein concentration, a slightly difference from lipase activity, which was a quadratic relationship. Size particle appears to have a similar influence as in lipase activity with high concentrations of protein in the middle values. In Figure 6**e**, it seems clear the interaction coefficient between temperature and water/solid ratio indicating a decrease in protein concentration with increase in the two variables. Slightly different from lipase activity, the protein concentration seems to be higher when the fermentation was carried on above 40% water/solid concentration. In Figure 6f**,** when the system is operated with the mean size particle (2.4 mm), higher concentrations of protein were achieved, similar to lipase activity. However, it showed no dependence from water/soli ratio.

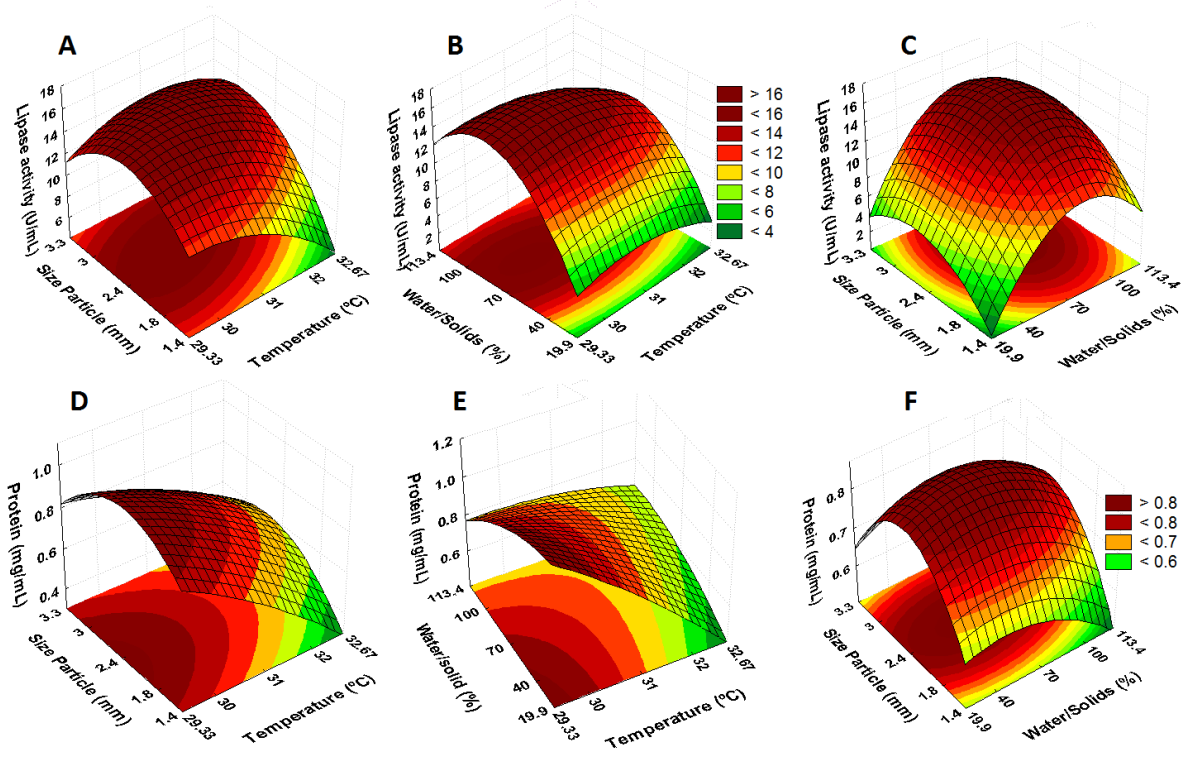

Figure 6 CCRD response surfaces for lipase activity a) Temperature and size particle; b) Water/solids and temperature; c) Size particle and water/solid ratio; and design response surface for protein: d) temperature and size particle; e) water/solid ratio and c) size particle and water/solid ratio.

Moisture, nature of solid substrate employed and heat and mass transfer characteristics within the substrate bed are the most important process parameters that affects the SSF (Singhania *et al.*, 2009; Rajendran and Thangavelu, 2013). Moisture plays an important role in SSF and some problems results from high moisture level due to low substrate porosity, impeding oxygen diffusion. Low moisture levels hinder microbial growth by decreasing the nutrients availability to the microorganism and by a lowering the substrate bump degree (Mahadik *et al.*, 2002; Gutarra *et al.*, 2005; Mahanta, Gupta and Khare, 2008; Chaari *et al.*, 2012; Rajendran and Thangavelu, 2013).

Mahanta, Gupta and Khare (2008) reported that 50% of moisture was the optimal giving maximum amount of protease and lipase production (6560 and 620 U/g of substrate, respectively) by solvent tolerant *Pseudomonas aeruginosa* PseA in SSF using *Jatropha curcas* seed cake as substrate. An increase and decrease in the moisture content significantly affected enzyme production. Mahadik *et al.* (2002) observed maximum lipase production from *A. niger* at about 71% moisture content with wheat bran as substrate. Vaseghi *et al.* (2013) produced *Rhizopus oryzae* lipase using sugarcane bagasse by SSF in a tray bioreactor and found that initial moisture content of 80% and 70% was optimum for the top and middle trays.

The same authors (Vaseghi *et al.*, 2013) evaluated the size particle to optimize the lipase production and obtained the highest lipolytic activity using particle size in the range of (0.335– 1 mm). Particle size above this range makes lipase activity decrease maybe due to the reduction in contact surface between substrate's particles and fungus solution, which slowed down microbial growth. Nevertheless, they observed that another important effect for microbial growth is the air supplied due to the aeration for microbial growth, which directly influences the respiration quotient. Thus, the use of very small particles (<0.18 mm) did not result in higher lipase activity, mainly due to the lack oxygen diffusion for the microorganism.

Moftah *et al.* (2012) studied the lipase and protease production by *Candida utilis* using olive oil cake in SSF and observed that increasing the particle size, both lipase and protease activities increased. A particle size higher than 800 μm was the most appropriate. Very small particles could result in substrate adhesiveness, resulting in oxygen transfer limitation and reduced microbial growth. However, larger particles offer better aeration efficiency due to increased interparticle space. Godoy *et al.* (2011) reported that the particle size showed a positive effect on lipase production by *Penicillium simplicissimum* in castor bean waste, showing better results with particles between 850 and 1,180 μm.

Sethi *et al.* (2013) produced lipase from *Aspergillus terreus* using mustard seed oil cake as a carbon source in SSF process and obtained maximum enzyme activity at 30 °C with all other conditions kept constant. Kumar *et al.* (2011) employing grease waste as a substrate for the production of lipase by a newly isolated fungal strain of *Penicillium chrysogenum* and observed that incubation temperature was an important factor for the lipase production. At high temperature, protease production happened denaturating the enzyme. The maximum enzyme yield was 42.5 U/ml at 30ºC.

Di Luccio *et al.* (2004) evaluated the effect of temperature, moisture and carbon supplementation on lipase production by *Penicillium simplicissimum* SSF of soy cake. They observed that temperature had a negative effect, not common considering that the mesophyllic microorganisms increases the metabolism with the temperature in the range investigated. They supposed that the negative effect could be related to the strong correlation between temperature and substrate moisture. The temperature elevation causes cake moisture decrease, generating an increase in water vapor pressure. They observed a strong correlation between moisture and temperature effects, which also affects negatively the activity in same order value as the other isolated variables. Proteolytic activity is another possibility related to a higher level denaturation of the produced lipase, induced by the temperature elevation.

3.2.1. Optimization and Prediction profile by Desirability function

The optimization made using desirability function approach used the polynomial models obtained in CCD study for prediction and establishment of a desirability profile for simultaneous optimization of lipase activity and protein. The Figure 7 represents the results obtained on prediction simulation for temperature, water/solids ratio and size particle effects for lipase activity and protein concentration. We observed that size particle behavior was similar for the two responses,

The desirability of individual response for lipase activity indicates that the optimum conditions were: temperature 30.3 ºC; water/solids ratio 90% and size particle of 2.35 mm achieving a desirability value of 0.94. For protein response, the optimum conditions were temperature 29.6 ºC, water/solids ratio 66.6 (%) and size particle of 2.82 and desirability value of 1. For simultaneous optimization, the best conditions were 29.6 ºC, water/solids ratio 90% and particle size 2.35 mm with a desirability value of 0.94. We can observe in desirability profile for water/solids ratio that optimum condition is constant between 70-90%. Therefore, we decided to reduce the water content in the fermentation medium from 90 to 70% and simulate the results to observe any changes in desirability value, which increased for 0.95, with small increase in lipase activity (16.07 U/mL) and no changes in protein concentration. Therefore, we decided to use the optimum conditions for SSF production of lipase as temperature 30ºC, water/solids ratio (70%) and particle size 2.4 mm.

The experimental validation in the optimum conditions obtained a result of 14.47 ± 1.28 U/mL of lipase activity and 0.91 ± 0.04 mg/mL of protein concentration on enzyme extract. The predicted results by the polynomial model were: 15.8 U/mL for lipase activity and 0.94 of protein concentration on enzyme extract. The results were slightly lower than the predicted by the model. However, they are situated on the limits of confiability for prediction (14.7 to 16.8 U/mL for lipase activity and 0.89 to 1 mg/mL for protein concentration) and therefore, we considered the polynomial model suitable for prediction.

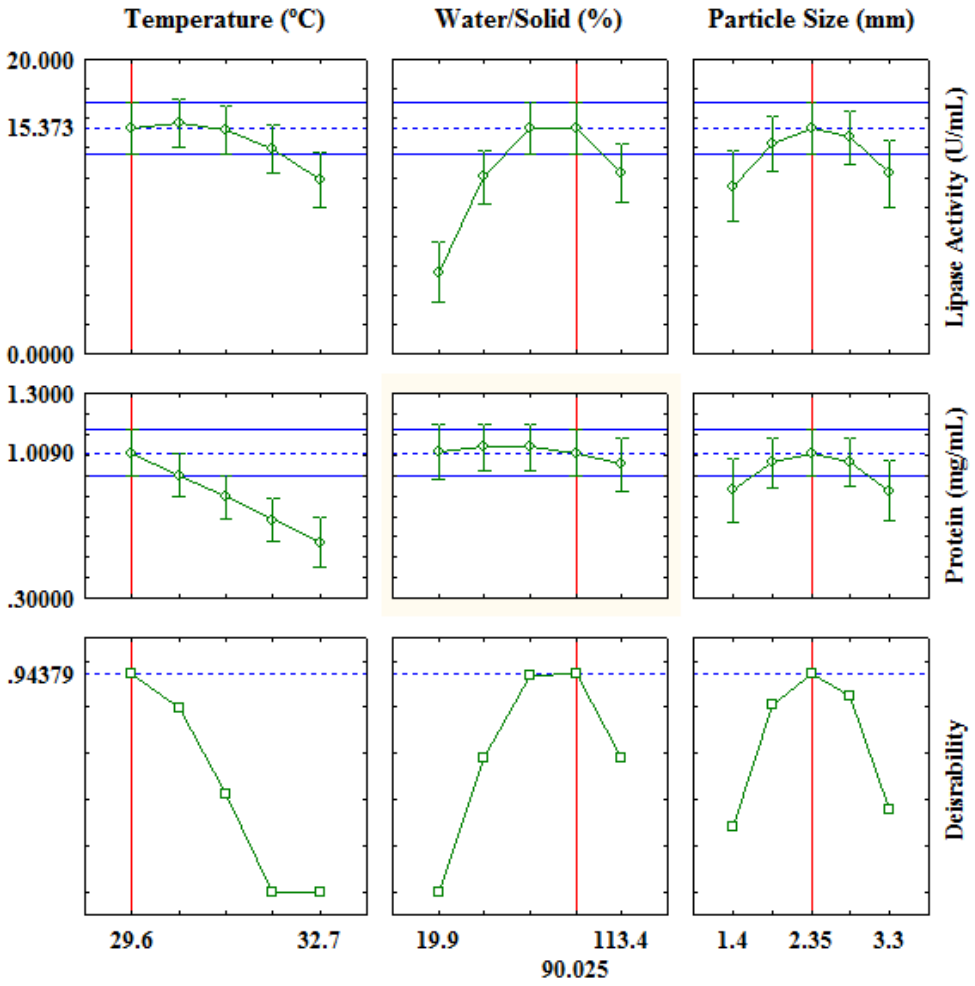

Figure 7 Predicted values and desirability profile of the CCD polynomial models.

3.3.Fermentation Time course

The SSF production of lipase using a wild strain of *Penicillium* sp. as biocatalyst and cottonseed meal and water as fermentation medium was submitted to a time course experiment to evaluate the best conditions determined on CCRD optimization. The results obtained (triplicate) were lipase activity (U/mL), protein (mg/mL) and specific activity (U/mg) for 24, 48, 72, 96 and 120 hours of fermentation (Figure 8a).

We observed that lipase activity and protein were slightly lower than in CCRD study using the same conditions. This fact can be attributed to differences in cottonseed meal batches used for time course study.

The highest peaks of lipase activity were in 48 hours and after that become to decrease but did not differ statically (Tukey test at 5% significance), being considered constant until 120

hours. Protein increased from 24 to 48 hours and still constant until 120 hours. Specific activity obtained its higher peak at 72 hours, but it was not statistically different from 48 hours.

Primary tests before substrate selection and SSF optimization showed that highest peaks of lipase activity for *Penicllium* sp. strain in wheat bran were obtained in 96 hours. After SSF optimization we observed a reduction in fermentation time from 96 to 48h which means a 50% decrease, improving the time productivity in SSF process.

The desirability optimization approach (Figure 8b) showed that 48 hours is the optimum fermentation time for obtain simultaneously the bests results for lipase activity, protein and specific activity. Therefore, we considered 48 hours as the best time for SSF production lipases using *Penicillium* sp. and cottonseed meal. This approach permitted an improvement on fermentation productivity from 13 to 27 U/g.h, also helping to reduce energy consumption on fermentation process.

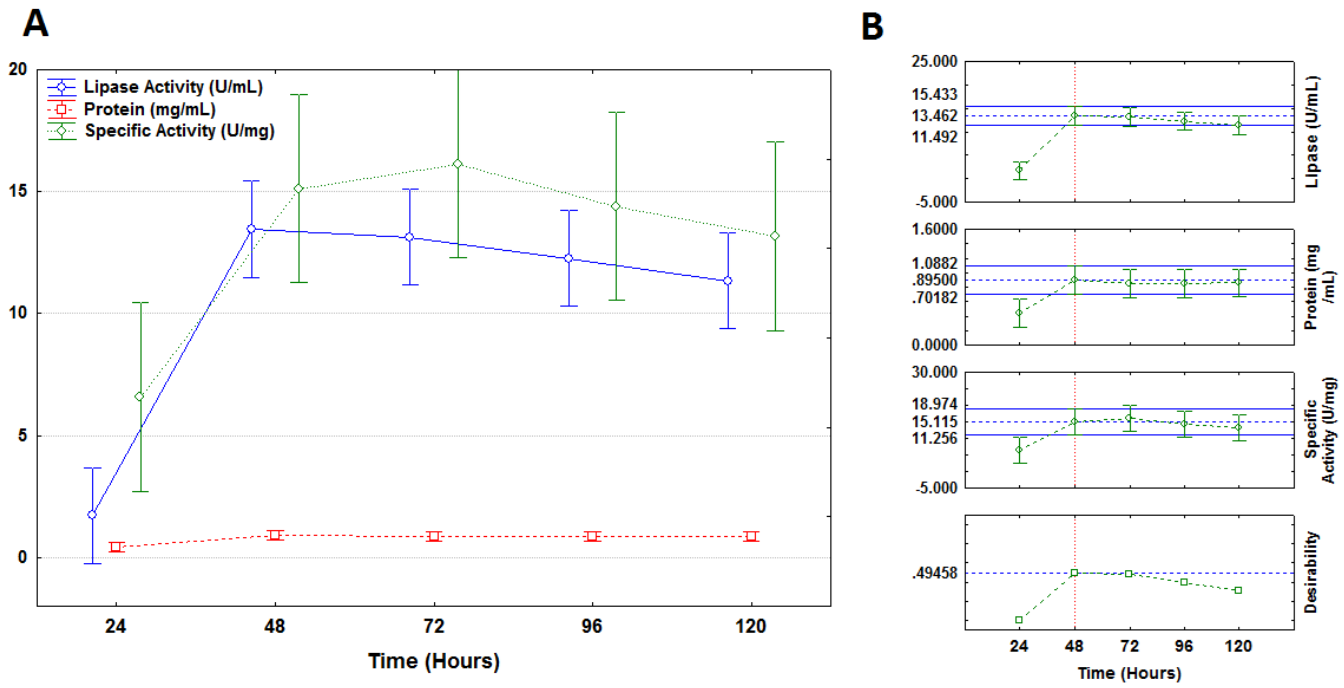

Figure 8 Experimental validation of optimum conditions: a) Time course of fermentation; b) Optimization of fermentation time by Desirability function.

Toscano *et al.* (2013) studied the lipase production by *Penicillium chrysogenum*, *Trichoderma harzianum* and *Aspergillus flavus* in wheat bran-olive oil, and wheat bran-castor oil cake and reported that fermentation time intensely affect enzyme production. Time increase from 72 h to 120 h resulted in a significant lipase activity increase for all the microorganisms. Maximum lipase activity reached at 120 h of fermentation. Past these fermentation time, the

enzyme activity decreases probably due to the nutrients depletion or enzyme denaturation caused by the interaction with other components present in the medium.

Gutarra *et al.* (2009) used a wild-type Brazilian strain of *Penicillium simplicissimum* in babassu cake SSF for production of acidic and thermostable lipase and obtained a maximum lipase activity of 19.6 U/g (35ºC, pH 7.0) after 72h. Di Luccio *et al.* (2004) obtained analogous values of lipase activity (21 U/g) after 48h of fermentation using same strain of *P. simplicissimum* in SM.

4. Conclusion

Application of raw material cost productivity as an input variable permitted the evaluation of commercial aspects in the SSF medium composition. This approach could be useful in research and develop of new processes for raw material selection with different characteristics and performances. Cottonseed meal was the best substrate for lipase production obtaining 89.2 U/g and 0.015 US$/$10^3$U as unitary cost.

The desirability function permitted a simultaneous optimization of different variables in SCMD and CCRD. The analysis also permitted a 20% reduction in water consumption in fermentation medium and a 50% reduction in fermentation time, duplicating the productivity and contributing to process sustainability and, therefore, indicates that it is an important tool for helping in develop processes with green engineering aspects.

Acknowledgements

The authors would like to thanks CNPq (Brazilian National Council for Scientific and Technology Development) for financial support; Bunge company for cottonseed meal and soybean meal donation.

REFERENCES

ANASTAS, P. T.; ZIMMERMAN, J. B. Peer Reviewed: Design Through the 12 Principles of Green Engineering. **Environmental Science & Technology,** v. 37, n. 5, p. 94A-101A, 2003/03/01 2003. ISSN 0013-936X. Disponível em: < http://dx.doi.org/10.1021/es032373g >.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry,** v. 72, n. 1–2, p. 248-254, 5/7/ 1976. ISSN 0003-2697. Disponível em: < http://www.sciencedirect.com/science/article/pii/0003269776905273 >.

CHAARI, F.*et al.* Statistical optimization for the production of lichenase by a newly isolated *Bacillus licheniformis* UEB CF in solid state fermentation using pea pomace as a novel solid support. **Industrial Crops and Products,** v. 40, p. 192-198, 11// 2012. ISSN 0926-6690. Disponível em: < http://www.sciencedirect.com/science/article/pii/S092666901200146X >.

DERRINGER, G.; SUICH, R. Simultaneous optimization of several response variables . **Journal of Quality Technology,** v. 12, p. 214-219, 1980.

DI LUCCIO, M.*et al.* Effect of temperature, moisture, and carbon supplementation on lipase production by solid-state fermentation of soy cake by *Penicillium simplicissimum*. **Applied Biochemistry and Biotechnology,** v. 113, n. 1-3, p. 173-180, 2004/03/01 2004. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1385/ABAB%3A113%3A1-3%3A173 >.

EDWINOLIVER, N. G.*et al.* Scale up of a novel tri-substrate fermentation for enhanced production of *Aspergillus niger* lipase for tallow hydrolysis. **Bioresource Technology,** v. 101, n. 17, p. 6791-6796, 9// 2010. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852410005687 >.

EMTIAZI, G.; HABIBI, M. H.; TAHERI, A. R. Production of thermostable extracellular lipase by *Pseudomonas* grown on cotton cake and cod removal of sunflower oil waste. **Fresenius Environmental Bulletin,** v. 12, n. 7, p. 704-708, 2003. Disponível em: < http://www.scopus.com/inward/record.url?eid=2-s2.0-0042286498&partnerID=40&md5=4710bedb482236532f14c453b2c7f861 >.

FAO. **Food and Agricultural commodities production (2012)** 2015.

FLEURI, L.*et al.* Production of fungal lipases using wheat bran and soybean bran and incorporation of sugarcane bagasse as a co-substrate in solid-state fermentation. **Food Science and Biotechnology,** v. 23, n. 4, p. 1199-1205, 2014/08/01 2014. ISSN 1226-7708. Disponível em: < http://dx.doi.org/10.1007/s10068-014-0164-7 >.

GODOY, M. G.*et al.* Adding value to a toxic residue from the biodiesel industry: production of two distinct pool of lipases from *Penicillium simplicissimum* in castor bean waste. **Journal of industrial microbiology & biotechnology,** v. 38, n. 8, p. 945-953, 2011. ISSN 1367-5435.

GUTARRA, M. E.*et al.* Lipase Production by Solid-State Fermentation. In: DAVISON, B.;EVANS, B., *et al* (Ed.). **Twenty-Sixth Symposium on Biotechnology for Fuels and**

Chemicals: Humana Press, 2005. cap. 10, p.105-116. (ABAB Symposium). ISBN 978-1-58829-697-9.

GUTARRA, M. L. E.*et al.* Production of an acidic and thermostable lipase of the mesophilic fungus *Penicillium simplicissimum* by solid-state fermentation. **Bioresource Technology,** v. 100, n. 21, p. 5249-5254, 11// 2009. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852409005872 >.

HARRINGTON, E. C. The desirability function. **Industrial Quality Control,** v. 21, p. 494, 1965.

KUMAR, S.*et al.* Use of evolutionary operation (EVOP) factorial design technique to develop a bioprocess using grease waste as a substrate for lipase production. **Bioresource Technology,** v. 102, n. 7, p. 4909-4912, 4// 2011. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852411000071 >.

LI, M. H.; ROBINSON, E. H. Use of Cottonseed Meal in Aquatic Animal Diets: A Review. **North American Journal of Aquaculture,** v. 68, n. 1, p. 14-22, 2006/01/01 2006. ISSN 1522-2055. Disponível em: < http://www.tandfonline.com/doi/abs/10.1577/A05-028.1 >. Acesso em: 2015/08/26.

LOPES, D. B.*et al.* Lipase and esterase: to what extent can this classification be applied accurately? **Food Science and Technology (Campinas),** v. 31, p. 603-613, 2011. ISSN 0101-2061. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0101-20612011000300009&nrm=iso >.

MACEDO, G.; PARK, Y.; PASTORE, G. Partial purification and characterization of an extracellular lipase from a newly isolated strain of *Geotrichum* sp. **Revista de Microbiologia,** v. 28, n. 2, p. 90-95, 1997. ISSN 0001-3714.

MAHADIK, N. D.*et al.* Production of acidic lipase by *Aspergillus niger* in solid state fermentation. **Process Biochemistry,** v. 38, n. 5, p. 715-721, 12/31/ 2002. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0032959202001942 >.

MAHANTA, N.; GUPTA, A.; KHARE, S. K. Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* PseA in solid-state fermentation using *Jatropha curcas* seed cake as substrate. **Bioresource Technology,** v. 99, n. 6, p. 1729-1735, 4// 2008. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852407002969 >.

MOFTAH, O.*et al.* Adding Value to the Oil Cake as a Waste from Oil Processing Industry: Production of Lipase and Protease by *Candida utilis* in Solid State Fermentation. **Applied Biochemistry and Biotechnology,** v. 166, n. 2, p. 348-364, 2012/01/01 2012. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1007/s12010-011-9429-2 >.

RAJENDRAN, A.; THANGAVELU, V. Utilizing Agricultural Wastes as Substrates for Lipase Production by *Candida rugosa* NCIM 3462 in Solid-State Fermentation: Response Surface Optimization of Fermentation Parameters. **Waste and Biomass Valorization,** v. 4, n. 2, p. 347-357, 2013/06/01 2013. ISSN 1877-2641. Disponível em: < http://dx.doi.org/10.1007/s12649-012-9140-8 >.

RAMACHANDRAN, S.*et al.* Oil cakes and their biotechnological applications – A review. **Bioresource Technology,** v. 98, n. 10, p. 2000-2009, 7// 2007. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852406003981 >.

RIGO, E.*et al.* Lipase production by solid fermentation of soybean meal with different supplements. **LWT - Food Science and Technology,** v. 43, n. 7, p. 1132-1137, 9// 2010. ISSN 0023-6438. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0023643810000824 >.

SALGADO, J.*et al.* Integrated Use of Residues from Olive Mill and Winery for Lipase Production by Solid State Fermentation with *Aspergillus* sp. **Applied Biochemistry and Biotechnology,** v. 172, n. 4, p. 1832-1845, 2014/02/01 2014. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1007/s12010-013-0613-4 >.

SANTIS-NAVARRO, A.*et al.* Production of lipases by solid state fermentation using vegetable oil-refining wastes. **Bioresource Technology,** v. 102, n. 21, p. 10080-10084, 11// 2011. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852411011680 >.

SETHI, B.*et al.* Lipase production by *Aspergillus terreus* using mustard seed oil cake as a carbon source. **Annals of Microbiology,** v. 63, n. 1, p. 241-252, 2013/03/01 2013. ISSN 1590-4261. Disponível em: < http://dx.doi.org/10.1007/s13213-012-0467-y >.

SINGH, A.; MUKHOPADHYAY, M. Overview of Fungal Lipase: A Review. **Applied Biochemistry and Biotechnology,** v. 166, n. 2, p. 486-520, 2012/01/01 2012. ISSN 0273-2289. Disponível em: < http://dx.doi.org/10.1007/s12010-011-9444-3 >.

SINGH, M. K.*et al.* Novel lipase from basidiomycetes *Schizophyllum* commune ISTL04, produced by solid state fermentation of *Leucaena leucocephala* seeds. **Journal of Molecular Catalysis B: Enzymatic,** v. 110, n. 0, p. 92-99, 12// 2014. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117714002859 >.

SINGHANIA, R. R.*et al.* Recent advances in solid-state fermentation. **Biochemical Engineering Journal,** v. 44, n. 1, p. 13-18, 4/15/ 2009. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X08003331 >.

SUN, H.*et al.* Partial substitution of fish meal with fermented cottonseed meal in juvenile black sea bream (*Acanthopagrus schlegelii*) diets. **Aquaculture,** v. 446, p. 30-36, 9/1/ 2015. ISSN 0044-8486. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0044848615002240 >.

SUN, H.*et al.* Chemical composition and in vitro antioxidant property of peptides produced from cottonseed meal by solid-state fermentation. **CyTA - Journal of Food,** v. 13, n. 2, p. 264-272, 2015/04/03 2014. ISSN 1947-6337. Disponível em: < http://dx.doi.org/10.1080/19476337.2014.948072 >. Acesso em: 2015/08/26.

TOSCANO, L.*et al.* Lipase Production Through Solid-State Fermentation using Agro-Industrial Residues as Substrates and Newly Isolated Fungal Strains. **Biotechnology &**

Biotechnological Equipment, v. 27, n. 5, p. 4074-4077, 2013/01/01 2013. ISSN 1310-2818. Disponível em: < http://dx.doi.org/10.5504/BBEQ.2012.0145 >. Acesso em: 2015/08/27.

VARGAS, G. D. L. P.*et al.* Optimization of lipase production by *Penicillium simplicissimum* in soybean meal. **Journal of Chemical Technology & Biotechnology,** v. 83, n. 1, p. 47-54, 2008. ISSN 1097-4660. Disponível em: < http://dx.doi.org/10.1002/jctb.1776 >.

VASEGHI, Z.*et al.* Production of active lipase by *Rhizopus oryzae* from sugarcane bagasse: solid state fermentation in a tray bioreactor. **International Journal of Food Science & Technology,** v. 48, n. 2, p. 283-289, 2013. ISSN 1365-2621. Disponível em: < http://dx.doi.org/10.1111/j.1365-2621.2012.03185.x >.

VEERABHADRAPPA, M. B.; SHIVAKUMAR, S. B.; DEVAPPA, S. Solid-state fermentation of Jatropha seed cake for optimization of lipase, protease and detoxification of anti-nutrients in Jatropha seed cake using *Aspergillus versicolor* CJS-98. **Journal of Bioscience and Bioengineering,** v. 117, n. 2, p. 208-214, 2// 2014. ISSN 1389-1723. Disponível em: < http://www.sciencedirect.com/science/article/pii/S138917231300265X >.

ZUBIOLO, C.*et al.* Morphological and physicochemical aspects of microbial lipase obtained from novel agroindustrial waste encapsulated in a sol–gel matrix. **Journal of Thermal Analysis and Calorimetry,** v. 120, n. 3, p. 1503-1509, 2015/06/01 2015. ISSN 1388-6150. Disponível em: < http://dx.doi.org/10.1007/s10973-015-4494-3 >.

CAPÍTULO 3. CHARACTERIZATION OF *PENICILLIUM* SP. LIPASE AND ULTRASOUND-ASSISTED HYDROLYSIS OF COTTONSEED OIL CATALYZED BY LIPASIC SOLID FERMENTED (LSF)

Camilo Barroso Teixeira*, Gabriela Alves Macedo

Biochemistry Laboratory, Food Science Department, Food Engineering College, University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil

Submitted to *Ultrasonics Sonochemistry*

ABSTRACT

The study evaluated the lipasic solid fermented (LSF) from a *Penicillium* sp. brazilian strain produced by cottonseed meal solid-state fermentation on cottonseed oil hydrolysis in two reaction systems: ultrasound (US) probe and shaking bath. Firstly, we determined the optimum and stability parameters of temperature and pH for lipase activity by central composite rotatable design (CCRD), specificity (chain size) and solvent stability. The optimum conditions were: 40ºC and pH 7 for activity and 30ºC and pH 3 for stability; high affinity with long chain fatty acids (p-NPP) and stable in hydrophobic solvent (hexane). A CCRD study evaluated the US and shaking bath assisted hydrolysis of cottonseed oil using as independent variables: % LSF, buffer/oil ratio for shaking bath and the same variables plus power density (W/mL) US probe system. The results showed that all variables were significant for oil hydrolysis with %LSF and buffer/oil ratio with positive effects for both systems. However, the US system showed a second order behavior for buffer/oil ratio, differently from orbital shaker reaction, which was linear. Besides that, the oil hydrolysis tends to increase in higher LSF concentration in US assisted reaction and to decrease in higher concentration of LSF in shaking bath. US power density had a negative effect indicating reaction inhibition. Desirability function optimization showed the possibility of improve hydrolysis yield by increasing buffer/oil ratio in both reaction systems. A time course experiment also optimized by desirability function showed that US probe system achieved 54% of oil hydrolysis yield in 30 minutes and 28.5% in 60 minutes for shaking bath.

Keywords: Lipase, ultrasound, solid fermented, cottonseed oil, hydrolysis.

RESUMO

O estudo avaliou o fermentado sólido lipásico (FSL) de um *Penicillium* sp. brasileiro produzido por farelo de algodão através da fermentação em estado sólido para aplicação na hidrólise de óleo de algodão em dois sistemas de reação: ultrassom (US) sonda e banho com agitação. Primeiramente, foram determinados os parâmetros ótimos e de estabilidade de temperatura e pH para a atividade do extrato de lipase por delineamento composto central rotativo (DCCR), especificidade (tamanho da cadeia) e estabilidade em solvente orgânico. As condições ótimas foram: 40ºC e pH 7 para a atividade e 30ºC e pH 3 para a estabilidade; o extrato apresentou elevada afinidade com ácidos gordos de cadeia longa (p-NPP) e estabilidade em solvente hidrofóbico (hexano). Um estudo CCRD avaliou a hidrólise de óleo de algodão assistida por US e em banho com agitação usando como variáveis independentes: LSF%, tampão/proporção de óleo para banho e as mesmas variáveis mais densidade de potência (W/mL) para sistema de sonda US. Os resultados mostraram que todas as variáveis foram significativas para a hidrólise do óleo com FSL% e proporção de tampão/óleo com efeitos positivos para ambos os sistemas. No entanto, o sistema de US mostrou um comportamento de segunda ordem para a proporção de tampão/óleo, diferente da reação em agitador orbital que era linear. Além disso, a hidrólise do óleo tende a aumentar na maior concentração na FSL para reação assistida por US e a diminuir em maior concentração de FSL no banho de agitação. Densidade de potência de US teve um resultado negativo indicando inibição de reação. Otimização pela função desejabilidade mostrou que há possibilidade de melhorar o rendimento da hidrólise, aumentando a proporção de tampão/óleo em ambos os sistemas de reação. Uma experiência ao longo do tempo, também otimizado pela função desejabilidade, mostrou que o sistema em US conseguiu 54% de rendimento de hidrólise do óleo em 30 minutos, e 28,5% em 60 minutos para agitação com banho.

Palavras-chave: lipase, ultrassom, sólido fermentado, óleo de algodão, hidrólise.

1. Introduction

Historically, vegetable and animal fats and oils are the most used renewable raw material by chemical industry (Biermann *et al.*, 2011) and fatty acids and glycerol are essential in oleochemical industry. The triglyceride hydrolysis, for release of free fatty acids and glycerol, represents an important chemical reaction to the natural oils and fats industrial processing (Rooney and Weatherley, 2001; Huang *et al.*, 2010). Fatty acids represents an important component of high-value products as coatings, adhesives, specially lubricating oils, shampoos and other personal care products (Murty, Bhat and Muniswaran, 2002). The use of lipase for fatty acids production is an energy-saving method, especially for producing high value-added products from heat sensitive fatty acids (Knezevic, Siler-Marinkovic and Mojovic, 1998; Rooney and Weatherley, 2001; Huang *et al.*, 2010). Usually, bioprocess products have better color and smell, with higher purity (no adverse reaction) than those commonly produced by chemical processes (Gandhi, 1997; Gonçalves *et al.*, 2012).

The combination of biocatalysis with physical technologies seems to be a green trend to develop and improve sustainable processes. Physical technologies improves the heat and mass transfers, besides the separation operations in bioprocesses and this combination may be a trend for green processes development due to its high efficiency and environmental harmlessness (Teixeira, Madeira Junior and Macedo, 2014). Ultrasound (US) is an emerging technology studied in different processes to enhance emulsification (Silva *et al.*, 2015), bioactive compounds extraction (M'hiri *et al.*, 2015) and catalysis (Cravotto *et al.*, 2015). Currently, enzymatic processes have used ultrasound broadly for esters production with desirable characteristics for the pharmaceutical, cosmetics, and food industry, production of biodiesel through the hydrolysis, glycerolysis and transesterification of vegetable oils. Moreover, ultrasound is a "green" technology due to its high efficiency, low instrumental necessity and significant processing time saving (Lerin *et al.*, 2014).

Numerous works found in the scientific database propose that the US releases energy through cavitation phenomena which improves mass transfer (substrate/enzyme), contributing to increase the enzyme catalytic activity (Lerin *et al.*, 2014). The cavitation bubbles breakdowns nearby the phase boundary of two immiscible liquids, the resulting shock wave provides an efficient stirring/mixing of the layers. Since the effects of cavitation can improve heterogeneous reactions and readily form transient reactive species, ultrasound is also a suitable tool in enzymatic reactions (Yachmenev, Blanchard and Lambert, 2004; Li *et al.*, 2005; Liu *et al.*, 2008). Furthermore, the use of ultrasound technology can increase the interfacial area resulting

in higher hydrolysis reaction rate. Lipases acts through the oil–water interface with aim on catalyze the reaction. Therefore, the total free interfacial area is an important requirement to enhance the reaction yield. (Huang *et al.*, 2010; Gonçalves *et al.*, 2012).

Most lipases applications reports the use of commercial immobilized enzymes, which represents a high cost for the process, although the possibility of enzyme recycle. An interesting approach recently reported for biodiesel production (Rasera *et al.*, 2012; Liu *et al.*, 2013; Soares *et al.*, 2013; Aguieiras *et al.*, 2014; Soares *et al.*, 2015), fat blends interesterification (Rasera *et al.*, 2012) and hydrolysis of oil in wastewater treatment (Damasceno, Cammarota and Freire, 2012; Damasceno, Freire and Cammarota, 2014; Duarte *et al.*, 2015) is the application of lipasic solid fermented (LSF) commonly denominated as dry fermented solids. The use of LSF as catalysts avoids the expensive steps of lipase extraction, purification and immobilization. Moreover, in solid-state fermentation (SSF) uses solid wastes from the vegetable oils processing (oil cakes) as raw material that are cheap and abundant in countries like Brazil. This new approach is an alternative method to decrease enzyme costs. In LSF, the solid particles acts as support that keeps the enzyme adsorbed on its structure (Aguieiras *et al.*, 2014).

This work aimed on application of a new LSF (from *Penicillium* sp. solid fermentation of cottonseed meal) on cottonseed oil hydrolysis assisted by US using a sonotrode and in shaking bath, comparing the effects of enzyme concentration and buffer/oil ratio for oil hydrolysis yield.

2. Experimental Methodology

2.1.Temperature e pH: Optimum and Stability

The evaluation of the optimum conditions of pH and of temperature for hydrolytic lipase activity and for enzymatic stability were carried out by a central composite rotatable design (CCRD) study using temperature and pH as independent variables and lipase activity and stability (4 hours) as responses. The Table 7 presents the experimental matrix with boundary conditions. The lipase activity was measured by titrimetric method. For stability, 1 mL of enzymatic filtrate after extraction was mixed to 1mL of phosphate buffer solution 0.1 M with different pH and temperature values during 4 hours and then measured the enzymatic activity in optimum conditions.

2.2.Amonium Sulphate Precipitation

The enzymatic extract filtrated was submitted to ammonium sulphate precipitation (80%). After homogenization, it was left for overnight under refrigeration and then centrifuged

twice at 15624g (4ºC), separating the precipitated and submitted to dialysis in cellulose membrane for 24 hour. After that, the dialyzed extract was submitted to liophilization.

2.3.Specificity (chain size)

Spectrophotometric method using p -NP (p- nitrophenol) accordingly to (Winkler and Stuckmann, 1979; Romero *et al.*, 2014) with some modifications. Reagents used were p- NPB (butirate), p- NPC (caprilate), p- NPL (laurate) , NPP (palmitate). The methodology consists on mixing 1mL of dissolved p -NP on isopropanol solution (3 mg/ml) with 9 ml with 0.1M pH7 phosphate buffer containing 2 % Triton X -100 and 0.5 % Arabic gum. We added 0.9 mL of solution mixture on test tubes and incubate at 37 ° C for 2 minutes. After that, 100 uL of enzymatic extract was added, homogenized and reacted for 1 minute. The reaction was paralyzed with 1 mL of 0.1 M NaOH. Make the reading in spectrophotometer at 410 nm of wavelength. The unit of activity was defined as U = μmol p -NP / min.

2.4.Organic solvent stability

The organic solvent stability was defined accordingly to Borkar *et al.* (2009). The enzymatic extract (1 mg/mL) dissolved in phosphate buffer pH7 was mixed with different solvents until 30% of acetone, methanol, ethanol, butanol and hexane. Each sample was left by 24 hours at 30ºC and then measured the residual activity using p-NPP as substrate on temperature and pH optimum conditions (item 2.1).

2.5.Lipasic solid fermented (LSF) Obtainment

After fermentation, the medium was removed from erlenmeyers and freezed at -18ºC without any fractionation or treatment. The lipase activity measured was 80U/g of cottonseed meal.

2.6. Oil Hydrolysis determination method

The cottonseed oil hydrolysis was estimated through free fatty acid determination by titrimetric method of basis-acid neutralization accordingly to Noor, Hasan and Ramachandran (2003) with some modifications. The reaction was stopped adding 10 mL of hexane/etanol mixture, added phenolphthalein and titrated with NaOH 0.3M. The total hydrolysis percentage (X) was determined accordingly to **Equation 5.**

$$X = \frac{V*M*Mm*100}{m*SI}$$ **Equation 5**

Where V is the volume of NaOH solution used on titration. M is the molarity of NaOH solution; Mm the molecular mass, m is the oil mass and S is the saponification number = 189-198 mg KOH/g of oil (Anvisa, 1999)

2.7.Shaking bath hydrolisys

The system was composed by LSF, cottonseed oil, phosphate buffer pH7 and glass beads on 125mL erlenmeyers under 150 rpm agitation in shaking bath at 40ºC for 20 minutes. We carried out a CCRD study with 2^2 runs, axial points and 3 replicates on central point. The input variables were buffer/oil ratio and enzymatic concentration. Oil hydrolisys was the response variable. The Table 9 presents the real and codified values of the variables.

2.8.Ultrasound assisted hydrolysis

The ultrasound assisted hydrolysis of cottonseed oil by LSF was carried out in ultrasonic cell disruptor (UNIQUE, Indaiatuba, SP, Brazil) belonging to supercritical technology laboratory (LASEFI) from Food Engineering School (FEA/UNICAMP). The reaction was conducted in 100mL falcon tube using a reaction system composed by phosphate buffer pH7 0.1M, cottonseed oil and *Penicillium* sp. LSF for 20 minutes. For reaction study and optimization, we applied a 2^3 CCRD with axial points and 3 replicates at central point. The input variables were power density, enzymatic concentration and buffer/oil ratio. The response variable was total oil hydrolysis. The Table 11 presents the real values for input (codified and real values) and response variables. The desirability function carried out the optimization of responses obtained on CCD studies in items 2.7 and 2.8.

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3. Results and Discussions

3.1.Temperature and pH: Optimum and stability

A CCRD study evaluated the optimum temperature and pH for activity and stability of lipase extract from *Penicillium* sp. SSF of cottonseed meal. The results are presented on the experimental matrix with independent and dependent variables (Table **7**). The variance analysis (ANOVA) in Table 8 validated the evaluation of polynomial models prediction of experimental results.

Table 7 Experimental matrix for optimum enzymatic activity and for stability

Runs	pH	Temperature (ºC)	**Lipase Activity (U/mL)**	**Stability (Relative activity %)**
1	4(-1)	40(-1)	**9.21**	**95.83**
2	4(-1)	60(+1)	**1.6**	**73.81**
3	8(+1)	40(-1)	**13.62**	**45.24**
4	8(+1)	60(+1)	**2.24**	**25**
5	3.2(-1.41)	50(0)	**3.21**	**94.64**
6	8.8(+1.41)	50(0)	**10.58**	**23.81**
7	6(0)	36(-1.41)	**13.78**	**100**
8	6(0)	64(+1.41)	**1.92**	**77.38**
9	6(0)	50(0)	**13.14**	**78.57**
10	6(0)	50(0)	**14.42**	**75.6**
11	6(0)	50(0)	**11.22**	**72.62**

In lipase optimum activity, the low p-value (0.00044) shows that the regression was statistically significant ($p<0.05$) compared to total residues of prediction, ensuring the suitability of the polynomial model with the most significant regression coefficients for fit the experimental data. In residues components, the lack of fit was not statistically significant compared to pure error, which represents that the data variability not explained by the model does not affect the prediction made by the polynomial model.

Table 8 Variance analysis (ANOVA) of CCD characterization of *Penicillium* sp. lipase extract.

Lipase Activity	**SS**	**df**	**MS**	**Fcal**	**Ftab**	**p**
Regression*	267.13	5	66.78	29.33	4.38	0.00044
Residues	13.66	6	2.28			
Lack of fit	8.47	4	2.12	0.82	19.24	0.61538
Pure error	5.19	2	2.59			
Total	280.79	10				
R^2	0.95					
Stability	**SS**	**df**	**MS**	**Fcal**	**Ftab**	**p**
Regression*	6658.467	4	2219.49	33.5588	4.12	0.00016
Residues	462.961	7	66.1373			
Lack of fit	445.260	5	89.052	10.0617	19.29	0.09286
Pure error	17.701	2	8.85063			
Total	7121.428	10				
R^2	0.94					

SS – Sum of Squares, DG – Degrees of freedom; MS - Mean Squares, Fcal- Fischer's calculated value; Ftab – Fischer's table value.

*Statistically significant at 95% of confidence.

For enzyme stability, it also resulted in low p-value (0.00016) showing that regression was statistically significant higher than the residues (95% of confidence), validating the suitability of the polynomial model with most significant coefficients for explain the experimental data. Evaluating the residues components, the lack of fit was not statistically higher than pure error, showing that the error from experimental data variability does not affect significantly the prediction by the polynomial model.

Accordingly to ANOVA results, we can use the polynomial models from prediction. The equations 6 and 7 represents optimum activity and stability functions, respectively, for *Penicillium* sp. lipase extract.

$$\textbf{Optimum activity}\ (\mathbf{U/mL}) = 1.94 * pH - 3.23 * (pH)^2 - 4.49 * temperature - 2.74 * (temperature)^2 + 12.93 \quad \textbf{(Equation 6)}$$

$$\textbf{Stability}\ (\%\textbf{relative activity}) = -25.07 * pH - 12.86 * (pH)^2 - 9.33 * temperature + 78.58 \quad \textbf{(Equation 7)}$$

We can observe in **Equation 6** the most significant regression coefficients ($p<0.005$ T-test) for optimum lipase activity. The linear and quadratic terms were significant, indicating a possible exponential relation of both variables. The interaction coefficient between temperature and pH was not significant, which means that the variables do not present a synergistic influence for lipase activity. Temperature (linear) had the highest coefficient, presenting a negative relation to lipase activity, followed by negative quadratic terms of pH, temperature (quadratic) and pH (linear).

In Equation 7, the stability, differently from optimum activity, presented pH (linear) as the highest regression coefficient, followed by pH (quadratic) and temperature (linear). The interaction term and temperature (quadratic) were not significant ($p<0.005$ T-test). The negative signal of pH shows that stability decreases with high pH, differently from lipase activity.

The Figure 9a shows the response surface as a representation of polynomial model for the pH and temperature coefficients on lipase activity. It is possible to observe that the lipase extract has higher activity in a wide range of pH (5-8) and temperature (40-50ºC). The lipase activity seems to decrease in low pH, differently from temperature, which seems to increase the activity in low temperatures.

The Figure 9b shows the response surface as a representation of polynomial model for the pH and temperature influence on lipase stability. Differently from optimum activity, the lipase had higher stability in low pH (4-6) but in a wide range of temperature (40-60 ºC) with a small decrease from 50 to 60ºC.

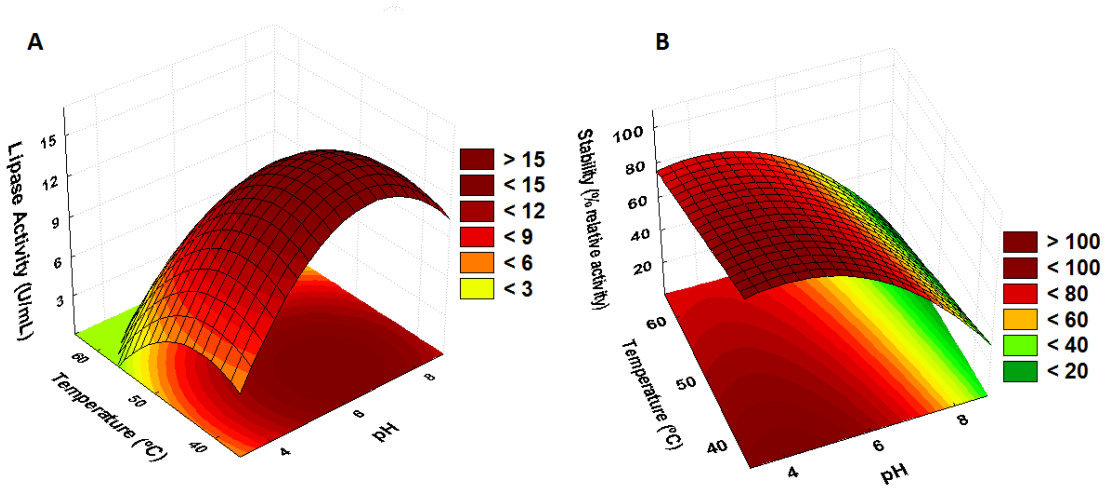

Figure 9 Response surfaces for pH and temperature of lipase characterization: a) Optimum activity; b) Stability

The desirability function showed that the *Penicillium* sp. lipase has higher activity at pH 7.4 and 43ºC (Figure 10a). The pH seems to present a range of optimum activity from 7 to 7.4 and temperature from 36 to 50ºC. For ensure the results, we executed an experimental validation in these conditions. The results obtained do not differ statistically (Tukey test with 95% of confidence) from pH 7-7.4 and temperature 40-43 ºC and therefore, we decided to use pH 7 and temperature 40ºC as operational conditions for measuring the enzyme stability.

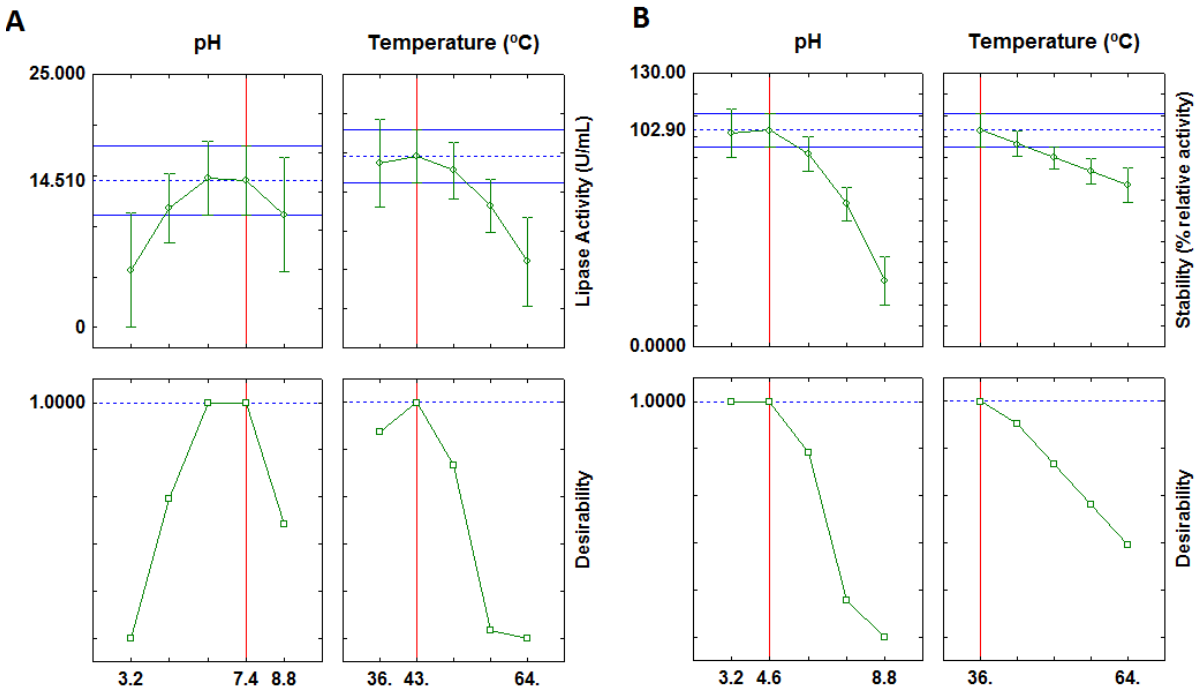

Figure 10 Profiles for predicted values and desirability function optimization of CCRD lipase characterization: a) Optimum activty; b) Stability.

The Figure 10b represents the desirability function profile for lipase stability response. The optimization showed that the best condition of pH and temperature for higher stability are 4.6 and 36ºC, respectively. The pH presents a range for optimum stability from 3.2 to 4.6. The lipase has higher stability in acidic conditions, differently from optimum activity and optimum temperature for stability agrees with the optimum temperature for activity.

The Figure 11 shows the pH and temperature characteristics for optimum activity of diverse lipases from *Penicillium* genus reported by Li, Li and Li (2010). It is possible to observe that majority of lipases has optimum activity in pH 6-8 and temperature 30-50 ºC, similar to our results, which seem to be a general characteristic from this genus.

Kumar *et al.* (2012) produced lipase from *P. chrysogenum* SNP5 on a mixture of grease waste and wheat bran with aim on get very specific lipase for oil waste remediation. The enzyme was stable in alkaline pH 7 to 9 with maximum activity at pH 8. The residual activity of lipase was 100% after 24h of incubation at 30 ºC. In thermal stability, it had 100% activity at 40 ºC. Chinaglia *et al.* (2014) evaluated the lipase from *P. solitum* 194A and found the termal stability for 10 minutes between 30 and 40 ºC with 100 % of residual activity and optimum pH 8.5-9.7.

Rigo *et al.* (2012) produced a lipase from a newly isolated *Penicillium crustosum* by solid-state fermentation and evaluated the enzyme extract, which presented optimal temperature of 37 ºC and pH of 9–10. The concentrated enzymatic extract showed more stability at 25ºC and pH 7.

Gutarra *et al.* (2009) evaluated a lipase from *P. simplicissimum* brazilian strain produced in babassu cake SSF supplemented with sugar cane molasses. The enzyme showed high activities in a wide range of temperatures (35–60 ºC) at an acidic pH range (4.0–6.0), with an optimum activity at 50 ºC and pH 4.0–5.0.

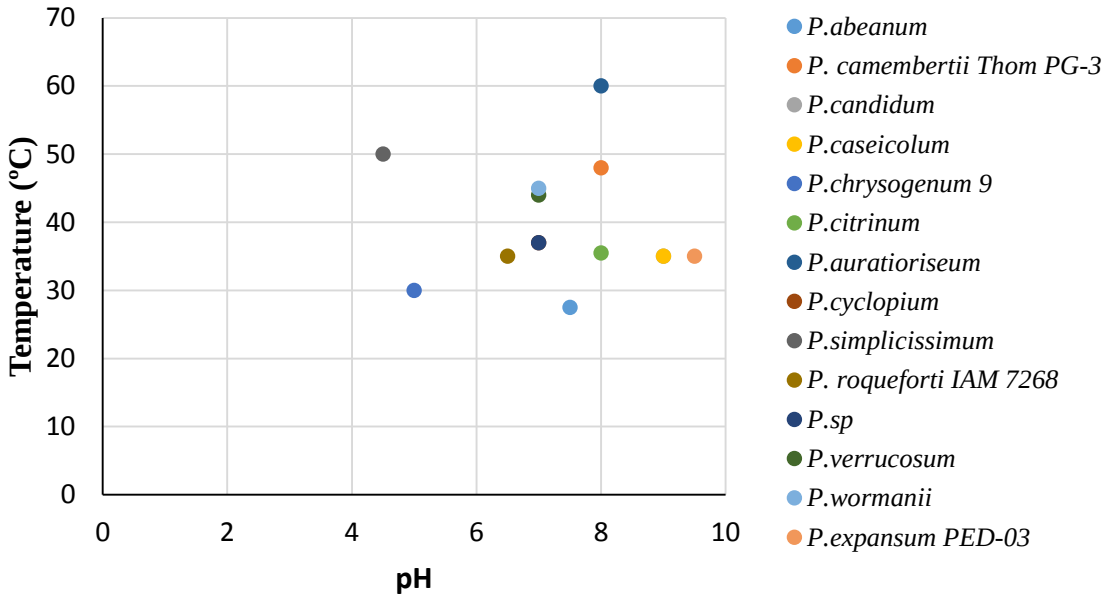

Figure 11 Optimum activities of different lipases from *Penicillium* species, published by different authors and reported by Li and Zong (2010).

3.2.Specificity (Chain size)

We can observe in **Figure 12** that the *Penicillium* sp. lipase has a higher affinity for palmitic acid as p-NPP had the major concentration of p-NP released, followed by laurate, caprylate and butyrate, in descending order of size chain. It means that the lipase had major affinity for higher size chain fatty acids, which is common to real lipases. Cottonseed oil present in cottonseed meal used for SSF production of the *Penicillium* sp.lipase has a higher concentration of palmitic acid (17-31 %) and none of laurate, caprylate and butyrate acids, which could explain the higher difference in acitivites from palmitic acid observed in Figure 13.

The lipase from *Penicillium crustosum produced by* Rigo *et al.* (2012) have specificity with diverse substrates in triglycerides from C4 to C18. The enzyme presented higher specificity to p-nitrophenyl laurate (C12:0).

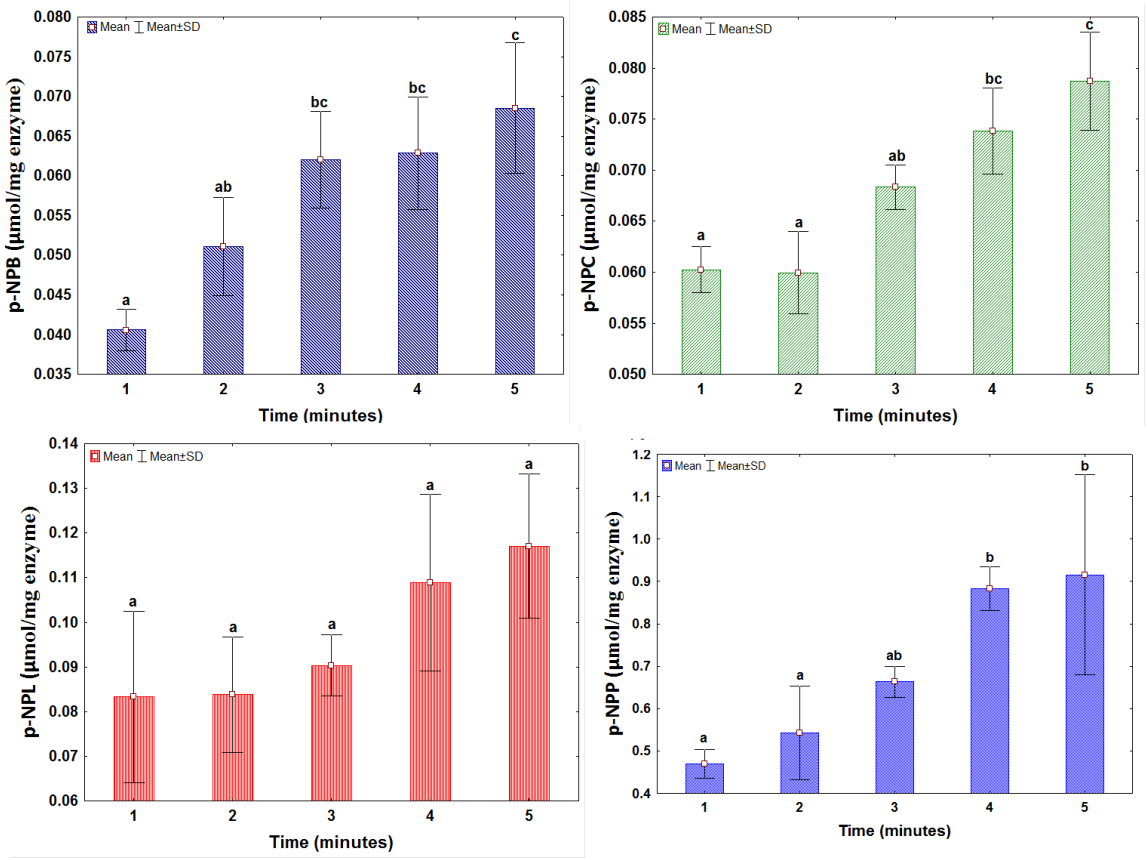

Figure 12 Chain size regioselectivity analysis with p-NPB (p-nitrophenol butyrate), p-NPC (p-nitrophenol caprylate), p-NPL (p-nitrophenol laurate) and p-NPP (p-nitrophenol palmitate) activities (means with same letter in the same graph are not different accordingly to Tukey test $p<0.05$)

Li and Zong (2010) analyzed the regiospecificity of different lipases from *Penicilia* genus in a review and observed that *P. candidum lipase showed* major affinity with long-chain palmitate (C16) when *p*-nitrophenyl fatty acid esters were used as the substrates (Ruiz *et al.*, 2001). On the contrary, lipase from *P. roqueforti* IAM 7268 showed high specificity toward short-chain fatty acid esters such as butyrate (C4) and caproate (C6) with *p*-nitrophenyl esters as the substrates (Mase, Matsumiya and Matsuura, 1995). Sugihara *et al.* (1996) reported that *P. abeanum* lipase showed lower activities toward ester bonds of polyunsaturated fatty acid esters as compared to those of other esters. Moreover, it displayed preferentially activities toward medium-chain fatty acid esters, which was also found in the case of lipases from *P. camembertii Thom* PG-3 (Lima *et al.*, 2004), *P. aurantiogriseum* (Lima *et al.*, 2004), *P. cyclopium* (Chahinian *et al.*, 2000) and *P. simplicissimum* (Gutarra *et al.*, 2009).

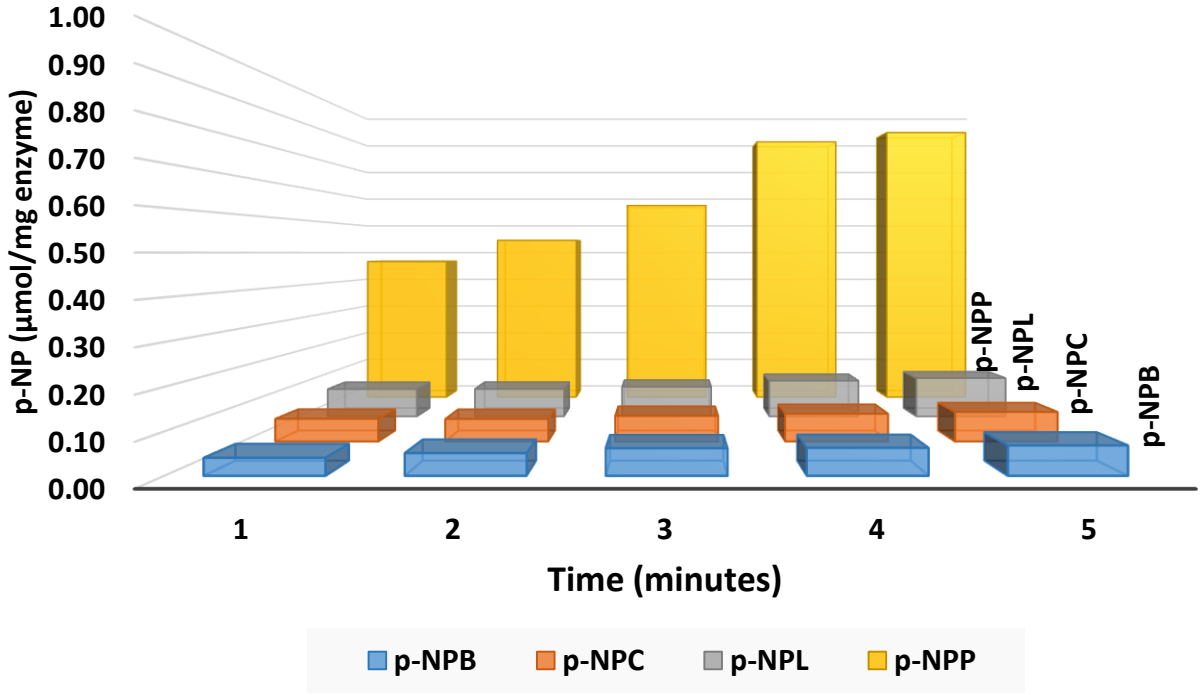

Figure 13 Resume of chain size regioselectivity analysis with p-NPB (p-nitrophenol butyrate), p-NPC (p-nitrophenol caprylate), p-NPL (p-nitrophenol laurate) and p-NPP (p-nitrophenol palmitate) activities.

3.3.Solvent stability

Evaluating the solvent stability of lipases indicates its capacity for act in organic synthesis in presence of organic solvents as n-heptane, hexane, and isopropanol. Besides that, the polar solvents stability also permits its application for alcoholysis reaction, like in biodiesel reaction for example. Therefore, we evaluated the *Penicillium* sp. lipase stability in acetone, hexane, ethanol, methanol comparing to phosphate buffer pH 7 for 24 hours of incubation at room temperature (25ºC) and then measured it activity with p-NPP reagent.

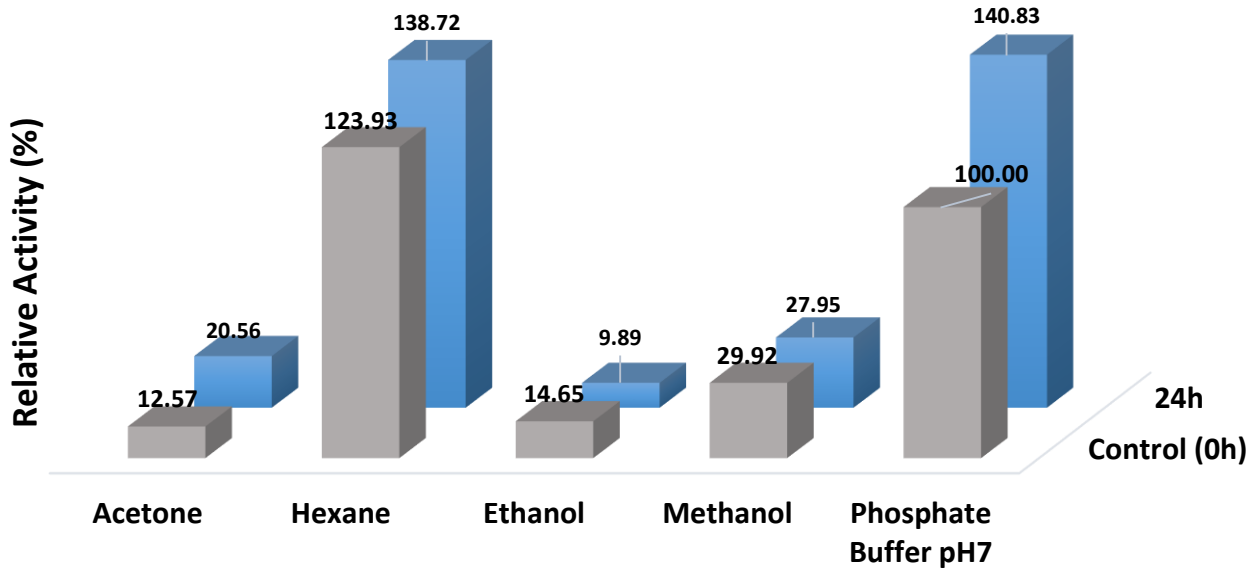

Figure 14 Solvent stability of *Penicillium* sp. lipase extract.

The Figure 14 shows the results for lipase stability in different solvents (polar and organic) with control samples (addition of enzyme in the moment of analysis) and samples of 24 incubation hours. We can observe a hyper activation in presence of hexane, with more than 20% higher than phosphate buffer and seems to keep the activity after 24h. Phosphate buffer had hyper activation after 24h. Acetone highly inhibits the lipase activity, almost 90%, like ethanol and methanol, which means that lipase has low stability and activity in polar solvents due to medium dehydration by ethanol, isoelectric protein molecules will attract each other to a sufficient degree by van der Waals forces to become insoluble in the ethanol-water mixtures used (Van Oss, 1989).

Romero *et al.* (2014) produced lipase by *Penicillium corylophilum* under basal and olive oil-induced conditions. The enzyme maintained a residual lipase activity after incubation in a wide pH range showing high stability in a defined acidic (3) to neutral pH (7) range and temperature (30-40 ºC). In presence of organic solvents such as methanol, ethanol, acetone,

butanol, hexanol, n -hexane, and heptane, the enzyme showed higher stability in aqueous acetone, methanol and ethanol than in n-hexane.

Penicillium aurantiogriseum lipase have lower activity of *p*-nitrophenyl palmitate (pNPP) in organic medium than in aqueous medium. The enzyme showed a good stability in organic solvents with high log *P* values, the best result being in *n*-heptane (114% residual activity) (Lima *et al.*, 2004).

3.4.Shaking bath hydrolysis

The CCRD experiment evaluated the hydrolysis of cottonseed oil catalyzed by lipasic solid fermented (LSF) in shaking bath with orbital shake using two independent variables (LSF concentration and buffer/oil ratio). The results of variance analysis are summarized in Table 10.

Table 9 Enzymatic hydrolysis of cottonseed oil in shaking bath.

Runs	%LSF (m/Vtotal)	Buffer/oil ratio (V/V)	**Hydrolysis (%)**
1	10(-1)	0.7(-1)	**6.05**
2	20(+1)	0.7(-1)	**7.56**
3	10(-1)	1.3(+1)	**8.53**
4	20(+1)	1.3(+1)	**10.91**
5	8(-1.41)	1(0)	**5.07**
6	22(1.41)	1(0)	**8.52**
7	15(0)	0.49(-1.41)	**5.70**
8	15(0)	1.5(1.41)	**10.65**
9	15(0)	1(0)	**8.70**
10	15(0)	1(0)	**8.63**
11	15(0)	1(0)	**8.34**

Table 10 Variance analysis of CCD for LSF hydrolysis of cottonseed oil in orbital shaker.

ANOVA	SS	df	MS	Fcal	Ftab	p
Regression*	32.03532	3	10.6784	26.44	4.34	0.00034
Residues	2.82704	7	0.40386			
Lack of fit	2.75349	5	0.5507	14.97	19.29	0.06378
Pure error	0.07355	2	0.03678			
Total SS	34.86236	10				
R^2	0.97					

SS – Sum of Squares, DG – Degrees of freedom; MS - Mean Squares, F - Fischer's calculated value; Ftab - . Fischer's table value

*Statistically significant at 95% of confidence.

The low p-value (0.00034) shows that the regression was statistically significant ($p<0.05$) compared to total residues of prediction, ensuring the suitability of the polynomial model with the most significant regression coefficients for fit the experimental data. In residues components, the lack of fit was not statistically significant compared to pure error, which represents that the data variability not explained by the model does not affect the prediction made by the polynomial model.

Considering the results of ANOVA, we can use the polynomial model (Equation 8) for prediction and simulation of LSF hydrolysis of cottonseed oil in dubnoff bath with orbital shaker.

$$\%\boldsymbol{Hidrolysis} = 1 * LSF - 0.55 * (LSF)^2 + 1.46 * buffer/oil\ ratio + 8.54$$

(Equation 8)

Only the quadratic term of buffer/oil ratio and the interaction term were not significant ($p<0.1$) for response. The buffer/oil ratio (linear) was the highest cofficient, followed by LSF concentration (linear) and LSF (quadratic), respectively.

The hydrolysis increases in high ratios of buffer due to increase in relation enzyme/substrate and diffusion rates, considering that buffer has a lower viscosity than cottonseed oil. The Figure 15a shows the response surface as a representation of the polynomial model for the %LSF and buffer/oil ratio terms on cottonseed hydrolysis. It is possible to observe that higher LSF concentration and buffer/oil ratio had the highest oil hydrolysis (11%) in 20 minutes of reaction.

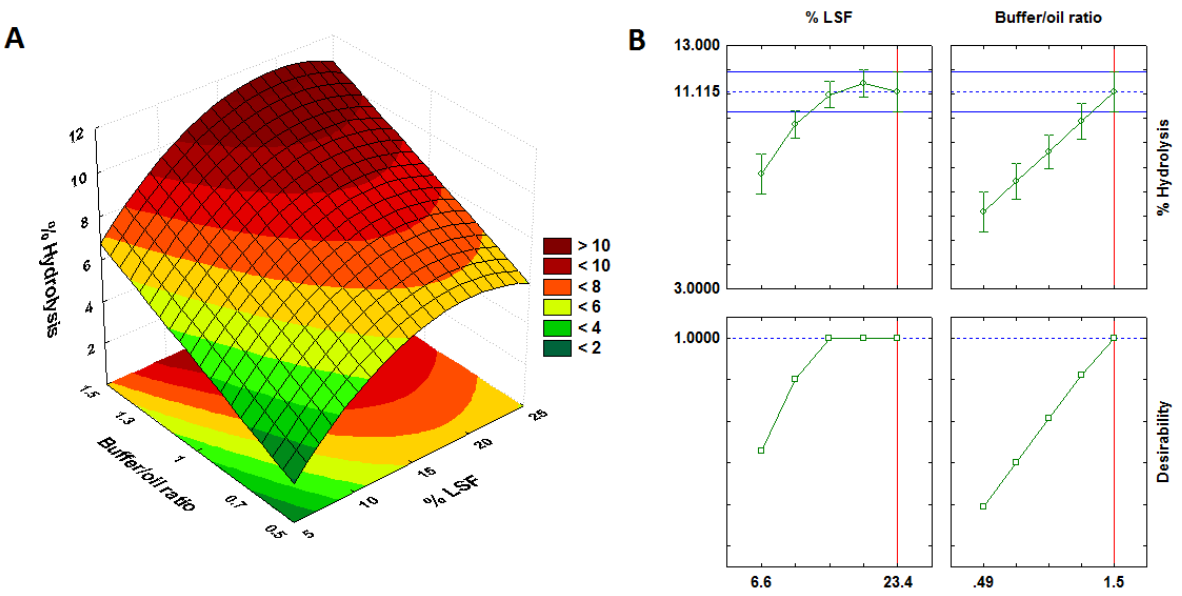

Figure 15 Cottonseed oil hydrolysis using LSF in agitated bath: a) Response surface; b) Prediction and desirability profiles.

The response surface indicates that the range with highest hydrolysis situates between 1.3-1.5 buffer/oil ratio and 15-25% of LSF concentration achieving more than 10% of hydrolysis in 20 minutes of reaction. The Figure 15b represents the prediction and profile of optimization by desirability function. The best conditions for achieve the highest hydrolysis yield is 23.4% of LSF and 1.5 of buffer/oil ratio reaching 11.11% of hydrolysis with a desirability value of 1. It seems that hydrolysis increases with high enzyme concentration and high buffer/oil ratio. The increase in the relation enzyme/substrate when increasing buffer/oil ratio would be responsible for oil hydrolysis enhancement, considering that the substrate concentration decreases in the reaction system. However, this fact would be prove by the interaction effect between LSF concentration and buffer/oil ratio, which did not occurr. A possible explanation may be the modification on diffusion rates when increases concentration of buffer, decreasing the viscosity of the reaction system.

Cammarota, Teixeira and Freire (2001) used a LSF from babassu cake containing *Penicillium restrictum* lipases in an upflow anaerobic sludge blanket reactor for pre-treat dairy wastewaters containing elevated fat and grease levels (868 mg l−1). The pre-treatment consisted of mixing the wastewater with the fermented cake for 12 h at 35◦C and stirring at 120 rpm. Using 0.1% (w/v) of LSF, turbidity and volatile suspended solids reduced by 75% and 90%, respectively, and 90% of chemical oxygen demand (COD) decreased.

Jung, Cammarota and Freire (2002) also used the same LSF from *Penicillium restrictum* to treat dairy wastewater containing different oil and grease contents in batch activated sludge systems using an enzymatic pre-hydrolysis stage with 0.2% (w/v) of LSF. Alberton *et al.* (2010) used the filamentous fungus *Rhizopus microsporus* CPQBA 312-07 DRM on a mixture of sugarcane bagasse and sunflower seed meal SSF to produce a LSF for treating a high-fat dairy wastewater an oil and grease level above 1300 mg/L. The LSF reduced the oil and grease levels to lower than 300 mg/L after 72 h at 35 °C. Zawadzki *et al.* (2013) used the same LSF in a packed-bed bioreactor to pretreat a meat and sausage processing factory high-fat wastewater. With residence time of 24 h, the process reduced 96 % of wastewater's oil and grease concentration and amplified its 5-day biochemical oxygen demand to chemical oxygen demand (BOD5/COD) ratio. The pretreated wastewater had a sufficiently high biodegradability for application in a traditional anaerobic digestion or activated sludge process. Duarte *et al.* (2015) evaluated the anaerobic biological treatment of a fish industry effluent at 30 ºC and 50 ºC, with and without preliminary hydrolysis at 30 ºC and 50 ºC, using a LSF rich in thermophilic hydrolase obtained by *Penicillium simplicissimum* SSF of babassu cake with optimal activity at 50 ºC.

3.5.Ultrasound assisted hydrolysis

The CCRD experiment evaluated the cottonseed hydrolysis catalyzed by LSF and assisted by US. The results can be observed in the experimental matrix with independent and dependent variables (Table 11). The least squares regression obtained a polynomial regression for prediction and the most significant coefficient (95% of confidence in T-student test) were used to compose the prediction model and then, evaluated by ANOVA (Table 12) for its suitability to fit the experimental data.

Table 11 Experimental matrix with real values for ultrasound assisted hydrolisys CCD.

Runs	Power Density (W/mL)	%LSF (m/Vtotal)	Buffer/oil ratio	**Hydrolysis (%)**
1	5(-1)	10(-1)	0.7(-1)	**6.16**
2	5(-1)	20(+1)	1.3(+1)	**11.36**
3	9(+1)	10(-1)	1.3(+1)	**7.29**
4	9(+1)	20(+1)	0.7(-1)	**5.85**
5	5(-1)	10(-1)	1.3(+1)	**7.71**
6	5(-1)	20(+1)	0.7(-1)	**9.24**
7	9(+1)	10(-1)	0.7(-1)	**4.24**
8	9(+1)	20(+1)	1.3(+1)	**8.54**
9	4(-1.68)	15(0)	1.0(0)	**7.25**
10	10.4(1.68)	15(0)	1.0(0)	**5.98**
11	7(0)	6.6(-1.68)	1.0(0)	**5.62**
12	7(0)	23.4(1.68)	1.0(0)	**9.60**
13	7(0)	15(0)	0.5(-1.68)	**5.42**
14	7(0)	15(0)	1.5(1.68)	**9.64**
15	7(0)	15(0)	1.0(0)	**6.34**
16	7(0)	15(0)	1.0(0)	**6.89**
17	7(0)	15(0)	1.0(0)	**7.07**

Table 12 CCD ANOVA of cottonseed oil hydrolysis by LSF assisted by US.

ANOVA	**SS**	**df**	**MS**	**Fcal**	**Ftab**	**p**
Regression*	52.209	6	8.701	34.031	3.21	0.0000043
Residues	2.556	10	0.255			
Lack of fit	2.272	8	0.284	1.996	19.37	0.376
Pure error	0.284	2	0.142			
Total SS	54.766	16				
R^2	0.95					

SS – Sum of Squares, DG – Degrees of freedom; MS - Mean Squares, Fcal- Fischer's calculated value; Ftab – Fischer's table value.

*Statistically significant at 95% of confidence.

The low p-value (0.0000043) obtained shows that the regression was statistically significant ($p<0.05$) when compared to residues, indicating that the polynomial model fits

satisfactorily the experimental data. The residues components evaluation shows that p-value (0.376) was not statically significant ($p<0.05$) indicating that lack of fit is sufficiently higher than pure error, which means that error from polynomial prediction is not affected by the error from experimental data repeatability. Therefore, we can considerate the polynomial model with the significant effects ($p<0.1$) suitable for prediction and simulation of the experimental data from cottonseed oil hydrolysis by LSF assisted by US **(**Equation 9**).**

$$\%Hydrolysis = -0.8 * Power\ intensity + 1.19 * LSF + 0.34 * (LSF)^2 + 1.21 * \frac{Buffer}{oil} ratio + 0.32 * \left(\frac{Buffer}{oil} ratio\right)^2 - 0.48 * Power\ intensity * LSF + 6.78$$

(**Equation 9)**

Only quadratic term of power intensity, power intensity*buffer/oil ratio and buffer/oil ratio*LSF terms were not statistically significant ($p<0.1$) for the oil hydrolysis. The buffer/oil ratio (linear) was the highest coefficient, followed by LSF (linear), power intensity (linear), power intensity*LSF, LSF (quadratic), buffer/oil ratio (quadratic). LSF (linear) and buffer/oil ratio (linear) have positive influence for oil hydrolysis, almost in the same value, 1.19 and 1.21, respectively, therefore, with same effect.

The negative interaction term of power intensity*LSF suggests that US power intensity inhibits the lipase, avoiding the hydrolysis in high power intensity. High temperatures were achieved in high US power intensity, which contributes for enzyme denaturation.

The Figure 16a, b and c shows the response surface as a representation of the polynomial model for the %LSF, buffer/oil ratio and US power intensity effects on enzymatic cottonseed oil hydrolysis. It is possible to observe in Figure 16a**,** that low US power intensity and high LSF concentration reached the highest oil hydrolysis yield (>12%). The US power intensity showed to influence negatively the lipase. Low US power intensity seems to increase the enzyme effect. However, US power intensity below 4 W/mL seems to avoid reaction system agitation.

The Figure 16b presents the representation of US power intensity and buffer/oil ratio terms on oil hydrolysis response. The reaction obtained the highest oil hydrolysis yield when operated in low US power intensity and high buffer/oil ratio. US power intensity does not seem to influence the buffer/oil ratio, resulting in a small decrease in oil hydrolysis (11-8 %) when the system worked with the highest buffer/oil ratio.

High buffer/oil ratio (low oil fraction) indicates the increase of the polar solvent (phosphate buffer) and decrease of substrate concentration (cottonseed oil), which increases substrate availability and drops the viscosity in the reaction system, permitting a higher propagation of US waves and a higher cavitation generation. However, this fact did not facilitate the enzymatic activity of the system as we see that the interaction effect between US power intensity and buffer/oil ratio was not significant for oil hydrolysis.

With respect to viscosity, it is necessary that energy overcome the cohesive forces present in the liquid to occur the cavitation. These forces increase the resistance to mass transfer and therefore require a larger energy for the phenomenon to happen. Therefore, high viscosity liquids have a reduction in the cavitation intensity. Liquids having high surface tension, such as water, have higher cavitational intensity (Gogate, Sutkar and Pandit, 2011).

Huang *et al.* (2010) observed that oil droplet size decreases in oil/water emulsion when the US power increases and low oil fraction is used. Leong *et al.* (2009) demonstrated that US could be a useful technology for producing nanoemulsions of triglyceride oils in water. The droplet size is a function of the emulsion composition and for reaching the minimum droplet size is necessary a certain shear intensity, or power density. They observed that for shorter residence times, the droplet size of a specific surfactant mixture is controlled by the specific energy input. This specific energy is a function of the power delivered to the emulsion and the residence time. They also verified that intense shear results do not hydrolyze the triglyceride oil.

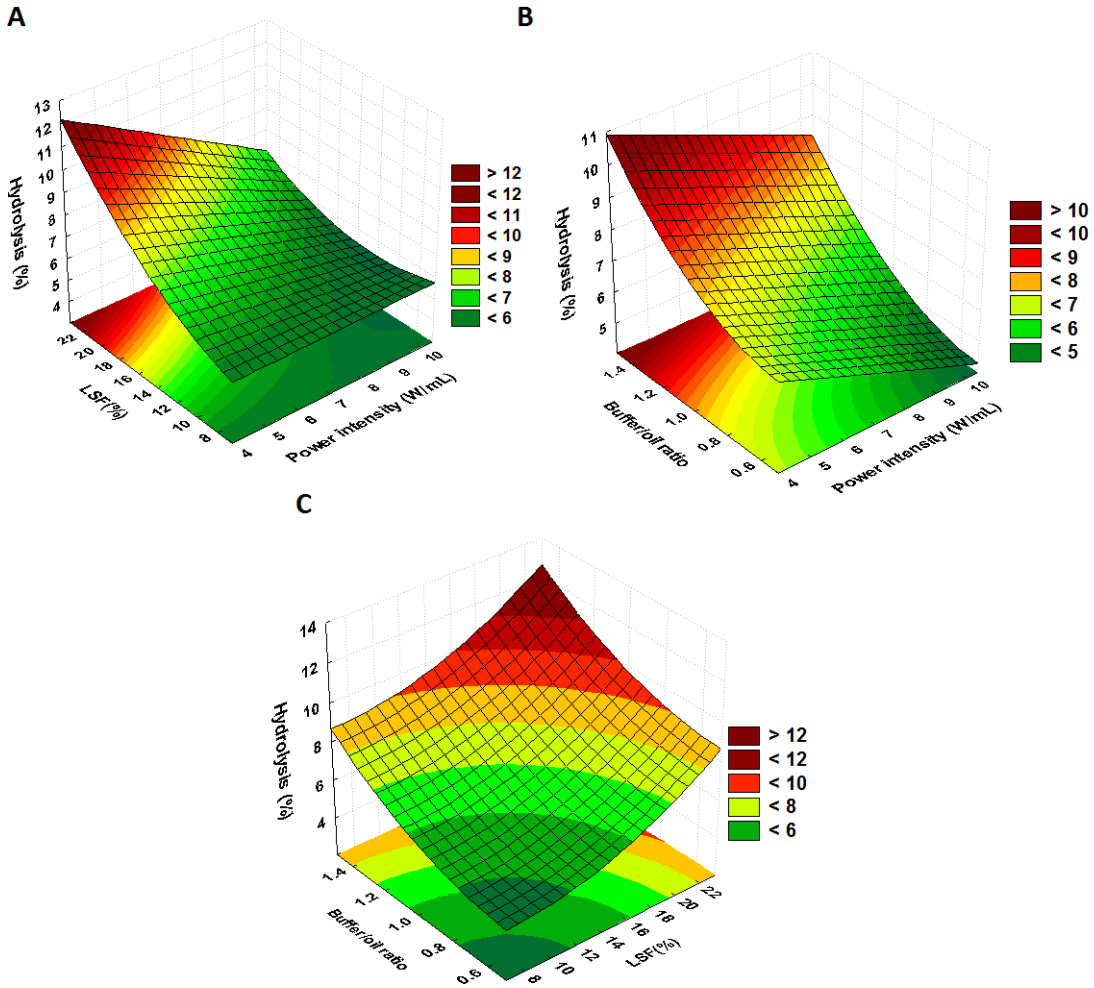

Figure 16 Response surfaces from CCRD study of cottonseed oil hydrolysis by LSF assisted by US: a) LSF and US power intensity effects; b) Buffer/oil ratio and US power intensity effects and c) Buffer/oil ratio and LSF effects.

The Figure 16c shows that both buffer/oil ratio and LSF concentration have positive effects for oil hydrolysis response obtaining the best results with highest values of each other. Besides that, both effects had the same magnitude effect, contributing almost equally for increase the oil hydrolysis yield. However, the interaction effect between them was not significant, which means that the relation enzyme/substrate does not have a significant effect for oil hydrolysis reaction.

In the optimization by desirability function (**Figure 17**) we can observed that best condition to maximize the cottonseed oil hydrolysis are: 7.2 W/mL of US power intensity, 23.4% LSF and 1.5 of buffer/oil ratio, achieving 12.5% of oil hydrolysis in 20 minutes of reaction. We can also observe in prediction profile that it is possible to enhance the hydrolysis increasing LSF concentration and buffer/oil ratio.

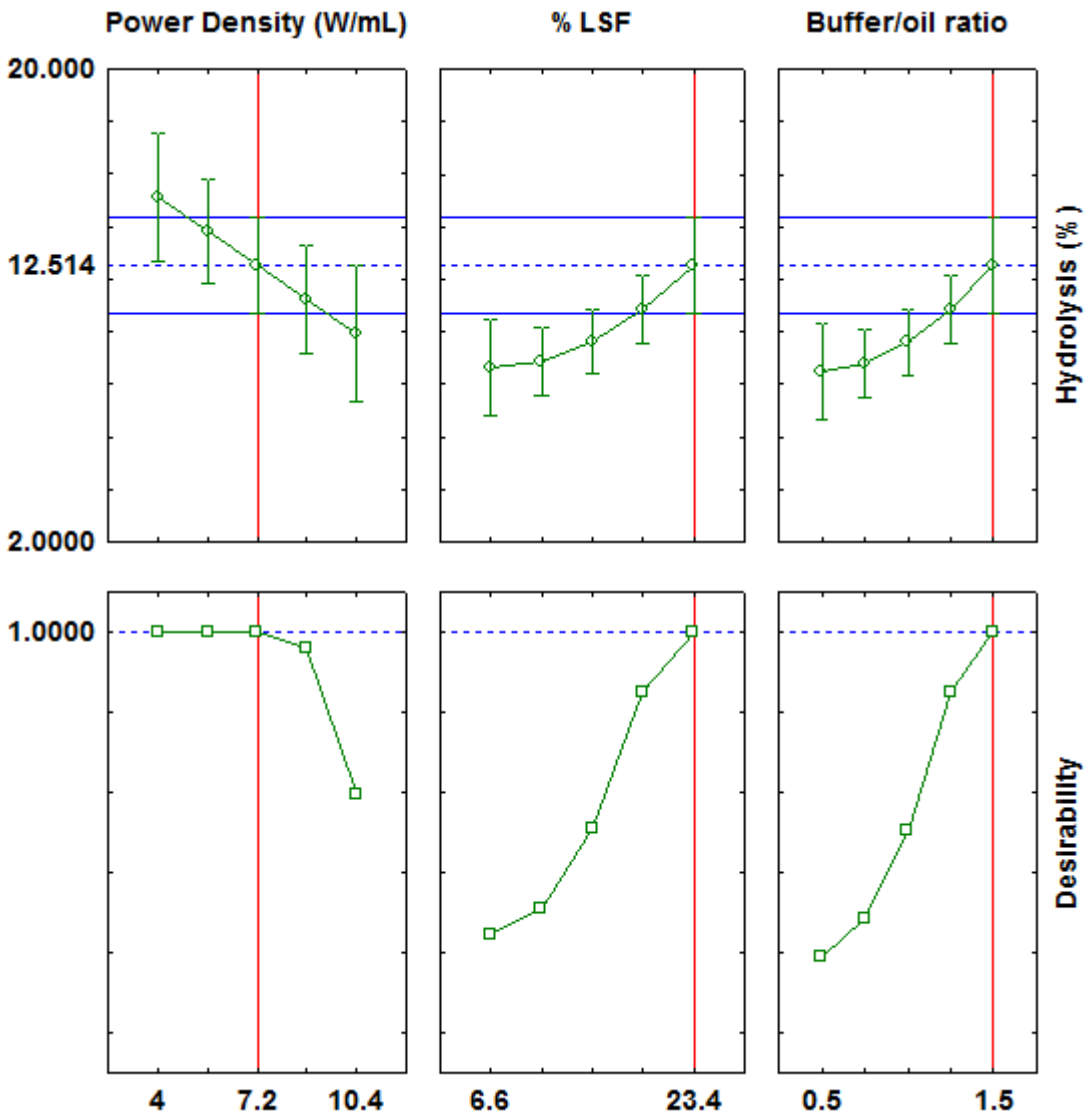

Figure 17 Prediction and desirability profiles for cottonseed oil hydrolysis by LSF assisted by US.

The desirability optimization can be useful for an industrial approach as we can observe the power density profile that shows it is possible to decrease power intensity without affecting the oil hydrolysis, which contributes for energy saving, therefore reducing the energy cost in the process.

Cucheval and Chow (2008) studied the emulsification mechanism and observed the oil–water interface (no pre-mix) with a high-speed camera. Transient cavitation is responsible for acoustic emulsification; however, there have been no measurements to relate the transient cavitation zone to the production of an emulsion. It has already been shown that the transient cavitation in probe systems is directly under the probe tip. High-speed observations showed that an emulsion could only be obtained if the interface was within a few millimetres of the probe

tip. These results strongly suggest that the transient cavitation zone is responsible for the acoustic emulsification of oil.

3.6.Comparative analysis on hydrolysis reaction

Comparing the effects of buffer/oil ratio and LSF concentration for both orbital shaker and US reactions, we observed that both variables have positive effects for the two reaction systems. However, the quadratic effect of buffer/oil ratio was statistically significant in US assisted reaction, differently from orbital shaker reaction. Besides that, the quadratic effect of LSF has a positive effect for US assisted reaction and a negative one for orbital shaker reaction, which means that oil hydrolysis tends to increase in higher LSF concentration in US assisted reaction and to decrease in higher concentration of LSF in orbital shaker.

Liu *et al.* (2008) performed a comparative study of soy oil lipase-catalyzed hydrolysis in solvent-free system carried out in shaking bath and in ultrasonic bath. They evaluated the influence of temperature, pH, enzyme concentration and water/oil ratio. Compared with that in shaking bath, optimum temperature and inactivation temperature of lipase in ultrasonic bath were about 5–10 °C higher, while pH effect in ultrasonic bath was similar; ultrasound also led to a smooth increase of reaction rate at relatively higher enzyme loading and less use of water to saturate hydrolysis substrate. In optimum conditions, the overall hydrolysis reaction rate in the ultrasonic bath process was above 2-fold than that in the shaking bath process.

Talukder *et al.* (2006) evaluated the hydrolysis of olive oil catalyzed by *Chromobacterium viscosum* lipase in a water/isooctane two-phase system under ultrasound and conventional stirring. The maximum activity of lipase in the ultrasonicated system was 1.75 times higher than that in the stirred system. The enzyme activity depends on ultrasonic power and volume ratio of isooctane to water. The stability of lipase at 25°C in the ultrasonicated system decreased faster in US than in the stirred system.

We evaluated the effect of buffer/oil ratio enhancement in both orbital shaker and US systems, keeping constant the LSF concentration on 20%, power intensity of 5 W/mL for US reaction and 120 RPM for orbital shaker and 20 minutes of reaction. The Figure 18 represents the results obtained experimentally and the predicted by the polynomial models.

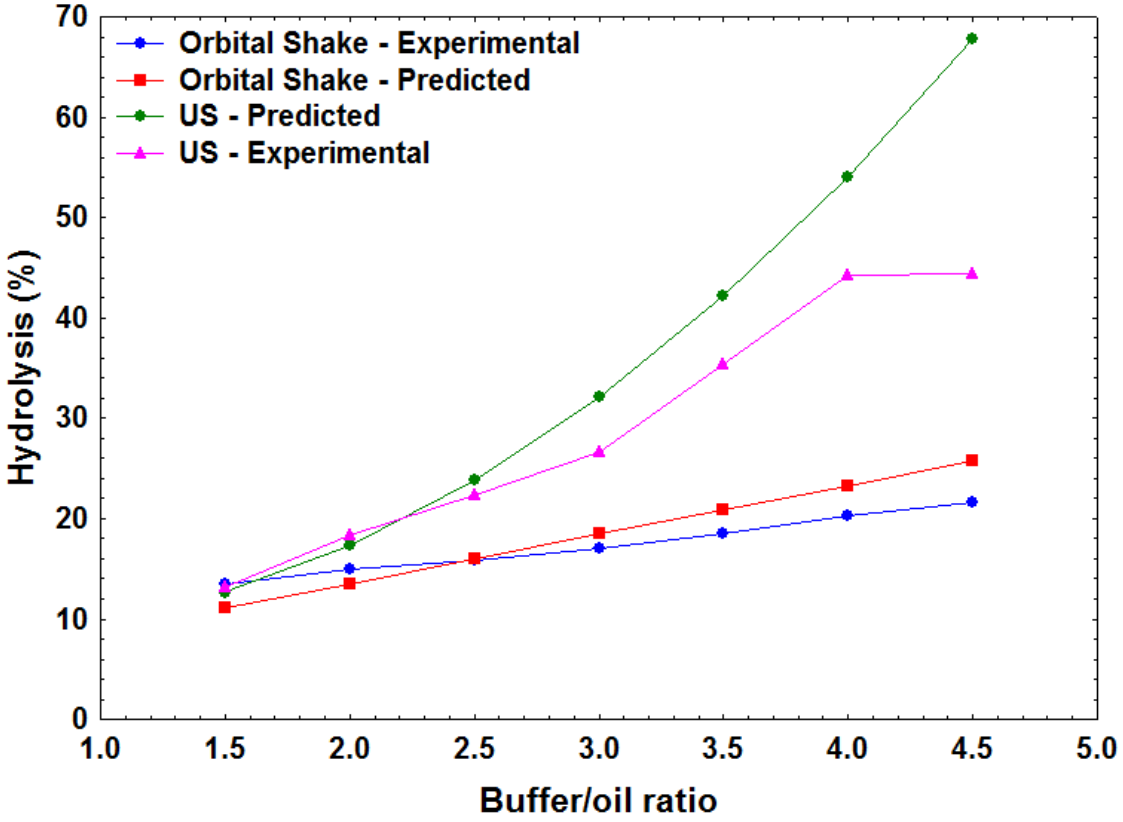

Figure 18 Effect of buffer/oil ratio enhancement on oil hydrolysis.

The buffer/oil ratio presents a higher intensity effect in US assisted reaction than in orbital shaker, as it is also possible to observe in polynomial models and in the Figure 18. The buffer/oil ration in orbital shaker presents only a linear effect, differently, in US presents also a quadratic effect, which could be attributed to the higher energy dissipation in the reaction system.

Ramachandran *et al.* (2006) evaluated the lipase oil hydrolysis assisted by US with a different approach. The lipase was added to reaction system after US emulsification. They observed that hydrolysis rate significantly enhanced with ultrasonic emulsification, with no interfacial area saturation with enzyme due to large interfacial area generated. A much narrower distribution of oil droplet diameter could be produced as compared to mechanical agitation. The hydrolysis occurs at the interface between the oil and the aqueous solution containing the enzyme. This interfacial area between the oil phase and the aqueous phase influences the rate of hydrolysis.

Huang *et al.* (2010) performed a comparative study of soy oil lipase-catalyzed hydrolysis in solvent-free using shaking bath and ultrasonic bath. They observed that the oil

phase and the aqueous phase interfacial area affects the hydrolysis rate. Ultrasonic shaking was more effective to disperse the oil in water, compared to shaking bath. Obtaining larger interfacial area and smaller drop size in ultrasonic bath. The initial rate of hydrolysis directly correlated with the interfacial area.

It can also be observed that the polynomial model for US reaction does not explain the oil hydrolysis results above 4 buffer/oil ratio, which presented a decrease in hydrolysis capacity, differently from orbital shaker results which agrees with the predicted data. The higher results obtained were 44% for US reaction in buffer/oil ratio of 4 and 25% for orbital shaker in buffer/oil ratio of 4.5.

Considering the results achieved in buffer/oil ratio enhancement, we evaluated the optimum reaction time for both US and orbital shaker reaction systems. The Figure 19 shows the result obtained until one hour of reaction. The US assisted reaction presents a higher variability in results than orbital shaker, which was uniform, with a minor standard deviation.

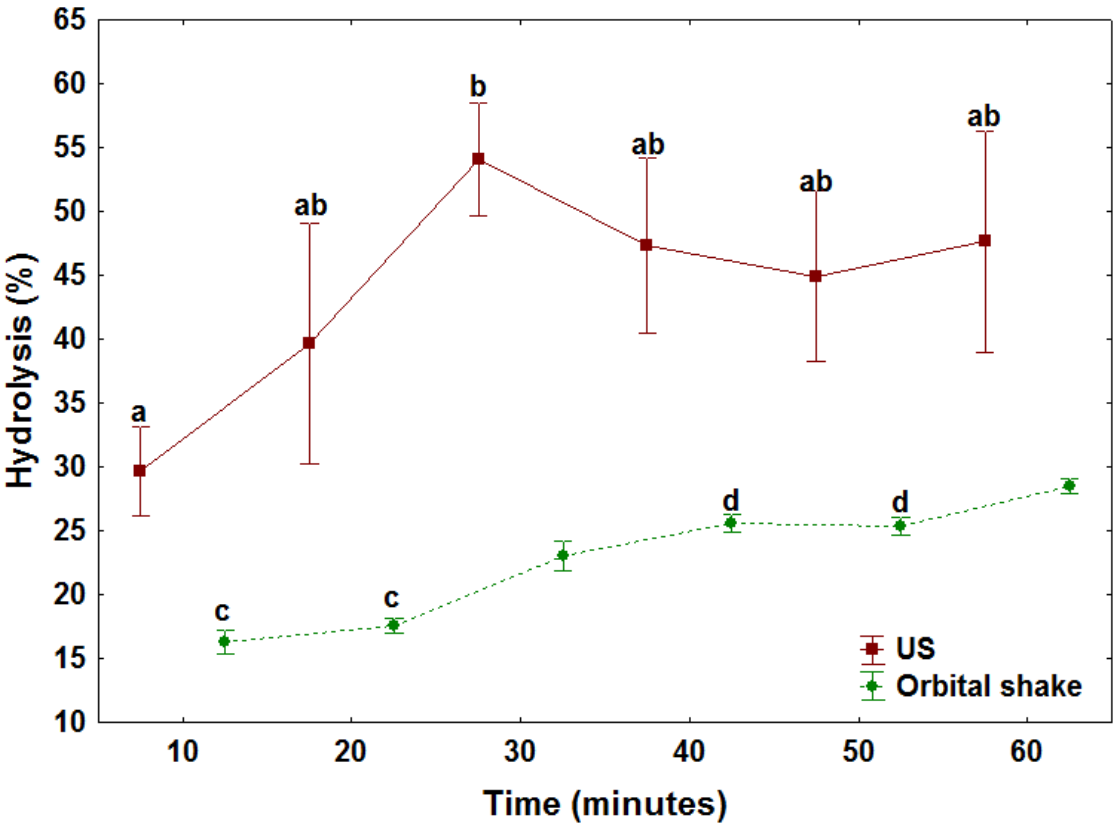

Figure 19 Reaction time evaluation in optimum conditions (means with same letter do not differ each other at Tukey test with 95% of confidence).

The reaction in US system seems to decrease after 30 minutes of reaction, differently from orbital shaker system, which stills increasing until 60 minutes of reaction in a first order reaction kinetics. In US system, only 30 and 10 minutes means were statistically different, according to Tukey test. In orbital shake system, only 10 and 20 minutes; and 40 and 50 minutes were no different. This difference is attributed to the high variability in US system in values of buffer/oil ratio above 4.

In Figure 20**,** the optimization by desirability function showed that the best time for oil hydrolysis is 30 minutes for US system and 60 minutes for orbital shake system, achieving a desirability value of 0.76 for US and 0.81 for orbital shake. The desirability value indicates that the orbital shake reaction obtained a better time reaction optimization than US reaction, which indicates that US system could have reached a higher value but did not, probably by a reaction limitation. The reaction in orbital shake system seems to continue increasing after 60 minutes, differently from US system which seems to decrease. It is also possible to observe the high variability in data from US system compared to orbital shake system.

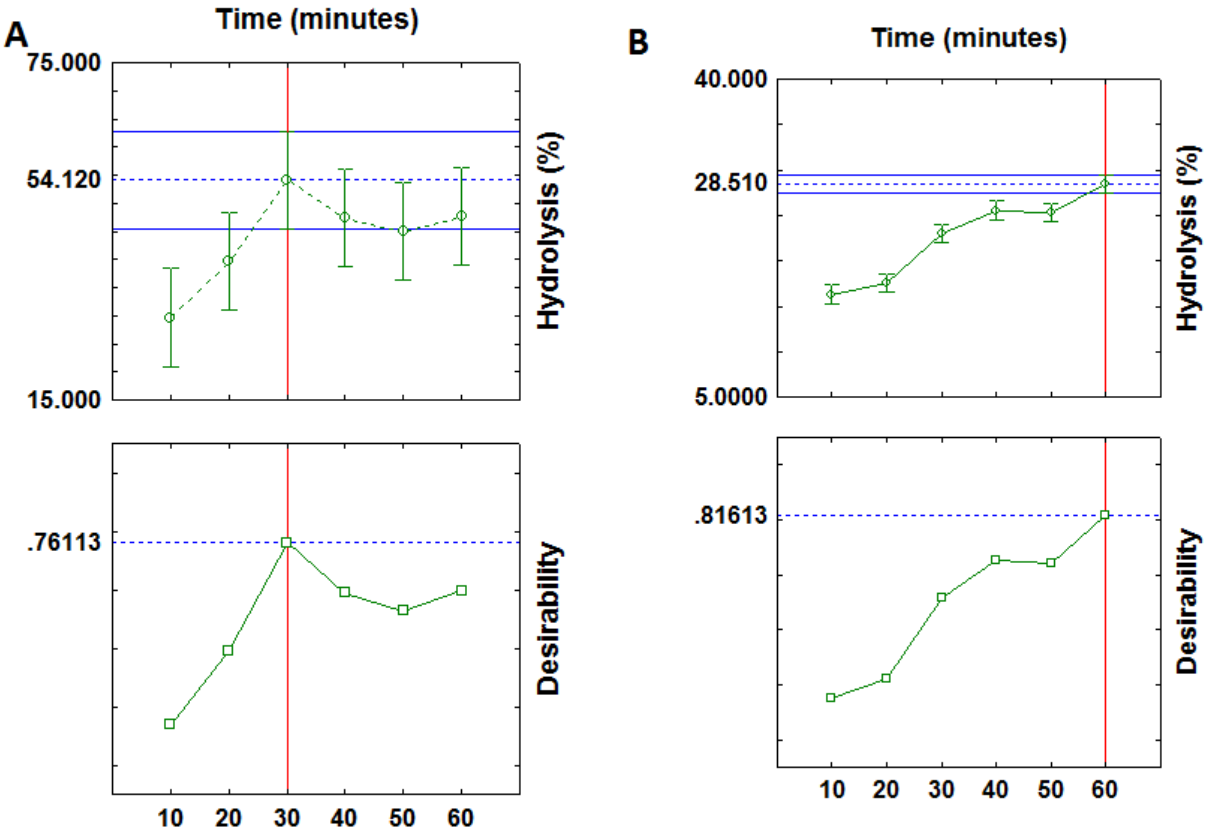

Figure 20 Prediction and desirability profiles for time course evaluation: a) US reaction; b) Orbital shaker reaction

Higher power ultrasound causes enzyme deactivation which is most likely due to the protein backbone reaction hydroxyl or hydrogen radicals generated by cavitation (Basto *et al.*, 2007; Fiametti *et al.*, 2011), which could lead to enzyme aggregation, thus obstructing the active sites and decreasing protein stability (Lima *et al.*, 2004; Fiametti *et al.*, 2011). Moreover, shear forces by US irradiation could contribute to enzyme inactivation (Özbek and Ülgen, 2000; Fiametti *et al.*, 2011). The US effects on enzyme behavior is not completely understood due to inconsistent results reported about inactivation and activation of enzymes upon US treatment. The sonication does not destroy the active site of an enzyme, unlike traditional heat denaturation, which had already been proved for α-amylase, horse radish peroxidase, laccase and alkaline phosphatase (Özbek and Ülgen, 2000; Rehorek, Tauber and Gübitz, 2004; Tauber, Gübitz and Rehorek, 2008; Fiametti *et al.*, 2011)

4. Conclusions

The cottonseed oil hydrolysis by *Penicillium* sp. LSF in thermostatic shaking bath and assisted by US showed that buffer/oil ratio and enzyme concentration have positive effects on oil hydrolysis for the two reaction systems. However, the US assisted reaction showed a second order behavior for buffer/oil ratio, differently from orbital shaker reaction, which was linear. Besides that, the oil hydrolysis tends to increase in higher LSF concentration in US assisted reaction and to decrease in higher concentration of LSF in shaking bath. Nevertheless, the US power seems to inhibit the enzyme, decreasing the oil hydrolysis yield. In time course evaluation, the US reaction did not change after 20-30 minutes, differently from shaking bath that stills increasing the oil hydrolysis yield with reaction time, which is possibly due to an enzyme thermo inactivation.

This study presents for the first time an application of a LSF in US system reaction for oil hydrolysis. The system using a US probe indicates that it is possible to obtain high cavitation with simultaneous heterogeneous system mixing for enzyme reaction. Although, further studies will be necessary to investigate and solve the limitations.

REFERENCES

AGUIEIRAS, E. C. G.*et al.* Biodiesel production from Acrocomia aculeata acid oil by (enzyme/enzyme) hydroesterification process: Use of vegetable lipase and fermented solid as low-cost biocatalysts. **Fuel,** v. 135, n. 0, p. 315-321, 11/1/ 2014. ISSN 0016-2361. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0016236114006358 >.

ALBERTON, D.*et al.* Production of a fermented solid containing lipases of *Rhizopus microsporus* and its application in the pre-hydrolysis of a high-fat dairy wastewater. **Food Technology and Biotechnology,** v. 48, n. 1, p. 28-35, 2010. Disponível em: < http://www.scopus.com/inward/record.url?eid=2-s2.0-77954055553&partnerID=40&md5=32a7365e8b60cdccbbbfb0ee2baa3de9 >.

ANVISA. **Resolução nº 482, de 23 de setembro de 1999, Regulamento técnico para fixação de identidade e qualidade de óleos e gorduras vegetais**. BRASIL, D. O. D. R. F. D. Brasília**:** 82-87 p. 1999.

BASTO, C.*et al.* Stability and decolourization ability of *Trametes villosa* laccase in liquid ultrasonic fields. **Ultrasonics Sonochemistry,** v. 14, n. 3, p. 355-362, 3// 2007. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417706000939 >.

BIERMANN, U.*et al.* Oils and Fats as Renewable Raw Materials in Chemistry. **Angewandte Chemie International Edition,** v. 50, n. 17, p. 3854-3871, 2011. ISSN 1521-3773. Disponível em: < http://dx.doi.org/10.1002/anie.201002767 >.

BORKAR, P. S.*et al.* Purification and characterization of extracellular lipase from a new strain: *Pseudomonas aeruginosa* SRT 9. **Brazilian Journal of Microbiology,** v. 40, p. 358-366, 2009. ISSN 1517-8382. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1517-83822009000200028&nrm=iso >.

CAMMAROTA, M. C.; TEIXEIRA, G. A.; FREIRE, D. M. G. Enzymatic pre-hydrolysis and anaerobic degradation of wastewaters with high fat contents. **Biotechnology Letters,** v. 23, n. 19, p. 1591-1595, 2001/10/01 2001. ISSN 0141-5492. Disponível em: < http://dx.doi.org/10.1023/A%3A1011973428489 >.

CHAHINIAN, H.*et al.* Production of Extracellular Lipases by *Penicillium cyclopium* Purification and Characterization of a Partial Acylglycerol Lipase. **Bioscience, Biotechnology, and Biochemistry,** v. 64, n. 2, p. 215-222, 2000/01/01 2000. ISSN 0916-8451. Disponível em: < http://dx.doi.org/10.1271/bbb.64.215 >. Acesso em: 2015/08/23.

CHINAGLIA, S.*et al.* Biochemistry of lipolytic enzymes secreted by *Penicillium solitum* and *Cladosporium cladosporioides*. **Bioscience, Biotechnology, and Biochemistry,** v. 78, n. 2, p. 245-254, 2014/02/01 2014. ISSN 0916-8451. Disponível em: < http://dx.doi.org/10.1080/09168451.2014.882752 >. Acesso em: 2015/08/22.

CRAVOTTO, G.*et al.* Organic reactions in water or biphasic aqueous systems under sonochemical conditions. A review on catalytic effects. **Catalysis Communications,** v. 63, p.

2-9, 3/10/ 2015. ISSN 1566-7367. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1566736714005160 >.

CUCHEVAL, A.; CHOW, R. C. Y. A study on the emulsification of oil by power ultrasound. **Ultrasonics Sonochemistry,** v. 15, n. 5, p. 916-920, 7// 2008. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S135041770800031X >.

DAMASCENO, F. R. C.; CAMMAROTA, M. C.; FREIRE, D. M. G. The combined use of a biosurfactant and an enzyme preparation to treat an effluent with a high fat content. **Colloids and Surfaces B: Biointerfaces,** v. 95, p. 241-246, 6/15/ 2012. ISSN 0927-7765. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0927776512001592 >.

DAMASCENO, F. R. C.; FREIRE, D. M. G.; CAMMAROTA, M. C. Assessing a mixture of biosurfactant and enzyme pools in the anaerobic biological treatment of wastewater with a high-fat content. **Environmental Technology,** v. 35, n. 16, p. 2035-2045, 2014/08/18 2014. ISSN 0959-3330. Disponível em: < http://dx.doi.org/10.1080/09593330.2014.890249 >. Acesso em: 2015/08/21.

DUARTE, J. G.*et al.* Enzymatic hydrolysis and anaerobic biological treatment of fish industry effluent: Evaluation of the mesophilic and thermophilic conditions. **Renewable Energy,** v. 83, p. 455-462, 11// 2015. ISSN 0960-1481. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960148115003365 >.

FIAMETTI, K. G.*et al.* Ultrasound irradiation promoted efficient solvent-free lipase-catalyzed production of mono- and diacylglycerols from olive oil. **Ultrasonics Sonochemistry,** v. 18, n. 5, p. 981-987, 9// 2011. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417710002257 >.

GANDHI, N. Applications of lipase. **Journal of the American Oil Chemists' Society,** v. 74, n. 6, p. 621-634, 1997/06/01 1997. ISSN 0003-021X. Disponível em: < http://dx.doi.org/10.1007/s11746-997-0194-x >.

GOGATE, P. R.; SUTKAR, V. S.; PANDIT, A. B. Sonochemical reactors: Important design and scale up considerations with a special emphasis on heterogeneous systems. **Chemical Engineering Journal,** v. 166, n. 3, p. 1066-1082, 2/1/ 2011. ISSN 1385-8947. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1385894710011654 >.

GONÇALVES, K. M.*et al.* Palm oil hydrolysis catalyzed by lipases under ultrasound irradiation – The use of experimental design as a tool for variables evaluation. **Ultrasonics Sonochemistry,** v. 19, n. 2, p. 232-236, 3// 2012. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417711001398 >.

GUTARRA, M. L. E.*et al.* Production of an acidic and thermostable lipase of the mesophilic fungus *Penicillium simplicissimum* by solid-state fermentation. **Bioresource Technology,** v. 100, n. 21, p. 5249-5254, 11// 2009. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852409005872 >.

HUANG, J.*et al.* Kinetic study on the effect of ultrasound on lipase-catalyzed hydrolysis of soy oil: Study of the interfacial area and the initial rates. **Ultrasonics Sonochemistry,** v. 17, n. 3,

p. 521-525, 3// 2010. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417709001850 >.

JUNG, F.; CAMMAROTA, M. C.; FREIRE, D. M. G. Impact of enzymatic pre-hydrolysis on batch activated sludge systems dealing with oily wastewaters. **Biotechnology Letters,** v. 24, n. 21, p. 1797-1802, 2002/11/01 2002. ISSN 0141-5492. Disponível em: < http://dx.doi.org/10.1023/A%3A1020621507944 >.

KNEZEVIC, Z. D.; SILER-MARINKOVIC, S. S.; MOJOVIC, L. V. Kinetics of lipase-catalyzed hydrolysis of palm oil in lecithin/izooctane reversed micelles. **Applied Microbiology and Biotechnology,** v. 49, n. 3, p. 267-271, 1998/03/01 1998. ISSN 0175-7598. Disponível em: < http://dx.doi.org/10.1007/s002530051167 >.

KUMAR, S.*et al.* Bioremediation of waste cooking oil using a novel lipase produced by *Penicillium chrysogenum* SNP5 grown in solid medium containing waste grease. **Bioresource Technology,** v. 120, p. 300-304, 9// 2012. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852412009224 >.

LEONG, T. S. H.*et al.* Minimising oil droplet size using ultrasonic emulsification. **Ultrasonics Sonochemistry,** v. 16, n. 6, p. 721-727, 8// 2009. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417709000303 >.

LERIN, L.*et al.* A review on lipase-catalyzed reactions in ultrasound-assisted systems. **Bioprocess and Biosystems Engineering,** v. 37, n. 12, p. 2381-2394, 2014/12/01 2014. ISSN 1615-7591. Disponível em: < http://dx.doi.org/10.1007/s00449-014-1222-5 >.

LI, C.*et al.* Effects of ultrasonic intensity and reactor scale on kinetics of enzymatic saccharification of various waste papers in continuously irradiated stirred tanks. **Ultrasonics Sonochemistry,** v. 12, n. 5, p. 373-384, 4// 2005. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417704000744 >.

LI, N.; ZONG, M.-H. Lipases from the genus *Penicillium*: Production, purification, characterization and applications. **Journal of Molecular Catalysis B: Enzymatic,** v. 66, n. 1–2, p. 43-54, 9// 2010. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117710001281 >.

LI, P. J.; LI, C. C.; LI, C. H. **Method for manufacturing coffee by solid state fermentation**: Google Patents 2010.

LIMA, V. M. G.*et al.* Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents. **Biochemical Engineering Journal,** v. 18, n. 1, p. 65-71, 4// 2004. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X03001657 >.

LIU, Y.*et al.* The effect of ultrasound on lipase-catalyzed hydrolysis of soy oil in solvent-free system. **Ultrasonics Sonochemistry,** v. 15, n. 4, p. 402-407, 4// 2008. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417707001526 >.

LIU, Y.*et al.* Biodiesel synthesis directly catalyzed by the fermented solid of *Burkholderia cenocepacia* via solid state fermentation. **Fuel Processing Technology,** v. 106, n. 0, p. 303-

309, 2// 2013. ISSN 0378-3820. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0378382012003062 >.

M'HIRI, N.*et al.* Effect of different operating conditions on the extraction of phenolic compounds in orange peel. **Food and Bioproducts Processing,** v. 96, p. 161-170, 10// 2015. ISSN 0960-3085. Disponível em: < http://www.sciencedirect.com/science/article/pii/S096030851500098X >.

MASE, T.; MATSUMIYA, Y.; MATSUURA, A. Purification and Characterization of *Penicillium roqueforti* IAM 7268 Lipase. **Bioscience, Biotechnology, and Biochemistry,** v. 59, n. 2, p. 329-330, 1995/01/01 1995. ISSN 0916-8451. Disponível em: < http://dx.doi.org/10.1271/bbb.59.329 >. Acesso em: 2015/08/23.

MURTY, V. R.; BHAT, J.; MUNISWARAN, P. K. A. Hydrolysis of oils by using immobilized lipase enzyme: A review. **Biotechnology and Bioprocess Engineering,** v. 7, n. 2, p. 57-66, 2002/04/01 2002. ISSN 1226-8372. Disponível em: < http://dx.doi.org/10.1007/BF02935881 >.

NOOR, I. M.; HASAN, M.; RAMACHANDRAN, K. B. Effect of operating variables on the hydrolysis rate of palm oil by lipase. **Process Biochemistry,** v. 39, n. 1, p. 13-20, 9/30/ 2003. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0032959202002637 >.

ÖZBEK, B.; ÜLGEN, K. Ö. The stability of enzymes after sonication. **Process Biochemistry,** v. 35, n. 9, p. 1037-1043, 5// 2000. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0032959200001412 >.

RAMACHANDRAN, K. B.*et al.* Kinetic study on hydrolysis of oils by lipase with ultrasonic emulsification. **Biochemical Engineering Journal,** v. 32, n. 1, p. 19-24, 11/1/ 2006. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X0600218X >.

RASERA, K.*et al.* Interesterification of fat blends using a fermented solid with lipolytic activity. **Journal of Molecular Catalysis B: Enzymatic,** v. 76, n. 0, p. 75-81, 4// 2012. ISSN 1381-1177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1381117711003274 >.

REHOREK, A.; TAUBER, M.; GÜBITZ, G. Application of power ultrasound for azo dye degradation. **Ultrasonics Sonochemistry,** v. 11, n. 3–4, p. 177-182, 5// 2004. ISSN 1350-4177. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1350417704000203 >.

RIGO, E.*et al.* Preliminary Characterization of Novel Extra-cellular Lipase from *Penicillium crustosum* Under Solid-State Fermentation and its Potential Application for Triglycerides Hydrolysis. **Food and Bioprocess Technology,** v. 5, n. 5, p. 1592-1600, 2012/07/01 2012. ISSN 1935-5130. Disponível em: < http://dx.doi.org/10.1007/s11947-010-0436-z >.

ROMERO, C. M.*et al.* Activity and Stability of Lipase Preparations from *Penicillium corylophilum*: Potential Use in Biocatalysis. **Chemical Engineering & Technology,** v. 37, n. 11, p. 1987-1992, 2014. ISSN 1521-4125. Disponível em: < http://dx.doi.org/10.1002/ceat.201300851 >.

ROONEY, D.; WEATHERLEY, L. R. The effect of reaction conditions upon lipase catalysed hydrolysis of high oleate sunflower oil in a stirred liquid–liquid reactor. **Process Biochemistry,** v. 36, n. 10, p. 947-953, 4// 2001. ISSN 1359-5113. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0032959201001303 >.

RUIZ, B.*et al.* Purification and characterization of an extracellular lipase from *Penicillium candidum*. **Lipids,** v. 36, n. 3, p. 283-289, 2001/03/01 2001. ISSN 0024-4201. Disponível em: < http://dx.doi.org/10.1007/s11745-001-0719-3 >.

SILVA, E. K.*et al.* Ultrasound-assisted formation of annatto seed oil emulsions stabilized by biopolymers. **Food Hydrocolloids,** v. 47, p. 1-13, 5// 2015. ISSN 0268-005X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0268005X1500003X >.

SOARES, D.*et al.* Biodiesel production from soybean soapstock acid oil by hydrolysis in subcritical water followed by lipase-catalyzed esterification using a fermented solid in a packed-bed reactor. **Biochemical Engineering Journal,** v. 81, p. 15-23, 12/15/ 2013. ISSN 1369-703X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1369703X13002696 >.

SOARES, D.*et al.* Analysis of multiphasic behavior during the ethyl esterification of fatty acids catalyzed by a fermented solid with lipolytic activity in a packed-bed bioreactor in a closed-loop batch system. **Fuel,** v. 159, p. 364-372, 11/1/ 2015. ISSN 0016-2361. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0016236115006675 >.

SUGIHARA, A.*et al.* Penicillium abeanum lipase: Purification, characterization, and its use for docosahexaenoic acid enrichment of tuna oil. **Journal of Fermentation and Bioengineering,** v. 82, n. 5, p. 498-501, // 1996. ISSN 0922-338X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0922338X97869917 >.

TALUKDER, M. M. R.*et al.* Ultrasonication enhanced hydrolytic activity of lipase in water/isooctane two-phase systems. **Biocatalysis and Biotransformation,** v. 24, n. 3, p. 189-194, 2006/01/01 2006. ISSN 1024-2422. Disponível em: < http://www.tandfonline.com/doi/abs/10.1080/10242420500132326 >. Acesso em: 2015/07/22.

TAUBER, M. M.; GÜBITZ, G. M.; REHOREK, A. Degradation of azo dyes by oxidative processes – Laccase and ultrasound treatment. **Bioresource Technology,** v. 99, n. 10, p. 4213-4220, 7// 2008. ISSN 0960-8524. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0960852407007043 >.

TEIXEIRA, C. B.; MADEIRA JUNIOR, J. V.; MACEDO, G. A. Biocatalysis combined with physical technologies for development of a green biodiesel process. **Renewable and Sustainable Energy Reviews,** v. 33, p. 333-343, 5// 2014. ISSN 1364-0321. Disponível em: < http://www.sciencedirect.com/science/article/pii/S136403211400094X >.

VAN OSS, C. J. On the mechanism of the cold ethanol precipitation method of plasma protein fractionation. **Journal of Protein Chemistry,** v. 8, n. 5, p. 661-668, 1989/10/01 1989. ISSN 0277-8033. Disponível em: < http://dx.doi.org/10.1007/BF01025606 >.

WINKLER, U. K.; STUCKMANN, M. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. **Journal of bacteriology,** v. 138, n. 3, p. 663-670, 1979. ISSN 0021-9193.

YACHMENEV, V. G.; BLANCHARD, E. J.; LAMBERT, A. H. Use of ultrasonic energy for intensification of the bio-preparation of greige cotton. **Ultrasonics,** v. 42, n. 1–9, p. 87-91, 4// 2004. ISSN 0041-624X. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0041624X04000101 >.

ZAWADZKI, R. A. F. O.*et al*. Continuous enzymatic prehydrolysis treatment of high-fat wastewater. **Food Technology and Biotechnology,** v. 51, n. 2, p. 293-300, 2013. Disponível em: < http://www.scopus.com/inward/record.url?eid=2-s2.0-84881505459&partnerID=40&md5=4b9ce9dba296bab23265ecec06db821f >.

GENERAL DISCUSSION OF THE RESULTS

This study aimed to develop a biocatalyst with lipolytic activity through solid-state fermentation (SSF) using agricultural by-products as substrate and a new strain of microorganism *Penicillium* sp. to evaluate the application on cottonseed oil hydrolysis assisted by ultrasound (US). The chapter 2 relates the study of SSF lipase production through the steps of substrate selection, physical parameter optimization and time course fermentation. The chapter 3 aimed on characterization of *Penicillium* sp. lipase and the application of lipasic solid fermented (LSF) on cottonseed oil hydrolysis assisted by ultrasound (US).

The results of Chapter 2 show the best formulation for SSF obtained by simplex centroid mixture design (SCMD) and optimized by desirability function. Evaluating lipase activity and cost/unit was possible to reach an efficient material use with the lowest cost between the raw materials studied: wheat bran (WB), soybean meal (SM) and cottonseed meal (CSM). The CSM seems to act as inductor for lipase biosynthesis due to its higher lipid concentration. However, the interaction between the three material did not showed to increase the enzyme activity. Due to the difference of enzyme activity, CSM also had the lowest cost/unit, even having the second higher cost of feedstock. CSM is the most suitable substrate obtaining the best results (69.2 U/g and 0.015 US$/10³U). After that, a CCRD study evaluated the effects of temperature, size particle and water volume for lipase activity, protein and specific activity. Results obtained showed that best performance was achieved in 30ºC, size particle of 2.4 mm and 90% of water. The CCRD optimization by desirability function permitted a 20% reduction in water consumption in fermentation medium and time course evaluation was possible to reduce 50% on fermentation time.

Application of raw material cost productivity as an input variable permitted the evaluation of commercial aspects in the SSF medium composition. This approach could be useful in research and development of new processes for raw material selection with different characteristics and performances. Besides that, the desirability function permitted a simultaneous optimization of different variables in SCMD and CCRD. The analysis also permitted a 20% reduction on water consumption in fermentation medium, and a 50% reduction on fermentation time, increasing the productivity and contributing to process sustainability. Therefore, it indicates that desirability function is a suitable multiple-response optimization method and an important tool for helping in process development with green engineering aspects.

Chapter 3 obtained the optimum conditions for lipase extract as 40ºC and pH 7 for activity and 30ºC and pH 3 for stability. The enzyme also presented high affinity with long chain fatty acids (p-NPP). It is stable in hydrophobic solvent (hexane) which indicates its possible application on organic synthesis and inhibited by polar solvents as ethanol and methanol, which indicates that is not so suitable for met- and ethanolysis reactions.

The LSF application focused on oil hydrolysis. A CCD study evaluated the US and shaking bath assisted hydrolysis of cottonseed oil using as independent variables: % LSF, buffer/oil ratio for shaking bath and the same variables plus power density (W/mL) for US probe system. All variables were significant for oil hydrolysis with %LSF and buffer/oil ratio with positive effects for both reaction systems. However, the US system showed a second order behavior for buffer/oil ratio, differently from orbital shaker reaction, which was linear. Moreover, the oil hydrolysis seems to increase in higher LSF concentration in US assisted reaction and to decrease in higher concentration of LSF in shaking bath. US power density had a negative effect indicating reaction inhibition. The US assisted reaction showed a second order behavior for buffer/oil ratio, differently from orbital shaker reaction, which was linear. Besides that, the oil hydrolysis tends to increase in higher LSF concentration in US assisted reaction and to decrease in higher concentration of LSF in shaking bath.

The desirability function optimization showed the possibility of improve hydrolysis yield by increasing buffer/oil ratio in both reaction systems. A time course experiment also optimized by desirability function showed that US probe system achieved 54% of oil hydrolysis yield in 30 minutes and 28.5% in 60 minutes for shaking bath. In time course evaluation, the US reaction did not change after 20-30 minutes, differently from shaking bath that still increasing the oil hydrolysis yield with reaction time, which is possibly due to an enzyme thermo inactivation.

This study presents for the first time an application of a LSF in US system reaction for oil hydrolysis. The system using a US probe indicates that it is possible to obtain high cavitation with simultaneous heterogeneous system mixing for enzyme reaction. However, further studies will be necessary to investigate and solve the limitations.

CONCLUSÃO GERAL

A aplicação de ultrassom em reações catalisadas por lipases pode representar uma possível integração das áreas de FES, oleoquímica e biocatálise para o desenvolvimento de uma bioeconomia, principalmente em países produtores agrícolas. Essa abordagem demonstra a possibilidade de utilizar subprodutos agrícolas oriundos da extração de óleos vegetais para gerar um biocatalisador utilizado na produção de compostos oleoquímicos comerciais, a partir dos mesmos óleos vegetais extraídos, contribuindo assim para uma sustentabilidade do processo.

A aplicação de misturas de substratos para FES pode ser uma alternativa para maximizar a atividade enzimática considerando-se que essas formulações possuem diferentes composições nutricionais. Além disso, a avaliação dos custos de substrato e sua relação com a produtividade enzimática contribui para a sustentabilidade no desenvolvimento de um processo de FES tendo em vista que mesmo ambientalmente favoráveis, esses processos necessitam ser economicamente viáveis para uma possível implementação comercial. Com a presente abordagem, foi possível obter na FES a maior atividade enzimática com menor custo de matéria-prima por unidade de produto.

A utilização de FSL é uma alternativa para diminuição nos custos de extração, purificação e imobilização de lipase. No entanto, sua aplicação é restrita devido à baixa atividade e estabilidade comparada com a enzima comercial purificada imobilizada. Além disso, aplicações mais refinadas exigem um grau de pureza maior. No entanto, representa uma alternativa promissora para síntese de biodiesel e hidrólise de óleo e gorduras.

Esse estudo relata pela primeira vez a aplicação de FSL na hidrólise de óleos vegetais assistido por ultrassom. A utilização de US direto através da sonda mostrou que é possível obter uma agitação do sistema sem necessitar de auxílio de agitação mecânica. Os efeitos da concentração enzimática e relação tampão/óleo são parecidos com os do banho de agitação e por isso, demonstram um mecanismo similar.

Os resultados obtidos representam um ponto inicial para estudos de aplicações de FSL em reações assistidas por ultrassom. É necessário uma investigação do processo para uma possível elucidação das limitações encontradas neste trabalho e otimização da performance da reação como taxa de conversão e tempo de reação por exemplo.

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Renewable and Sustainable Energy Reviews 33 (2014) 333–343

Contents lists available at ScienceDirect

Renewable and Sustainable Energy Reviews

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Biocatalysis combined with physical technologies for development of a green biodiesel process

Camilo Barroso Teixeira*, Jose Valdo Madeira Junior, Gabriela Alves Macedo

Food Science Department, College of Food Engineering, University of Campinas (UNICAMP), P.O. Box 6121, 13083-862 Campinas, SP, Brazil

ARTICLE INFO

Article history:
Received 12 June 2013
Received in revised form
29 December 2013
Accepted 31 January 2014

Keywords:
Biodiesel
Enzymatic transesterification
Microwave
Ultrasound
Membrane reactor

ABSTRACT

Biodiesel derived from the transesterification of vegetables oils or animal fats with alcohol is composed of saturated and unsaturated long chain alkylesters. The process has some technical problems that must be resolved to reduce the high cost of operation. Limitation of mass and heat transfers, reaction equilibrium, batch mode operation and product purification affects conversion yield, time of reaction, productivity and energy cost. This paper highlights some recent advances in process innovation for the biodiesel industry to develop a sustainable continuous process, environmentally benign and cost effective. Eco-friendly physical technologies as microwave, ultrasound and membrane reactors and their possible combination have successfully improved the enzymatic transesterification for biodiesel production.

Contents

1. Introduction

The world trend of sustainable technologies development has contributed to green chemistry emergence, which directed the responsibility of the scientific community toward developing new, improved and sustainable industrial processes. For such a trend, biofuels are considered mainstream due to energy importance, environmental pollution and necessity of fossil fuel substitution. Biodiesel, or fatty acid alkyl esters, has been considered an environmentally friendly alternative fuel for diesel engines [1]. It has some advantages like greenhouse gas emissions reduction, use of renewable sources and applicability in the existing transport sector [2].

Commercial biodiesel is produced by transesterification of vegetable oils and animal fats with methanol or ethanol on stirred tank reactors in the presence of base or acid catalysts. This process has some operating problems which include time of reaction, energy consumption and low productivity that contributes for increasing the production cost. For solving these issues, the biodiesel researches have focused on developing process intensification technologies [3]. These studies propose some eco-friendly technologies for reducing operational costs and simplifying the continuous process of the biodiesel production in large scale. Technologies such as microwaves, ultrasound, enzymatic catalysis and membrane reactors have been studied recently (Fig. 1) and used in combined mode with promising results.

* Corresponding author. Tel.: +55 19 3521 2175; fax +55 19 3521 1513.
E-mail address: teixeira.camilo@gmail.com (C.B. Teixeira).

http://dx.doi.org/10.1016/j.rser.2014.01.072
1364-0321

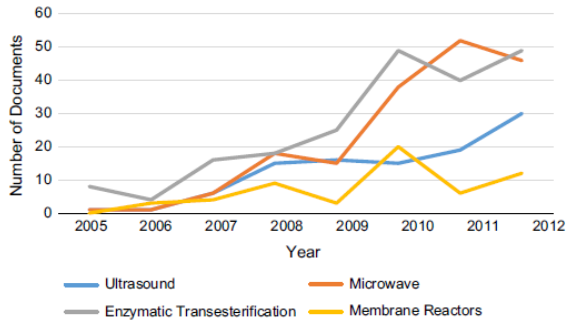

Fig. 1. Number of documents published for biodiesel production [115].

Microwave radiation has been used as an alternative heating method which showed more efficiency in all catalytic models, generating a faster reaction activation energy compared to conventional heating. As a consequence, short reaction times with a reduced energy consumption are obtained [4]. Besides it, microwave reactors have been developed for continuous mode operation.

Considering the immiscible problem between alcohol and oil, ultrasonic irradiation could be an alternative tool for increasing the mass transfer of liquid–liquid heterogeneous systems [5]. With increased liquid–liquid mass transfer, oils and methanol are easily emulsified, and cavitation contributes to the catalytic reaction. Under ultrasonic irradiation, the transesterification can be carried out at a low temperature, and smaller amounts of catalyst and alcohol are needed. Ultrasound, as well as microwaves, showed good performance with heterogeneous, homogeneous and enzymatic catalysis.

In terms of catalysis, the enzymatic transesterification, considered as a green process due to biochemical characteristics of the biocatalyst, presents some advantages like compatibility with variations in the feedstock quality, fewer process stages (heterogeneous catalysis), higher quality glycerol, easy phase separation (no emulsification from soaps), reduced energy cost and wastewater volumes [2]. Nevertheless, the reaction time of enzymatic transesterification is still much longer than homogeneous catalysis and for that reason it is considered an important research target in biodiesel process development.

Although the major biodiesel production cost is due to feedstock, the downstream process for biodiesel purification is considered expensive. The washing step to remove free glycerol, soap, alcohol excess and residual catalyst uses a large volume of water, generating a wastewater stream that must be treated [6–8]. The multiple downstream processes expends time and require additional cost [9]. The intensification technologies comprise the application of novel reactors or coupled reaction–separation processes combined to optimize the reaction rate, reduce the residence time and the number of operation units in whole process [3,9]. In membrane reactors, the simultaneous reaction and separation step not only reduces the unit operations but also avoids the reaction equilibrium limitation, besides the possibility of application in continuous mode.

This work presents some advantages and recent advances on the application of microwave, ultrasound, enzymatic catalysis and membrane reactors and its combination for overcoming the technical limitations in conventional biodiesel processing like mass and heat transfer; reaction equilibrium and purification. This process integration are useful for the implantation of a continuous mode operation as a sustainable process, due to its simultaneous environmental safety and operational cost reduction.

2. Microwave-assisted process

It has been reported in biodiesel transesterification that yield and reaction time vary greatly when heating by microwave irradiation instead of using conventional heating methods [10–14]. The selective heating of microwave irradiation requires less energy compared to conventional heating methods. It is possible to perform reactions faster, efficiently and safely using a microwave apparatus [15,16]. Besides, it is considered to be a sustainable technology due to its environment-friendly characteristic and its reduced energy consumption.

Microwaves are non-ionizing radiations *i.e.*, electromagnetic waves of low energy that which cannot ionize the atoms crossed. In spite of its induction molecular agitation capacity, such as ion migration or dipole rotation; microwaves cannot change the molecular structure [17]. This results in molecular attrition and collisions, which generates localized heating and thereby accelerating the chemical reaction, giving high conversion in a short time [14]. Compared with conventional heating, microwave irradiation requires less energy when solvents with higher dielectric loss factors are used [15,18]. According to this principle, the microwave heating has been studied for substitution of conventional heating in biodiesel production.

Microwave irradiation can be applied with different kinds of catalysts and feedstock, generating a satisfactory yield conversion and reaction time. Besides the homogeneous catalysts have the best conversion results, heterogeneous catalysis has showed same performance with microwave heating. It has the advantage of an easy catalyst separation step, which could reduce the operation cost and the number of unit operations in the whole process. Zhang et al. [19] developed an efficient microwave-assisted transesterification approach to produce biodiesel from yellow horn (*Xanthoceras sorbifolia* Bunge.) oil with a heteropolyacid (HPA) catalyst, namely, $Cs_{2.5}H_{0.5}PW_{12}O_{40}$. The optimization study obtained the maximum yield of fatty acid methyl esters (FAME) of 96.22% in 10 min using temperature of 60 °C, molar ratio of methanol:oil; 12:1, catalyst concentration of 1% (w/w of oil) and minimum recycle number of nine times. These results showed that the microwave method was more efficient compared to conventional heating.

Hsiao et al. [12] evaluated another heterogeneous base catalyst on the microwave-assisted transesterification of soybean oil. The results showed that nanopowder calcium oxide (nano CaO) combined with microwaves were greater than conventional heating in soybean oil transesterification. The results achieved 96.6% of conversion in 60 min using a methanol/oil molar ratio of 7:1, 3.0 wt% catalyst, and a reaction temperature of 60 °C.

Chen et al. [20] described the influence of microwave power on transesterification of waste-cooking-oil biodiesel. They discovered that conversion yields raises with reaction power. However, the high power microwave could damage organic molecules. The optimal reaction conditions were 0.75 wt% CH_3ONa catalyst, a methanol-to-oil molar ratio of 6, a reaction time of 3 min, and energy power of 750 W.

Microwave irradiation also has been applied in simultaneous extraction and reaction like the transesterification of algae oil, which has intracellular oil accumulation. Patil et al. [21] studied the application of microwave irradiation on the simultaneous extraction and transesterification (*in situ*) of dry algal biomass. The results achieved a high oil/lipid extraction yield from dry algal biomass and an efficient conversion to biodiesel simultaneously in optimal conditions: dry algae to methanol (wt/vol) ratio of 1:12, catalyst concentration about 2 wt%, and reaction time of 4 min.

Although high performance reported, microwaves need to be applied in continuous mode for its industrial approach. The continuous-flow process of biodiesel using commercially-available

Table 1
Resume of recent studies of microwave-assisted transesterification process for biodiesel production.

Reference	Catalyst/oil	Temperature (°C)	Yield (%)	Reaction time (min)
[11]	Alkali/rapeseed oil	40–50	88.3–93.7	3–5
[110]	KOH/dry algal	190	26.3	10
[111]	Sodium potassium tartrate doped zirconia/soybean oil	65	97.29	30
[112]	Acid and alkali/*Jatropha* seed	–	97.29	48
[19]	Heteropolyacid/yellow horn oil	60	96.22	10
[12]	nanopowder calcium oxide/soybean oil	65	96.6	60
[113]	NaOH/macroalgae oil	–	–	3
[13]	NaOH/waste palm oil	–	97%	0.5

scientific microwave equipment offers a fast, easy route to study the capacity and reaction performance. The operation permits the system reaction under atmospheric conditions and use of new or used vegetable oil. Energy consumption calculation suggests that the continuous-flow microwave process for the transesterification reaction is more energy-efficient than using conventional heating equipment [22].

The recent research on microwave-assisted transesterification shows its efficient application on continuous processes with minor energy consumption and faster reaction than conventional heating. It seems to be a robust technology due to the possibility of application with heterogeneous catalysis and with any raw material. Moreover, microwave heating is considered an environmentally friendly process. The summary of results from recent studies using microwave-assisted transesterification process for biodiesel production is presented in Table 1.

3. Ultrasonic-assisted process

Ultrasonic irradiation can induce an effective emulsification with improved mass transfer in biodiesel transesterification so that the rate of ester formation under ultrasonic mixing conditions is higher than traditional stirring conditions [23–26]. Research indicated that ultrasonic mixing is efficient, faster and economically functional with many advantages compared to the conventional stirring [5,26,27]. It is considered a green process since it is an eco-friendly technology with environmentally harmless.

The ultrasonic technique has been used for the transesterification reaction in biodiesel production aiming on improvement of yield conversion, reaction time and energy consumption reduction. Recent studies have shown that ultrasound could substitute the conventional stirring method and be combined with homogeneous or heterogeneous catalysis with its application on continuous mode operation.

Ultrasound has initially been applied in homogeneous catalysis with successful results by Thanh et al. [28] that produced biodiesel from canola oil with methanol in basic catalysis at room temperature, using a low frequency ultrasound (20 kHz) obtaining a conversion of 99% in 50 min in optimal conditions and Santos et al. [29], who studied the optimization of soybean oil methanolysis under ultrasound irradiation in batch mode and observed that the methanol to oil ratio showed major positive effect in the reaction than the catalyst concentration, which could be related to reaction equilibrium deviation and mass transfer limitation.

Despite high conversion yield, the homogeneous system needs a catalyst separation step, which could be simplified in heterogeneous catalysis. Kumar et al. [30] performed a transesterification assisted by ultrasonication under atmospheric conditions. The solid catalyst (Na/SiO_2) and ultrasound reduced the reaction time compared to the conventional batch process, resulting in 98.53% biodiesel conversion in 15 min, achieved in optimal conditions of molar ratio oil to methanol 1:9, catalyst concentration of 3 wt% of oil.

Industrial design have developed and converted batch for continuous process due to some benefits of productivity. Batch mode operation presents some limitations generated by reaction equilibrium and mass transfer resistance. Thanh et al. [31] performed an alkaline alcoholysis of waste cooking oils (WCO) with methanol in a continuous ultrasonic reactor of low-frequency; 20 kHz, in a two-step solvent addition. The FAME yield was extremely high even at the short residence time in the ultrasonic reactor (less than 1 min for the two steps) at room temperature. Using the approach of two-step solvent addition, the reaction rate was faster with small alcohol concentration. The authors considered that conversion rates are faster with small reagent concentration, generating a better performance of catalyst in a continuous process.

Although ultrasound utilization is related to mass transfer improvement, another recent application has presented the ultrasound usability in process control, with the same principle of sonar. For the biodiesel process, during the alcoholysis reaction, the separated glycerin sediments at the bottom and forms a heterogeneous phase. Low-intensity ultrasound pulse echoes use the density of glycerin and methyl ester difference to measure the time of flight (TOF) of a sound wave, which gives information on the glycerin separation situation during the reaction. Monitoring the variation of glycerin over time can be used to determine the glycerin separation start time, glycerin separation rate or separation end time. It can be used for establishing the reaction end or analytical determination of various parameters on transesterification reaction [32,33].

The effects of high-intensity ultrasound irradiation and temperature on glycerin separation start time and separation rate during the soybean oil transesterification were studied by Koc and McKenzie [33]. They evaluated the ultrasonication time and temperature on glycerin separation start time and separation rate (responses measured by ultrasound monitoring). The conditions that obtained the lowest starting times for glycerin separation were reaction temperature of 50 °C, ultrasonication of 5 min and ultrasonication rate of 90%.

The most recent works published for application of ultrasound in transesterification to biodiesel has focused on utilization of new heterogeneous catalysts and new sources of non-edible oils. Choedkiatsakul et al. [34] investigated the application of calcium oxide (CaO) and potassium phosphate (K_3PO_4) and Badday et al. [35] applied a gamma alumina supported tungstophosphoric as heterogeneous catalysts. Guo et al. [36] performed the alcoholysis in the presence of a Brønsted acidic ionic liquid-based catalyst, demonstrating the versatility of ultrasound technique combined with various catalysts. The same characteristic is observed for the types of raw materials used: Manh et al. [37] used different blends of tung, palm and canola oils; Encinar et al. [38] used castor oil and Paiva et al. [39] used babassu oil.

The ultrasound technique showed its adequation for biodiesel process presenting vantages compared to conventional stirring as efficiency of emulsification and energy input reduction; besides its

Table 2
Resume of recent studies of ultrasound-assisted transesterification process for biodiesel production.

Reference	Catalyst/oil	Frequency (kHz)	Yield (%)	Time reaction (min)
[114]	KOH/soybean	611	90	30
[39]	Alkali/babassu palm		97	10
[15]	Alkali/*Pongamia pinnata*	60	96–97	5
[31]	KOH/Canola	20	99	50
[28]	KOH/waste cooking	20	93.8	56
[35]	Tungstophosphoric acid/ *Jatropha* seed		84	50
[30]	Na–SiO_2/*Jatropha curcus*	24	98.53	15

fitness with all kinds of feedstock, catalyst and operation modes. Ultrasound also is considered as a green process due to its harmless behavior for environment.

The summary of recent studies using an ultrasound-assisted transesterification process for biodiesel production can be observed in Table 2.

4. Enzymatic catalysis

Biocatalysis has been considered a trend for sustainable synthesis technology due to biologic origin of the catalyst, selectivity and the possibility of reusing agro-industrial residues for biocatalyst production, which classifies the method as a green process. Enzymatic catalysis has been applied for biodiesel which starts its industrial scale operation in China. This is the first industrial scale with lipase (EC 3.1.1.3) as the catalyst [40]. These enzymes are obtained from most fungi and bacteria fermentation such as *Rhizomucor miehei*, *Rhizopus oryzae*, *Candida antarctica*, *Candida rugosa*, *Pseudomonas cepacia* and *Thermomyces lanuginosa*, but the commercial immobilized lipase B from *C. antarctica* (Novozym 435) is the most commonly used enzyme and the most expensive catalyst available on the market [41]. Lipase could be immobilized on solid supports both extra- or intra-cellular forms [42]. For that reason, it is considered a heterogeneous catalyst and could simplify the catalyst separation step, beyond the possibility of lipase recycling and application in continuous mode operation. It has been applied for biodiesel synthesis in free- and solvent systems as ionic liquids [43], organic co-solvents [44], supercritical fluids [45] and most recently with glymes [46].

Enzymatic transesterification eliminates the disadvantages of the alkali process by generating the product with high purity, with few downstream operations [47]. It has been reported in the literature that enzymatic transesterification of lipids for biodiesel production is considered technically feasible but the high cost of lipases is the main limitation for a commercially feasible enzymatic production of biodiesel. The technology to re-use enzymes is not enough to be competitive. However, literature data documenting the enzymatic biodiesel productivity together with the development of new immobilization technologies indicates that enzyme catalysis can become cost effective compared to chemical processing [2].

Recent studies have been focusing on improving catalysis performance and stability of the enzyme with aiming on reducing the lipase cost in the biodiesel conversion process. Some different approaches have been developed for application mode of lipases. Solid fermented, whole-cell biocatalyst and immobilized lipase in different supports are the mainly studied modes.

The application of fermented solid was created for reduce cost in lipase production and could be used as a catalyst in batch and continuous operation [48,49]. The solid fermentation of agricultural residues permits that enzyme have a low price compared to commercial enzymes due to its application as fermented solid avoids the extraction, purification and immobilization steps in enzyme production with satisfactory catalytic results in transesterification reaction [49]. Moreover, this approach has potential as a sustainable solution due to utilization of residues from the feedstock for catalyzing the biodiesel synthesis.

Salum et al. [49] produced a solid fermented lipase from *Burkholderia cepacia* LTEB11 in solid fermentation of sugarcane bagasse and sunflower seed meal that was used to catalyze the biodiesel synthesis in a fixed-bed reactor. The results showed a high conversion of 95% after 46 h, obtained at 50 °C, with an alcohol:oil molar ratio of 3:1, in two steps alcohol addition.

Using the same approach, Liu et al. [50] produced a *B. cenocepacia* solid fermented lipase and used it in soybean oil ethanolysis with *tert*-butanol as co-solvent. It was obtained in optimum conditions a highest biodiesel conversion of 86% in 96 h, 45 °C reaction temperature, 200 rpm speed rate, 4:1 alcohol/oil molar ratio, 1.5 wt% fermented solid concentration (based on oil weight, g), 40% *tert*-butanol concentration (based on oil volume, v/v) and 5 wt% moisture content (based on oil weight, g).

The whole-cell biocatalyst is an another approach studied. It is produced by expression of the enzyme on the microbial cells surfaces; intracellular lipase bacteria or fungi and/or different immobilization techniques of fungal mycelium in different supports for continuous mode operation. This approach permits the application of intracellularly-accumulated lipases as whole-cell biocatalysts which avoids the complex procedures of extraction, purification, and immobilization in lipase production process [51]. There are several recent works reporting the utilization of bacteria, yeast and fungi as whole-cell biocatalysts in biodiesel process [52–56].

Genetic engineering has been applied for modeling and has developed new catalysts by cloning and expressing lipase gene in different fungi and bacteria with aim to improve the catalytic activities and stability. The recombinants microbial are used as whole-cell biocatalyst in transesterification reaction immobilized in different supports in continuous reactors.

Gao et al. [54] developed a recombinant *E. coli* expressing novel alkaline lipase-coding gene from *Proteus* sp. for olive oil methanolisys which reached a conversion yield of 100% in 12 h reaction, temperature of 15 °C, which was the lowest temperature catalysis in biodiesel transesterification.

Adachi et al. [57] developed an *Aspergillus oryzae* whole-cell biocatalyst by expressing the lipase gene of *C. antarctica* lipase B (r-CALB) with high esterification activity. The two step reaction consisted first in a hydrolysis of soybean and palm oils using a *C. rugosa* lipase and then submitted to esterification with immobilized r-CALB catalysis which achieved a methyl ester content of more than 90% after 6 h with the addition of 1.5 M methanol. The r-CALB maintained a 90% methyl ester content even after the 20th batch.

Another *A. oryzae* whole-cell biocatalyst developed by Adachi et al. [58] co-expresses two lipase genes in the same cell to improve biodiesel transesterification: a *Fusarium heterosporum* lipase (*FHL*) and a mono- and di-acylglycerol lipase B (*mdlB*). The results obtained showed the best performance of reaction using the lipase-coexpressing whole-cell compared to lipase-mixing method and two step reactions, reaching the best conversion rate and the best ester concentration (98%).

Yan et al. [59] also developed a biocatalyst by expression of two synergistic lipases, *C. antarctica* lipase B and *Thermomyces lanuginosus* lipase on the *Phichia pastoris* cell surface. Results showed a high conversion rate (95.4%) and good operational stability.

Among several modes of application, the immobilization remains the most studied one for enzymatic catalysis in biodiesel process (Fig. 2). Several solid materials, such as ceramics, kaolinites, silica, cellulose, polymers, zeolites and mesoporous matrixes

have been used as support for enzymes immobilization [60–63]. Moreover, several methods have been studied for enzyme immobilization: adsorption; covalent binding; cross-linking and containment behind a barrier (micro-encapsulation, entrapment and confinement). The immobilization merit relies to the activity and the stability preservation of lipase [64].

The packed-bed reactor (PBR) generally leads to higher productivity than a continuous stirred-tank reactor. Moreover, the volumes are reduced, the technology is less expensive (no mobile parts) and enzyme support attrition is avoided [65–67].

Hama et al. [68] created a packed-bed reactor (PBR) system with a fungus whole-cell biocatalyst. Immobilized in polyurethane foam biomass support particles (BSPs), lipase-producing *R. oryzae* cells were cultivated in an airlift bioreactor. The soybean oil methanolysis reached the highest ester content of 90% maintaining around 80% after the 10th cycle.

Wang et al. [69] developed a biodiesel process based on soybean oil methanolysis in a packed bed reactor system using a lipase–Fe_3O_4 nanoparticle biocomposite catalyst obtaining a 88% conversion rate for 192 h, decreasing to approximately 75% after 240 h of reaction.

The application of lipases immobilized in PBR could be linked in continuous downstream separation. Hama et al. [70] created a packed-bed reactor on a bench scale which allows the separation of glycerol byproduct in continuous process. To separate soluble glycerol present in the biodiesel, adsorptive purification using ion-exchange resin was applied to the PBR system. The optimization discovered that the PBR could operate for a long time generating high methyl ester content and an efficient glycerol removal. Hence, this developed model incorporating the enzymatic PBR and glycerol separating system is promising for practical biodiesel production.

The glycerol separation is considered in some new approaches as Xu et al. [71] which developed a two-stage enzymatic ethanolysis in a packed bed reactor using an experimental immobilized lipase (NS 88001) and Novozym 435 to perform reaction of transesterification (first step) and esterification (second one) respectively. The reactions were carried out in a simulated series of reactors considering the separation of the glycerol and water between passes in the first and second stages. Recent advances in enzyme catalysis for biodiesel indicate that some approaches have been tested for application processes in continuous operation. However, some authors considered that the use of the enzyme in the soluble form has lower cost since immobilization process is more expensive [72]. In order to reuse for several cycles, increasing the profitability of the biocatalyst; recent studies are focusing on new materials and new methods that are able to decrease the cost and increase the efficiency and capacity of lipase. It is possible that in a few years the enzymatic catalysis will be economically feasible for biodiesel production on a large scale. The summary of recent studies using enzymatic transesterification process for biodiesel production can be observed in Table 3.

5. Membrane reactors

Reaction and separation are conducted on different stages of the process with different equipment in most chemical processes using continuous stirred-tank reactor (CSTR), plug-flow reactor (PFR), batch reactor and distillation column with diverse configuration [3,73–76]. Some reactive-separation technologies have been studied as possible alternatives with low capital investment and reduced operational cost for continuous operating in biodiesel production. Some reactors have been designed and used as a single device for reaction and separation in transesterification: centrifugal contactor separators [73], reactive distillation [77], reactive absorption [78], reactive extraction [79] and membrane

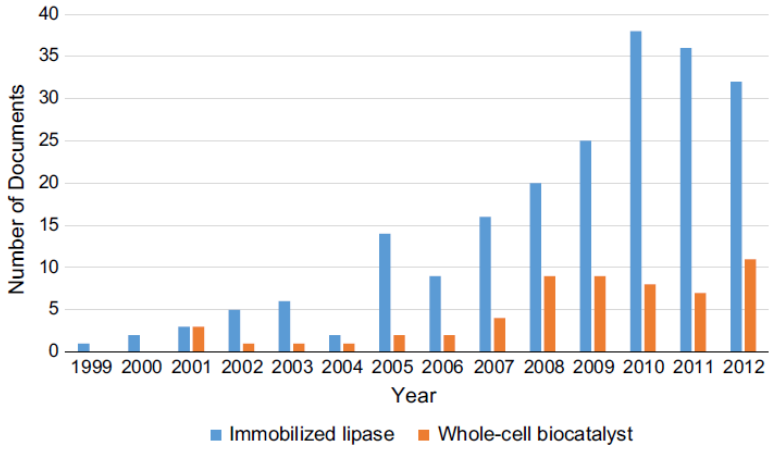

Fig. 2. Number of documents published for biodiesel produced by enzymatic catalysis [115].

Table 3
Resume of recent studies of enzymatic transesterification for biodiesel production.

Reference	Catalyst/oil	Immobilization support	Temperature (°C)	Yield (%)	Time reaction (h)
[70]	*C. antarctica* lipase/shirashime oil	Macroporous acrylic resin	30	98.9	–
[67]	*C. antarctica* lipase/high oleic sunflower	Lewatit VP OC	60	96.5	49
[69]	*P. cepacia* lipase/soybean	Fe_3O_4 nanoparticle in cotton	40	100	24
[54]	Recombinant *E. coli* cell/vegetable oils	–	15	100	12
[58]	Recombinant *A. oryzae* cell/soybean	Reticulated polyurethane foam	30	98	80

reactors [80] are some examples. This process integration is aimed on improving the productivity, with energy reduction, excluding the need for solvents generating an efficient process with green engineering characteristics [76,81].

The membrane reactor was defined by Cao, Tremblay [82] as a system which combines membrane separation and chemical reactions. It is used to simultaneously perform a reaction and membrane separation of products in the same operation unit. This approach permits to solving the equilibrium reaction limitation, enhancing mass and heat transfer conversion rate, besides the possibility of application in continuous mode operation. Furthermore, the membrane has the ability to function as a support for the solid catalyst (heterogeneous) avoiding an additional step of separating the additional catalyst. In the conventional process, a large amount of water used in the neutralization of alkaline catalyst purification and alkyl esters creates a step of treating waste water, which is the main problem in the process of homogeneous catalysis [83].

The membrane technology has been used in various processes of biorefining and bioenergy production, including: separation and purification of individual molecules from biomass, removal of fermentation inhibitors, enzyme recovery from hydrolysis processes, membrane bioreactors for bioenergy and chemical production, such as bioethanol, biogas and acetic acid, bioethanol dehydration, bio-oil and biodiesel production, and algae harvesting [84].

The application of membrane reactors can be based on three different principles. Based on the size of the oil droplets, the catalytic membrane, pervaporation. The application of catalytic membrane can be combined with catalyst in two ways: by integrating a catalyst and without incorporating of catalyst [9]. For the production of biodiesel membrane technologies are used based on principles of oil droplet size and catalytic membrane. The catalytically active membrane appears to be the best option for the production of biodiesel because fewer purification is necessary since catalytically inert membrane requires an additional purification due to the presence of a mixture of glycerol, methanol catalysts and FAME in the permeate flow.

To control the membrane reactor, it is necessary to know the variables with the most significant effects for the process. The parameters to be controlled are effect of reaction temperature, the alcohol to oil ratio, catalyst concentration, reactant flow rate, transmembrane pressure (TMP), the pore size and membrane thickness [9]. There are some physical–chemical characteristics of membrane that permits its application in biodiesel transesterification. The membrane offers a barrier to lipophilic substances present in lipid feedstocks. This difference offers reliability in the production of biodiesel that parallels the use of distillation in petroleum processes. The vapor–liquid interface determines the mass transfer mechanism in distillation, like the lipophilic–hydrophilic phase boundary does in the membrane reactor. The membrane serves to retain the smallest lipophilic droplets within the reactor considering the oil droplet principle [82].

The material characteristic of inorganic ceramic membranes offers resistance to chemical attack and thermal stability [85]. Resistance to corrosion is considered fundamental when the system is operated with base or acid catalysts.

In the conversion of vegetable oil by the transesterification process, the reversible reaction between the reactant and the product designates that the conversion for biodiesel is greatly dependent on the proportion of reactants and the conditions of the transesterification process. According to Le Chatelier's principle, big concentration of alcohol is necessary to switch the reaction equilibrium for the product side and rise the conversion yield [9,86].

The most recent studies on membrane reactors for biodiesel have focused on the optimization of process parameters to improve the performance of the reactor. Some implementation strategies are used in addition to investigation of mechanisms associated with the thermodynamic phase equilibrium of ternary mixture between alcohol, triglycerides and alkyl esters. Cheng and Yen [87] studied the effects of operating parameters, including methanol-to-oil molar ratio, catalyst concentration, temperature and the reaction time course for the reactant composition in the oil–FAME–MeOH ternary phase diagram. The results obtained indicate that increasing the residence time of the whole system within the two-phase zone improves the separation through membranes.

Studies to improve the recovery of methanol through the knowledge of the variation of process parameters are also performed and utilized to optimize the performance of membrane reactors. Baroutian and Aroua [88] studied the methanol recovery by means of continuous distillation after membrane separation and concluded that operational parameters including heating temperature, permeate flow rate and reactants ratio have significant effects on the rate of methanol recovery.

Falahati and Tremblay [89] studied the effect of membrane flux and residence time on the membrane reactor performance for different feedstock. Low free fatty acid (FFA) oils (FFA $< 1\%$), *i.e.*, canola, corn, sunflower and unrefined soy oils, and high FFA waste cooking oil (FFA $= 5\%$) were alkali transesterified. The membrane reactor could be operated at the upper limit of the flux tested (70 $L/m^2/h$) and a residence time of 60 min.

Some authors have been studying the use of membranes as a downstream process in a separation unit only. The reaction is carried on in a batch reactor and separated by membrane filtration. Reyes et al. [8] developed a new semi-continuous strategy to produce and refine FAME at a low methanol-to-oil molar ratio using a ceramic membrane filtration process. The sequential batch coupled with the membrane reactor (SBMR) strategy was based on the operation of consecutive reactions and refining cycles; the latter operated only when a 70% FAME conversion yield was reached. This permitted an operation with a high permeate flux, due to the low viscosity of FAME compared to vegetable oil. The use of a stoichiometric methanol-to-oil molar ratio in the transesterification increases the accumulation of mono and diglycerides in a conventional batch reactor (CBR). However, this conventional batch reactor coupled with a membrane system (CBR–MS) allows the permeation of these compounds. The SBMR separated 99% of glycerol and reduced 79% and 78% of mono and diglycerides in FAME, respectively, compared to CBR. Therefore, the phase equilibrium during transesterification is a main factor to be considered in the implementation of a FAME separation–refining process, since the ceramic membrane is not able to remove MG and DG, but separation of these compounds is possible using the adequate operational strategy.

The evaluation of membrane performance and its behavior in transesterifcation of biodiesel have been studied in different approaches. For this purpose, a mathematical simulation of the operation of transesterification has been a brew of crucial importance to determine the merits of each approach or operational configuration. Cheng et al. [90] studied the modeling, simulation and experimental validation of biodiesel process using a membrane reactor integrated with a prereactor. The mathematical modeling considered and included the equations of transesterification kinetics, phase equilibrium, mass balance for both prereactor and tubular ceramic membrane derived from mass balances of both the feed side and the permeate side, attached with the mass transfer across the membrane. It was evaluated the integrated reactor performance according to the permeated biodiesel flux, selectivity of methanol-to-oil ratio in the feed, the initial reaction time in the prereactor, the volume ratio of the prereactor to tube membrane and the tube membrane length. The results showed

that the prereactor can be used for the purpose of carrying out a substantial part of the transesterification reaction in the early stage. The subsequent membrane reactor could separate the unreacted emulsified oil from the product stream. The process was validated experimentally and fit considerably well with the simulated data by adjusting the operating conditions, including the initial reaction time in the prereactor and the tube membrane length.

The biodiesel production by catalytic membrane reactor is a new technology which can be an alternative to solve the actual limitations related to conventional biodiesel production processes. The technology is considered environmentally friendly with lower cost invest requirement, overcoming the limitation caused by the chemical equilibrium of the reaction, with high flexibility related to the type of raw material used, generating products according to international standards [9].

6. Process combination

Recent work involving the technologies mentioned in this article has been recently applied in combined mode in order to increase efficiency and optimize performance in the transesterification reaction for biodiesel production. This approach is based on the combination of the mechanisms involved for each technology. Microwave for heat transfer, ultrasound to mass transfer and membrane reactors for the process of simultaneous reaction and separation. All are applied to continuous operation mode in the transesterification reaction for biodiesel production with green process characteristics. In addition, some studies describe the possible application in enzymatic catalysis: microwave [91–93]; ultrasound [94–96] and membrane reactors [97,98] have been recently studied in combination with enzymatic catalysis for different products obtainment.

The possible combination of microwave irradiation and enzymatic transesterification for biodiesel transesterification was reported by Nogueira and Carretoni [99] and the results showed that the microwave method increased the lipase activity but the time exposure led to enzyme deactivation. Da Rós and Freitas [100] also combined enzymatic ethanolysis with a microwave heating system. Using palm oil and a *Pseudomonas fluorescens* lipase immobilized on an epoxy polysiloxane–polyvinyl alcohol hybrid composite, they obtained the optimal conditions ranging from 8 to 15 W (according to reaction temperature), 8:1 ethanol-to-oil molar ratio at 43 °C reaching 97.56% of palm oil conversion in a 12 h reaction, conforming to a six fold increase compared to the conventional heating assisted-process. This work showed that microwave irradiation accelerated the enzyme-catalyzed reaction and no destructive effects on the enzyme properties were observed, such as stability and substrate specificity. In addition, the microwave irradiation permits uniform heating of the entire reaction volume. The approach presented a low energy demand and a faster conversion of palm oil into biodiesel. The resume of the results is presented in Fig. 3

The enzymatic transesterification has also been combined with ultrasound irradiation for biodiesel production as Yu and Tian [101] studied the transesterification of soybean oil and methanol using Novozym 435 lipase under ultrasonic irradiation and vibration. The results showed that the combination of ultrasonic with vibration increased the lipase catalytic activity and improved the time of reaction, reaching a 96% yield of fatty acid methyl ester in 4 h.

Similar results were also obtained by Batistella and Lerin [102] who studied the enzymatic soybean oil ethanolysis in organic solvent under ultrasonic irradiation. The reaction with two commercial immobilized lipases in an ultrasonic bath was performed.

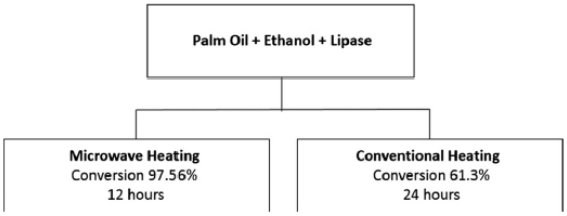

Fig. 3. Study developed by Da Rós and Freitas [100].

The results showed high reaction yields (~90 wt%) obtained at mild irradiation power supply (~100 W) and temperature (60 °C) in a relatively short reaction time, 4 h, using Lipozyme RM as catalyst. The use of Novozym 435 led to lower conversions (about 57%) nevertheless, the enzyme activity was stable after eight cycles of use, showing a reduction in product conversion after the fourth cycle.

Tupufia and Jeon [103] reported in coconut oil alcoholysis that the alkali reaction was about 2 orders of magnitude faster than the lipase reaction, however resulted in saponification/partial solidification. They also observed that the ratios of the kinetic constants during enzymatic transesterification are in agreement with reaction stoichiometry, resulting in purified products. The resume of the results are presented in Fig. 4.

Most studies report only the application of microwave or ultrasound. However, it is possible that these technologies can be applied in combined mode whereas the mechanisms of each one is different. Recently, Hsiao et al. [27] employed ultrasonic mixing and microwave irradiation to assist soybean oil alkaline transesterifcation with the objective to enhance the fatty acid ester yield and time reaction. Results showed that the optimal time of reaction was 1 min of ultrasonic mixing and 2 min of microwave irradiation. The optimal process conditions for 97.7% of conversion rate were: catalyst concentration, 1.0 wt%; reaction temperature, 60 °C and methanol:oil molar ratio, 6:1. The protocol established results in a 3-min reaction. The authors suggested that the ultrasonic mixing improved the performance of microwave irradiation which can be considered a synergistic effect between the mechanism of mass and heat transfer. The resume of results is presented in Fig. 5. Utilizing the same approach combining microwave and ultrasound in sequence for biodiesel synthesis, Gole and Gogate [104] transesterified the high value acid Nagchampa oil in a two-step synthesis method. The first step comprises of esterification for acid neutralization and the second, an alkali transesterification. Both steps carried out under microwave and ultrasound irradiation. The reaction time for the esterification and transesterification using ultrasound alone was 60 min and 20 min, respectively, and it reduced to only 15 min and 6 min for the sequential approach. The innovation is that the required excess of alcohol is significantly reduced (ratio 1:4), which can lead to a substantial energy saving in the downstream separation.

There are studies that report the application of these technologies combined with catalytic membranes and ultrafiltration membranes [105–107] for several bioprocesses. Zhang et al. [16] studied the performance of conventional heating and microwave-assisted method for biodiesel production using cation ion-exchange resin particles (CERP)/PES catalytic for transesterification of waste cooking oil (WCO). The experimental results showed that microwave irradiation exhibited a remarkable enhanced effect for esterification compared with that of the traditional heating method, reaching 97.4% under the optimal conditions of reaction temperature 60 °C, methanol/acidified oil mass ratio, 2:1, catalytic membrane (annealed at 120 °C) loading, 3 g, microwave power, 360 W and reaction time 90 min.

and membrane reactors for simultaneous reaction–separation may be a practical improvement, considering the evolutions in intensification for industrial process. This present work showed which have been published recently in the combination of this physical technologies with enzymatic transesterification using lipases as biocatalysts with the possibility of an approach using an enzymatic membrane reactor coupled with a system of ultrasound and microwave. This experimental application for biodiesel production shows that its potential is real and that the results collaborate to reduce operating costs of the process, making the product more competitive and affordable for the market.

Acknowledgment

The authors would like to thank the Brazilian National Council for Scientific and Technological Development (CNPq) for the financial support.

References

[1] Fukuda H, Hama S, Tamalampudi S, Noda H. Whole-cell biocatalysts for biodiesel fuel production. Trends Biotechnol 2008;26:668–73.
[2] Nielsen PM, Brask J, Fjerbaek L. Enzymatic biodiesel production: technical and economical considerations. Eur J Lipid Sci Technol 2008;110:692–700.
[3] Qiu Z, Zhao L, Weatherley L. Process intensification technologies in continuous biodiesel production. Chem Eng Process: Process Intensif 2010;49:323–30.
[4] Hernando J, Leton P, Matia MP, Novella JL, Alvarez-Builla J. Biodiesel and FAME synthesis assisted by microwaves: homogeneous batch and flow processes. Fuel 2007;86:1641–4.
[5] Ji J, Wang J, Li Y, Yu Y, Xu Z. Preparation of biodiesel with the help of ultrasonic and hydrodynamic cavitation. Ultrasonics 2006;44(Suppl.):e411–e414.
[6] Demirbaş A. Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey. Energy Convers Manag 2003;44:2093–109.
[7] Karaosmanoğlu F, Cigizoglu KB, Tüter M, Ertekin S. Investigation of the refining step of biodiesel production. Energy Fuels 1996;10:890–5.
[8] Reyes I, Ciudad G, Misra M, Mohanty A, Jeison D, Navia R. Novel sequential batch membrane reactor to increase fatty acid methyl esters quality at low methanol to oil molar ratio. Chem Eng J 2012;197:459–67.
[9] Shuit SH, Ong YT, Lee KT, Subhash B, Tan SH. Membrane technology as a promising alternative in biodiesel production: a review. Biotechnol Adv 2012;30:1364–80.
[10] Azcan N, Danisman A. Alkali catalyzed transesterification of cottonseed oil by microwave irradiation. Fuel 2007;86:2639–44.
[11] Azcan N, Danisman A. Microwave assisted transesterification of rapeseed oil. Fuel 2008;87:1781–8.
[12] Hsiao M-C, Lin C-C, Chang Y-H. Microwave irradiation-assisted transesterification of soybean oil to biodiesel catalyzed by nanopowder calcium oxide. Fuel 2011;90:1963–7.
[13] Lertsathapornsuk V, Pairintra R, Aryusuk K, Krisnangkura K. Microwave assisted in continuous biodiesel production from waste frying palm oil and its performance in a 100 kW diesel generator. Fuel Process Technol 2008;89:1330–6.
[14] Lidström P, Tierney J, Wathey B, Westman J. Microwave assisted organic synthesis—a review. Tetrahedron 2001;57:9225–83.
[15] Kumar R, Ravi Kumar G, Chandrashekar N. Microwave assisted alkali-catalyzed transesterification of *Pongamia pinnata* seed oil for biodiesel production. Bioresour Technol 2011;102:6617–20.
[16] Zhang H, Ding J, Zhao Z. Microwave assisted esterification of acidified oil from waste cooking oil by CERP/PES catalytic membrane for biodiesel production. Bioresour Technol 2012;123:72–7.
[17] Manco I, Giordani L, Vaccari V, Oddone M. Microwave technology for the biodiesel production: analytical assessments. Fuel 2012;95:108–12.
[18] Liu J, Takada R, Karita S, Watanabe T, Honda Y, Watanabe T. Microwave-assisted pretreatment of recalcitrant softwood in aqueous glycerol. Bioresour Technol 2010;101:9355–60.
[19] Zhang S, Zu Y-G, Fu Y-J, Luo M, Zhang D-Y, Efferth T. Rapid microwave-assisted transesterification of yellow horn oil to biodiesel using a heteropolyacid solid catalyst. Bioresour Technol 2010;101:931–6.
[20] Chen K-S, Lin Y-C, Hsu K-H, Wang H-K. Improving biodiesel yields from waste cooking oil by using sodium methoxide and a microwave heating system. Energy 2012;38:151–6.
[21] Patil PD, Gude VG, Mannarswamy A, Cooke P, Munson-McGee S, Nirmalakhandan N, et al. Optimization of microwave-assisted transesterification of dry algal biomass using response surface methodology. Bioresour Technol 2011;102:1399–405.
[22] Barnard TM, Leadbeater NE, Boucher MB, Stencel LM, Wilhite BA. Continuous-flow preparation of biodiesel using microwave heating. Energy Fuels 2007;21:1777–81.
[23] Colucci J, Borrero E, Alape F. Biodiesel from an alkaline transesterification reaction of soybean oil using ultrasonic mixing. J Am Oil Chem Soc 2005;82:525–30.
[24] Hanh HD, Dong NT, Okitsu K, Nishimura R, Maeda Y. Biodiesel production by esterification of oleic acid with short-chain alcohols under ultrasonic irradiation condition. Renew Energy 2009;34:780–3.
[25] Hanh HD, Dong NT, Starvarache C, Okitsu K, Maeda Y, Nishimura R. Methanolysis of triolein by low frequency ultrasonic irradiation. Energy Convers Manag 2008;49:276–80.
[26] Stavarache C, Vinatoru M, Maeda Y, Bandow H. Ultrasonically driven continuous process for vegetable oil transesterification. Ultrason Sonochem 2007;14:413–7.
[27] Hsiao M-C, Lin C-C, Chang Y-H, Chen L-C. Ultrasonic mixing and closed microwave irradiation-assisted transesterification of soybean oil. Fuel 2010;89:3618–22.
[28] Thanh LT, Okitsu K, Sadanaga Y, Takenaka N, Maeda Y, Bandow H. A two-step continuous ultrasound assisted production of biodiesel fuel from waste cooking oils: a practical and economical approach to produce high quality biodiesel fuel. Bioresour Technol 2010;101:5394–401.
[29] Santos FFP, Rodrigues S, Fernandes FAN. Optimization of the production of biodiesel from soybean oil by ultrasound assisted methanolysis. Fuel Process Technol 2009;90:312–6.
[30] Kumar D, Kumar G, Poonam Singh CP. Ultrasonic-assisted transesterification of Jatropha curcus oil using solid catalyst, Na/SiO_2. Ultrason Sonochem 2010;17:839–44.
[31] Thanh LT, Okitsu K, Sadanaga Y, Takenaka N, Maeda Y, Bandow H. Ultrasound-assisted production of biodiesel fuel from vegetable oils in a small scale circulation process. Bioresour Technol 2010;101:639–45.
[32] Bulent Koc A. Ultrasonic monitoring of glycerol settling during transesterification of soybean oil. Bioresour Technol 2009;100:19–24.
[33] Koc AB, McKenzie EH. Effects of ultrasonication on glycerin separation during transesterification of soybean oil. Fuel Process Technol 2010;91:743–8.
[34] Choedkiatsakul I, Ngaosuwan K, Assabumrungrat S. Application of heterogeneous catalysts for transesterification of refined palm oil in ultrasound-assisted reactor. Fuel Process Technol 2013;111:22–8.
[35] Badday AS, Abdullah AZ, Lee K-T. Ultrasound-assisted transesterification of crude Jatropha oil using alumina-supported heteropolyacid catalyst. Appl Energy 2013;105:380–8.
[36] Guo W, Li H, Ji G, Zhang G. Ultrasound-assisted production of biodiesel from soybean oil using Brønsted acidic ionic liquid as catalyst. Bioresour Technol 2012;125:332–4.
[37] Manh D-V, Chen Y-H, Chang C-C, Chang C-Y, Hanh H-D, Chau N-H, et al. Effects of blending composition of tung oil and ultrasonic irradiation intensity on the biodiesel production. Energy 2012;48:519–24.
[38] Encinar JM, González JF, Pardal A. Transesterification of castor oil under ultrasonic irradiation conditions. Preliminary results. Fuel Process Technol 2012;103:9–15.
[39] Paiva EJM, da Silva MLCP, Barboza JCS, de Oliveira PC, de Castro HF, Giordani DS. Non-edible babassu oil as a new source for energy production—a feasibility transesterification survey assisted by ultrasound. Ultrason Sonochem 2013;20:833–8.
[40] Du W, Li W, Sun T, Chen X, Liu D. Perspectives for biotechnological production of biodiesel and impacts. Appl Microbiol Biotechnol 2008;79:331–7.
[41] Adamczak M, Bornscheuer UT, Bednarski W. The application of biotechnological methods for the synthesis of biodiesel. Eur J Lipid Sci Technol 2009;111:800–13.
[42] Robles-Medina A, González-Moreno PA, Esteban-Cerdán L, Molina-Grima E. Biocatalysis: towards ever greener biodiesel production. Biotechnol Adv 2009;27:398–408.
[43] Lai J-Q, Hu Z-L, Wang P-W, Yang Z. Enzymatic production of microalgal biodiesel in ionic liquid [BMIm][PF6]. Fuel 2012;95:329–33.
[44] Chattopadhyay S, Karemore A, Das S, Deysarkar A, Sen R. Biocatalytic production of biodiesel from cottonseed oil: standardization of process parameters and comparison of fuel characteristics. Appl Energy 2011;88:1251–6.
[45] Ciftci ON, Temelli F. Enzymatic conversion of corn oil into biodiesel in a batch supercritical carbon dioxide reactor and kinetic modeling. J Supercrit Fluids 2013;75:172–80.
[46] Tang S, Jones CL, Zhao H. Glymes as new solvents for lipase activation and biodiesel preparation. Bioresour Technol 2013;129:667–71.
[47] Fukuda H, Kondo A, Noda H. Biodiesel fuel production by transesterification of oils. J Biosci Bioeng 2001;92:405–16.
[48] Liu Y, Li C, Meng X, Yan Y. Biodiesel synthesis directly catalyzed by the fermented solid of *Burkholderia cenocepacia* via solid state fermentation. Fuel Process Technol 2012;106:303–9.
[49] Salum TFC, Villeneuve P, Barea B, Yamamoto CI, Côcco LC, Mitchell DA, et al. Synthesis of biodiesel in column fixed-bed bioreactor using the fermented solid produced by *Burkholderia cepacia* LTEB11. Process Biochem 2010;45:1348–54.
[50] Liu Y, Li C, Meng X, Yan Y. Biodiesel synthesis directly catalyzed by the fermented solid of *Burkholderia cenocepacia* via solid state fermentation. Fuel Process Technol 2013;106:303–9.

[51] Matsumoto TM, Takahashi ST, Kaieda MK, Ueda MU, Tanaka AT, Fukuda HF, et al. Yeast whole-cell biocatalyst constructed by intracellular overproduction of *Rhizopus oryzae* lipase is applicable to biodiesel fuel. Appl Microbiol Biotechnol 2001;57:515–20.

[52] Adachi D, Koda R, Hama S, Yamada R, Nakashima K, Ogino C, et al. An integrative process model of enzymatic biodiesel production through ethanol fermentation of brown rice followed by lipase-catalyzed ethanolysis in a water-containing system. Enzyme Microb Technol 2013;52:118–22.

[53] Andrade GSS, Freitas L, Oliveira PC, de Castro HF. Screening, immobilization and utilization of whole cell biocatalysts to mediate the ethanolysis of babassu oil. J Mol Catal B: Enzymatic 2012;84:183–8.

[54] Gao B, Su E, Lin J, Jiang Z, Ma Y, Wei D. Development of recombinant *Escherichia coli* whole-cell biocatalyst expressing a novel alkaline lipase-coding gene from Proteus sp. for biodiesel production. J Biotechnol 2009;139:169–75.

[55] Jin Z, Han S-Y, Zhang L, Zheng S-P, Wang Y, Lin Y. Combined utilization of lipase-displaying *Pichia pastoris* whole-cell biocatalysts to improve biodiesel production in co-solvent media. Bioresour Technol 2013;130:102–9.

[56] Yoshida A, Hama S, Tamadani N, Noda H, Fukuda H, Kondo A. Continuous production of biodiesel using whole-cell biocatalysts: sequential conversion of an aqueous oil emulsion into anhydrous product. Biochem Eng J 2012;68:7–11.

[57] Adachi D, Hama S, Nakashima K, Bogaki T, Ogino C, Kondo A. Production of biodiesel from plant oil hydrolysates using an *Aspergillus oryzae* whole-cell biocatalyst highly expressing *Candida antarctica* lipase B. Bioresour Technol 2013;135:410–6.

[58] Adachi D, Hama S, Numata T, Nakashima K, Ogino C, Fukuda H, et al. Development of an *Aspergillus oryzae* whole-cell biocatalyst coexpressing triglyceride and partial glyceride lipases for biodiesel production. Bioresour Technol 2011;102:6723–9.

[59] Yan Y, Xu L, Dai M. A synergetic whole-cell biocatalyst for biodiesel production. RSC Adv 2012;2:6170–3.

[60] Costa L, Brissos V, Lemos F, Ramôa Ribeiro F, Cabral JMS. Enhancing the thermal stability of lipases through mutagenesis and immobilization on zeolites. Bioprocess Biosyst Eng 2009;32:53–61.

[61] Gonçalves APV, Lopes JM, Lemos F, Ramôa Ribeiro F, Prazeres DMF, Cabral JMS, et al. Zeolites as supports for enzymatic hydrolysis reactions. Comparative study of several zeolites. J Mol Catal B: Enzymatic 1996;1:53–60.

[62] Macario A, Moliner M, Corma A, Giordano G. Increasing stability and productivity of lipase enzyme by encapsulation in a porous organic–inorganic system. Microporous Mesoporous Mater 2009;118:334–40.

[63] Yagiz F, Kazan D, Akin AN. Biodiesel production from waste oils by using lipase immobilized on hydrotalcite and zeolites. Chem Eng J 2007;134:262–7.

[64] Macario A, Verri F, Diaz U, Corma A, Giordano G. Pure silica nanoparticles for liposome/lipase system encapsulation: application in biodiesel production. Catal Today 2013;204:148–55.

[65] Balcão VM, Paiva AL, Xavier Malcata F. Bioreactors with immobilized lipases: state of the art. Enzyme Microb Technol 1996;18:392–416.

[66] Rao NN, Lütz S, Würges K, Minör D. Continuous biocatalytic processes. Org Process Res Dev 2009;13:607–16.

[67] Séverac E, Galy O, Turon F, Monsan P, Marty A. Continuous lipase-catalyzed production of esters from crude high-oleic sunflower oil. Bioresour Technol 2011;102:4954–61.

[68] Hama S, Yamaji H, Fukumizu T, Numata T, Tamalampudi S, Kondo A, et al. Biodiesel-fuel production in a packed-bed reactor using lipase-producing *Rhizopus oryzae* cells immobilized within biomass support particles. Biochem Eng J 2007;34:273–8.

[69] Wang X, Liu X, Zhao C, Ding Y, Xu P. Biodiesel production in packed-bed reactors using lipase–nanoparticle biocomposite. Bioresour Technol 2011;102:6352–5.

[70] Hama S, Tamalampudi S, Yoshida A, Tamadani N, Kuratani N, Noda H, et al. Enzymatic packed-bed reactor integrated with glycerol-separating system for solvent-free production of biodiesel fuel. Biochem Eng J 2011;55:66–71.

[71] Xu Y, Nordblad M, Woodley JM. A two-stage enzymatic ethanol-based biodiesel production in a packed bed reactor. J Biotechnol 2012;162:407–14.

[72] Cesarini S, Diaz P, Nielsen PM. Exploring a new, soluble lipase for FAMEs production in water-containing systems using crude soybean oil as a feedstock. Process Biochem 2013;48:484–7.

[73] Abduh MY, van Ulden W, Kalpoe V, van de Bovenkamp HH, Manurung R, Heeres HJ. Biodiesel synthesis from *Jatropha curcas* L. oil and ethanol in a continuous centrifugal contactor separator. Eur J Lipid Sci Technol 2013;115:123–31.

[74] Chen Y-H, Huang Y-H, Lin R-H, Shang N-C. A continuous-flow biodiesel production process using a rotating packed bed. Bioresour Technol 2010;101:668–73.

[75] Harvey AP, Mackley MR, Seliger T. Process intensification of biodiesel production using a continuous oscillatory flow reactor. J Chem Technol Biotechnol 2003;78:338–41.

[76] Kiss AA, Bildea CS. A review of biodiesel production by integrated reactive separation technologies. J Chem Technol Biotechnol 2012;87:861–79.

[77] Noshadi I, Amin NAS, Parnas RS. Continuous production of biodiesel from waste cooking oil in a reactive distillation column catalyzed by solid heteropolyacid: optimization using response surface methodology (RSM). Fuel 2012;94:156–64.

[78] Kiss AA, Bildea CS. Integrated reactive absorption process for synthesis of fatty esters. Bioresour Technol 2011;102:490–8.

[79] Zakaria R, Harvey AP. Direct production of biodiesel from rapeseed by reactive extraction/in situ transesterification. Fuel Process Technol 2012;102:53–60.

[80] Shi W, He B, Cao Y, Li J, Yan F, Cui Z, et al. Continuous esterification to produce biodiesel by SPES/PES/NWF composite catalytic membrane in flow-through membrane reactor: experimental and kinetic studies. Bioresour Technol 2013;129:100–7.

[81] Malone MF, Huss RS, Doherty MF. Green chemical engineering aspects of reactive distillation. Environ Sci Technol 2003;37:5325–9.

[82] Cao P, Tremblay AY, Dubé MA, Morse K. Effect of membrane pore size on the performance of a membrane reactor for biodiesel production. Ind Eng Chem Res 2007;46:52–8.

[83] Atadashi IM, Aroua MK, Aziz AA. Biodiesel separation and purification: a review. Renew Energy 2011;36:437–43.

[84] He Y, Bagley DM, Leung KT, Liss SN, Liao B-Q. Recent advances in membrane technologies for biorefining and bioenergy production. Biotechnol Adv 2012;30:817–58.

[85] Atadashi IM, Aroua MK, Abdul Aziz AR, Sulaiman NMN. Membrane biodiesel production and refining technology: a critical review. Renew Sustain Energy Rev 2011;15:5051–62.

[86] Othman R, Mohammad AW, Ismail M, Salimon J. Application of polymeric solvent resistant nanofiltration membranes for biodiesel production. J Membr Sci 2010;348:287–97.

[87] Cheng L-H, Yen S-Y, Su L-S, Chen J. Study on membrane reactors for biodiesel production by phase behaviors of canola oil methanolysis in batch reactors. Bioresour Technol 2010;101:6663–8.

[88] Baroutian S, Aroua MK, Raman AAA, Sulaiman NMN. Methanol recovery during transesterification of palm oil in a TiO2/Al2O3 membrane reactor: experimental study and neural network modeling. Sep Purif Technol 2010;76:58–63.

[89] Falahati H, Tremblay AY. The effect of flux and residence time in the production of biodiesel from various feedstocks using a membrane reactor. Fuel 2012;91:126–33.

[90] Cheng L-H, Yen S-Y, Chen Z-S, Chen J. Modeling and simulation of biodiesel production using a membrane reactor integrated with a prereactor. Chem Eng Sci 2012;69:81–92.

[91] Rós PM, Castro H, Carvalho AF, Soares CF, Moraes F, Zanin G. Microwave-assisted enzymatic synthesis of beef tallow biodiesel. J Ind Microbiol Biotechnol 2012;39:529–36.

[92] Shi Y-G, Li J-R, Chu Y-H. Enzyme-catalyzed regioselective synthesis of sucrose-based esters. J Chem Technol Biotechnol 2011;86:1457–68.

[93] Yadav GD, Lathi PS. Intensification of enzymatic synthesis of propylene glycol monolaurate from 1,2-propanediol and lauric acid under microwave irradiation: kinetics of forward and reverse reactions. Enzyme Microb Technol 2006;38:814–20.

[94] Bashari M, Eibaid A, Wang J, Tian Y, Xu X, Jin Z. Influence of low ultrasound intensity on the degradation of dextran catalyzed by dextranase. Ultrason Sonochem 2013;20:155–61.

[95] Huang D, Jiang X, Zhu H, Fu X, Zhong K, Gao W. Improved synthesis of sucrose fatty acid monoesters under ultrasonic irradiation. Ultrason Sonochem 2010;17:352–5.

[96] Zheng M-M, Wang L, Huang F-H, Dong L, Guo P-M, Deng Q-C, et al. Ultrasonic pretreatment for lipase-catalyzed synthesis of phytosterol esters with different acyl donors. Ultrason Sonochem 2012;19:1015–20.

[97] Chakraborty S, Drioli E, Giorno L. Development of a two separate phase submerged biocatalytic membrane reactor for the production of fatty acids and glycerol from residual vegetable oil streams. Biomass Bioenergy 2012;46:574–83.

[98] Staniszewski M. Steady states of an enzymatic membrane reactor with product retention for a system of non-cooperating enzymes—model predictions. Desalination 2010;261:80–8.

[99] Nogueira BM, Carretoni C, Cruz R, Freitas S, Melo Jr PA, Costa-Félix R, et al. Microwave activation of enzymatic catalysts for biodiesel production. J Mol Catal B: Enzymatic 2010;67:117–21.

[100] Da Rós P, Freitas L, Perez V, de Castro H. Enzymatic synthesis of biodiesel from palm oil assisted by microwave irradiation. Bioprocess Biosyst Eng 2012:1–9.

[101] Yu D, Tian L, Wu H, Wang S, Wang Y, Ma D, et al. Ultrasonic irradiation with vibration for biodiesel production from soybean oil by Novozym 435. Process Biochem 2010;45:519–25.

[102] Batistella L, Lerin LA, Brugnerotto P, Danielli AJ, Trentin CM, Popiolski A, et al. Ultrasound-assisted lipase-catalyzed transesterification of soybean oil in organic solvent system. Ultrason Sonochem 2012;19:452–8.

[103] Tupufia SC, Jeon YJ, Marquis C, Adesina AA, Rogers PL. Enzymatic conversion of coconut oil for biodiesel production. Fuel Process Technol 2013;106:721–6.

[104] Gole VL, Gogate PR. Intensification of synthesis of biodiesel from non-edible oil using sequential combination of microwave and ultrasound. Fuel Process Technol 2013;106:62–9.

[105] Uragami T, Kishimoto J, Miyata T. Membrane reactor for acceleration of esterification using a special ionic liquid with reaction and separation and microwave heating. Catal Today 2012;193:57–63.

[106] Xu M, Wen X, Yu Z, Li Y, Huang X. A hybrid anaerobic membrane bioreactor coupled with online ultrasonic equipment for digestion of waste activated sludge. Bioresour Technol 2011;102:5617–25.

[107] Yu Z, Wen X, Xu M, Huang X. Characteristics of extracellular polymeric substances and bacterial communities in an anaerobic membrane bioreactor coupled with online ultrasound equipment. Bioresour Technol 2012;117:333–40.

[108] Badenes SM, Lemos F, Cabral JMS. Performance of a cutinase membrane reactor for the production of biodiesel in organic media. Biotechnol Bioeng 2011;108:1279–89.
[109] Ko M, Park H, Hong S, Yoo Y. Continuous biodiesel production using in situ glycerol separation by membrane bioreactor system. Bioprocess Biosyst Eng 2012;35:69–75.
[110] Patil P, Reddy H, Muppaneni T, Ponnusamy S, Sun Y, Dailey P, et al. Optimization of microwave-enhanced methanolysis of algal biomass to biodiesel under temperature controlled conditions. Bioresour Technol 2013;137:278–85.
[111] Li Y, Ye B, Shen J, Tian Z, Wang L, Zhu L, et al. Optimization of biodiesel production process from soybean oil using the sodium potassium tartrate doped zirconia catalyst under microwave chemical reactor. Bioresour Technol 2013;137:220–5.
[112] Jaliliannosrati H, Amin NAS, Talebian-Kiakalaieh A, Noshadi I. Microwave assisted biodiesel production from *Jatropha curcas* L. seed by two-step in situ process: optimization using response surface methodology. Bioresour Technol 2013;136:565–73.
[113] Cancela A, Maceiras R, Urrejola S, Sanchez A. Microwave-assisted transesterification of macroalgae. Energies 2012;5:862–71.
[114] Mahamuni NN, Adewuyi YG. Optimization of the synthesis of biodiesel via ultrasound-enhanced base-catalyzed transesterification of soybean oil using a multifrequency ultrasonic reactor. Energy Fuels 2009;23:2757–66.
[115] Scopus. ⟨http://www.scopus.com⟩; 02.13.13.