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Uma revisão entre tecnologias de células íntegras e lipases imobilizadas para produção de biodiesel e ésteres de sabor

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Resumo

As lipases são enzimas versáteis que catalisam diversas reações e podem ser aplicadas em várias áreas da indústria. No entanto, a biocatálise enfrenta um obstáculo econômico para poder competir com as rotas convencionais. Diferentes estratégias têm sido estudadas com o objetivo de reduzir o custo dos biocatalisadores e melhorar a atividade e estabilidade catalítica. A imobilização enzimática é uma das estratégias mais eficientes para tornar a aplicação de enzimas competitiva em larga escala industrial, proporcionando reutilização contínua, facilidade de separação do meio reacional e maior eficiência do processo. No entanto, apesar dos avanços tecnológicos alcançados, os biocatalisadores ainda são caros para usos industriais devido às etapas de recuperação, purificação e imobilização de enzimas. Nesse contexto, surge a tecnologia de células integras (CI) como uma forma de imobilização, na qual as próprias células, geralmente cultivadas em um suporte, são aplicadas no processo de biotransformação, contendo as proteínas de interesse aderidas à sua superfície, permitindo fácil separação, reutilização e dispensando a etapa de purificação. Embora a tecnologia de células inteiras tenha se tornado uma ferramenta valiosa para muitos processos de biotransformação, existem alguns inconvenientes inerentes, que impediram seu avanço em escala industrial, e muitos estudos foram realizados com o objetivo de otimizar seu desempenho. Este artigo apresenta uma revisão entre as tecnologias de células íntegras e de lipases imobilizadas, principalmente relacionadas às sínteses de biodiesel e de ésteres de sabor, uma vez que estas são amplamente relatadas na literatura.

Palavras-chave: Enzima. Lipases. Imobilização. Biocatalisadores de células íntegras. Síntese de biodiesel. Ésteres de sabor.

Abstract

A review between whole cells and immobilized lipases technologies for biodiesel and flavor esters production

Lipases are versatile enzymes that catalyze diverse reactions which allow their applications in various areas of the industry. However, biocatalysis faces an economical obstacle to be able to compete with conventional routes. Different strategies have been studied aiming to reduce the biocatalysts cost and to improve their catalytic activity and stability. Enzymatic immobilization is one of the most efficient strategies for making the enzyme application competitive on a large industrial scale, providing their continuous reuse, ease of

separation of the reactional medium and higher process efficiency. Nevertheless, despite the technological advances achieved, biocatalysts are still costly for industrial uses due to enzyme recovery, purification, and immobilization steps. In this context comes the whole cells (WC) technology as a form of immobilization in which the cells themselves, usually grown on a support, are applied in the biotransformation process, containing the proteins of interest adhered to their surface, allowing easy separation, reuse and dispensing the purification step. Although whole cells technology has become a valuable tool for many biotransformation processes, there are some inherent drawnbacks associated that hindered its advance in industrial scale, and many studies have been performed aiming to optimize their performance. This paper presents a review between whole cells and immobilized lipases technologies, mainly related to biodiesel and flavor esters synthesis, since these are largely reported in literature.

Keywords: Enzyme. Lipases. Immobilization. Whole cells biocatalysts. Biodiesel synthesis. Flavor esters.

Resumen

Una revisión entre las tecnologías de células enteras y lipasas inmovilizadas para la producción de biodiésel y ésteres de sabor

Las lipasas son enzimas versátiles que catalizan diversas reacciones que permiten su aplicación en diversas áreas de la industria. Sin embargo, la biocatálisis se enfrenta a un obstáculo económico para poder competir con las rutas convencionales. Se han estudiado diferentes estrategias con el objetivo de reducir el costo de los biocatalizadores y mejorar su actividad catalítica y estabilidad. La inmovilización enzimática es una de las estrategias más eficientes para hacer que la aplicación de enzimas sea competitiva en gran escala industrial, proporcionando su reutilización continua, facilidad de separación del medio de reacción y mayor eficiencia del proceso. Sin embargo, a pesar de los avances tecnológicos logrados, los biocatalizadores siguen siendo costosos para usos industriales debido a los pasos de recuperación, purificación e inmovilización de enzimas. En este contexto, surge la tecnología de células enteras (WC) como una forma de inmovilización en la que las propias células, generalmente cultivadas sobre un soporte, se aplican en el proceso de biotransformación, conteniendo las proteínas de interés adheridas a su superficie, permitiendo una fácil separación, reutilización y dispensar la etapa de purificación. Mismo que la tecnología de células enteras se ha convertido en una herramienta valiosa para muchos procesos de biotransformación, existen algunos inconvenientes inherentes asociados que afectaron su avance a escala industrial, y se han realizado muchos estudios con el objetivo de optimizar su desempeño. Este artículo presenta una revisión entre las tecnologías de células enteras y lipasas inmovilizadas, principalmente relacionadas con la síntesis de biodiesel y ésteres de sabor, ya que estos están ampliamente reportados en la literatura.

Palabras clave: enzima. Lipasas. Inmovilización. Biocatalizadores de células enteras. Síntesis de biodiesel. Ésteres aromatizantes.

Introduction

Enzymes are highly efficient biocatalysts and widely employed in biotechnological areas. Hydrolases are dominant in the enzyme market due to their low cost, availability, reaction conditions and specificity, representing 75% of the enzymes used on an industrial scale. Among hydrolases, proteases, lipases and amylases are the most impacting commercially (RIOS *et al.*, 2018). The global market value for industrial enzymes in 2019 was US$ 5,6 billion and lipases represent about 10%, corresponding to the third most traded group of enzymes, behind proteases and carbohydrates. Until 2027, the annual growth expected is 6.4%. (YU *et al.*, 2016; TACIN *et al.*, 2019; GRAND VIEW RESEARCH, 2020).

Lipases (EC 3.1.1.3) are enzymes belonging to the group of serine hydrolases, their most common substrate are long chain triglycerides (usually with more than 10 carbon atoms) and may act on the ester bond of various compounds. Their versatility provides applicability in several areas, being one of the most widely used in the industry, and often acting in a chemo- region and enantioselective form (BHARATHI *et al.,* 2019). The

highlight of this enzyme is due to its specificity, stability, recognition of several substrates and cofactor independence. Moreover, they can be employed at high substrate concentrations, being able to catalyze chemical reactions beyond the acyl group transfer, acting not only in their natural environment (aqueous solutions), but also in organic media and producing high yields with low levels of contamination (PENHA *et al.*, 2016; RIOS *et al.*, 2018; BHARATHI *et al.*, 2019; CAO *et al.*, 2020). The most relevant areas, in which lipases are used, include: food, pharmaceutical, fine chemistry, detergent formulation, cosmetic, textil and biodiesel production. Addionally, their applications may overtake the industrial area, including the deterioration of dairy products and oils, thus contributing environmentally to several sectors (PEIL *et al.*, 2016; PENHA *et al.*, 2016; BHARATHI *et al.*, 2019; CAO *et al.*, 2020; KUMAR *et al.*, 2019).

Among the major components of lipases are the oxyanion hole, the binding site, disulfide bonds and the lid, when present. Normally, these enzymes exhibit interfacial activation (reactions usually occur at the water-lipid interface) and, for this reason, it is observed that in many cases the reaction kinetics cannot be described by the Michaelis-Menten equations. The lid, in aqueous medium, by interacting with hydrophobic areas around the active site, changes its conformation and allows access to the site in hydrophobic surfaces due to the change in the dielectric environment in the surface region. In certain lipases, the lid cannot inactivate the enzyme because it is not large enough. The flexibility of the lid allows conformational changes to occur without affecting the enzymatic activity of lipase (BARRIUSO *et al.*, 2016; CORTEZ *et al.*, 2017; RIOS *et al.*, 2018). Open conformation (access to the active site released) is more stable than closed (access to the active site blocked), but this does not influence lipase specificity or selectivity. The oxyanion hole influences the efficiency of catalysis as it is a component that stabilizes the intermediate ion formed during the process. Disulfide bridges confer conformational stability to the enzymes. The binding site is where the substrate binds, usually hydrophobic, located near the surface, consisting of a serine, an aspartate or glutamate and a histidine (REIS *et al.*, 2009; RIOS *et al.*, 2018).

When interfacially activated, lipases adsorb on hydrophobic structures, usually their substrates. However, there may be some problems with this feature, lipase-lipase dimer formation may occur, bound to the hydrophobic parts of each enzyme, or lipases may adsorb to other hydrophobic substrate structures. Also, considering interfacial activation, the addition of an organic solvent provides better stability and mass transfer, since it decreased the system viscosity. Surface properties, such as hydrophobicity and charge distribution, are the main factors that confer stability to these enzymes in the presence of organic solvents (RODRIGUES *et al.*, 2019). Lipases are widely used in several processes involving triglycerides, being able to catalyze reactions such as esterification, transesterification, interesterification, acidolysis and alcohololysis. An interesterification consists of a combination of esterification products for a new esterification. Glycerolysis is an example of interesterification between triacylglycerol and glycerol (resulting from an esterification process). An acidolysis is the transfer of the acyl group between a triacylglycerol and a carboxylic acid. In alcoholysis, acyl group transfer occurs between a triacylglycerol and an alcohol. In transesterification there is the exchange of acyl groups between two esters (CORTEZ *et al.*, 2017; PRABANINGTYAS *et al.*, 2018; RIOS *et al.*, 2018).

Lipases may be produced by plant, animal, or microbial cells. Microorganism production is more advantageous from an industrial point of view due to the potential for large-scale application, specificity, easy handling, broad catalytic activity, regular supply, readily genetically engineered and greater stability than those obtained from animal and plant cells (PEIL *et al.*, 2016). Such aspects make the use of microbial lipases more economically viable than other sources (PEIL *et al.*, 2016). Considering the various possible origins, the molecular mass of these enzymes can vary from 20 to 75 kDa, the optimum working pH is in the range of 4 to 9 and the optimum working temperature between 25 and 70°C. Thus, it is observed that, due to the wide range of working conditions presented, the standardization of methodologies is considerably difficult for lipases (RIBEIRO *et al.*, 2011; RIOS *et al.*, 2018).

Fungi (yeasts and filamentous fungi) are the main sources of lipases for biodiesel production and other industrial applications. The potential of filamentous fungi for industrial application and lipase production is based on their ability to grow on simple and accessible substrates, sometimes under adverse conditions, and their ability to produce extracellular enzymes. In addition, the vast number of researches about these microorganisms allows the advance in the exploration of their potential in the production of lipases and other enzymes. Among the fungi that are sources of commercial lipases, it is possible to highlight the genera *Aspergillus sp.*, *Geotrichum sp.*, *Mucor sp.*, *Penicillium sp.*, *Rhizomucor sp.* and *Rhizopus sp.* (AGUIEIRAS *et al.*, 2015; CORTEZ *et al.*, 2017; SÁ *et al.*, 2017).

The production of these enzymes from microorganisms occurs mainly by submerged fermentation. Although solid-state fermentation has advantages such as benefit environmental sustainability, simplicity and low substrate cost, reduced water and energy demands, high productivity and produt concentration, some limitations make it difficult to scale up of this system. Examples of limitations include high media heterogeneity that reduces substrate accessibility, low efficiency in mass and heat transfer, and difficulty in controlling process parameters such pH, oxygen, humidity, temperature and cell growth (COLLA *et al.*, 2016; GEOFFRY; ACHUR, 2018; GODOY *et al.*, 2018; MANDARI *et al.*, 2019). On the other hand, characteristics of the submerged fermentation, such as better nutrient distribution in the reactor, more rigorous monitoring of process variables and possibility of automation, make this process more efficient and productive and make it currently dominant in industrial production. However, a disadvantage is related to high cost of installation and maintenance of the equipment used to submerged fermentation (COLLA *et al.*, 2016; FURINI *et al.*, 2018; GEOFFRY; ACHUR, 2018; GODOY *et al.*, 2018; MANDARI *et al.*, 2019).

Nevertheless, despite of the many advantages and widespread application perspective, the use of lipases in industrial processes encounters some obstacles, such as low productivity and high cost. The low biocatalyst productivity is related to low activity and low stability, responsible by long reaction times and low reusability, respectively (BARÃO *et al.*, 2014; AGUIEIRAS *et al.*, 2015). In addition, the steps associated to efficient recovery, purification and reusability of enzymes are among the most critical and challenging aspects, which render them excessively expensive for industrial exploitability (BILAL *et al.*, 2018).

To overcome the obstacles, improving performance and making the use of lipases economically viable, one of the most widely used strategies is enzymatic immobilization, considered in recent years the most promising methodology for making the enzyme application competitive on a large industrial scale (BILAL *et al.*, 2018). Immobilization is a generic term used to describe the retention and stabilization of a biomolecule within a reactor or analytical system. In the case of enzymes, immobilization consists in the confinement of the protein molecule in the pores or on the surface of a solid support insoluble in the mobile phase (which may be an aqueous or organic phase). The enzyme support system must maintain physical properties of the support and the biological activity of the enzyme. Thus, immobilization is considered one of the most efficient tools for altering the specificity, activity and stability of enzymes, providing their continuous reuse in reactor, ease of separation of the reactional medium and higher process efficiency (MATEO *et al.*, 2007; BARÃO *et al.*, 2014).

This review had as its starting point the establishment of the criteria to be used for the selection of references. First, the search descriptors were defined, namely: whole cells; whole cell lipases; Rhizopus oryzae lipase; immobilization; whole cell biodiesel; synthesis of whole cell biodiesel; synthesis of whole cell flavor esters. The second step was the search for references using the Science Direct platform, as it is a reliable repository and contains qualified publications. In addition, the Research Gate website was also used to select articles. Preference was given to studies published in highly qualified journals on the Sucupira platform. During the search, it was possible to find 210 publications, where the abstracts and results were analysed, observing if they met the search criteria. Thus, for the present study, 96 publications were selected because they are closer to the theme and have high scientific relevance. The discussions covered in this review are related to enzyme immobilization, immobilization of whole cells, and their uses in the production of biodiesel and flavor esters.

Immobilized lipases

The immobilization of lipases aims to improve catalytic properties of the enzymes, pH and thermal stability, solvent resistance, and its reuse (AGUIEIRAS *et al.*, 2015; ZDARTA *et al.*, 2018; ZAITSEV *et al.*, 2019). A determining factor in immobilization is the choice of support. Some supports can be too costly to use on an industrial scale or do not offer sufficient improvement in enzyme properties. Therefore, several studies have been conduced focusing on supports development, using more efficient and economically favorable alternatives. In recent years, innovative materials proposals have emerged with variable application characteristics. The supports can be of various types, flexible, rigid, porous or not, macroporous particles, nanoparticles, membranes, among others (MOHAMAD *et al.*, 2015; CIPOLATTI *et al.*, 2017; LIU *et al.*, 2018).

Hydrophobic supports are the mostly used for lipase immobilization, due to the mechanism of interfacial activation in the presence of a hydrophobic interface. However, enzymes physically adsorbed to the support

do not ensure high operational stability. The structure and properties of the support can be of great influence on the final performance of the enzyme (MATTE *et al.*, 2017; MANOEL *et al.*, 2015). The tendency of lipases interact with hydrophobic structures can cause some problems, such as dimer formation and other aggregates. In these cases, it is necessary to take some precautions during the immobilization process. Detergents may be used to break aggregates, but care must be taken to ensure that enzymes withstand the use of detergents and do not become inactive. Another problem that may occur is when porous supports are used. Interfacial activation is hampered due to limited access in these areas; and in these cases, lipases will be activated only if anhydrous environment is employed. The use of hydrophobic supports serves as a solution for both problems, as in this case external interfaces are not necessary; the lid moves in contact with the support itself, releasing access to the active lipase site (SANTOS *et al.*, 2014; MANOEL *et al.*, 2016; ZHANG *et al.*, 2017). As a general rule, the more hydrophobic the support, greater is the interaction with lipase and, therefore, higher is the immobilization process, although there are examples of lipases that do not have high affinity to highly hydrophobic supports (CARVALHO *et al.*, 2013; MANOEL *et al.*, 2016; MATTE *et al.*, 2017).

Hydrophobic supports allow the separation of lipases from other enzymes since the immobilization process is not unfavorable at even low ionic strength of the medium. Contrary behavior is generally observed for other enzymes. For high ionic strength, on the other hand, the lipase immobilization process happens at low rates, due to, in this condition, the active form (open form) is disadvantaged (MANOEL *et al.*, 2015).

Advantages of this type of support include simplicity in the immobilization protocol, ability to act over a wide pH range and high enzyme stability (TACIAS-PASCACIO *et al.*, 2016). The disadvantages are based on the possibility of desorption of the enzyme under certain conditions, which may lead to their inactivation. The addition of organic solvents is a cause of desorption on hydrophobic supports. Compounds with detergent properties also serve as agents desorbents. For example, fatty acids, di- or monoglycerides and phospholipids, which are common substrates and products of lipase catalyzed reactions, have detergent properties and in such cases the application of this type of support is limited. Several studies are being conducted to minimize desorption problems for hydrophobic supports (TACIAS-PASCACIO *et al.*, 2016; VIRGEN-ORTÍZ *et al.*, 2017a; RODRIGUES *et al.*, 2019). A few examples of hydrophobic supports reported for lipase immobilization include Mesoporous poly-methacrylate particles (BASSI *et al.*, 2016), Magnetic poly (styrene divinylbenzene) particles functionalized with oleic acid (BENTO *et al.*, 2017), Polyvinyl alcohol microspheres (BERGAMASCO *et al.*, 2013) and Hydroxyapatite (DIMITRIJEVIĆ *et al.*, 2012).

Another method of immobilization is intermolecular crosslinking. This method is mainly applied to make enzyme desorption difficult. Glutaraldehyde was the first crosslinking reagent employed; but this low molecular weight reagent can only work in a massive enzyme intermolecular crosslinking if the immobilized enzyme molecules are close to each other (RODRIGUES *et al.*, 2019). However, such condition may change their stability properties. A possible solution to this problem would be to apply a high molecular mass crosslinker reagent, such as dextran aldehyde. For this agent, there are studies proving efficient irreversible immobilization (RODRIGUES *et al.*, 2019; LIU *et al.*, 2018).

Poly-ionic polymers, such as polyethylenimine or dextran sulfate, are alternatives to physical crosslinking. The results with the use of these agents are favorable, providing adequate stability and low desorption rate. However, even more satisfactory results are found when glutaraldehyde is added to the preparation, since under such conditions the enzyme is coated with poly-ionic polymers, providing improvements in enzymatic activity, stability and decreased desorption rate (VIRGEN-ORTÍZ *et al.*, 2017b; RODRIGUES *et al.*, 2019).

Heterofunctional supports are another alternative to overcome the lipase desorption problem. For the application of this type of support, the first immobilization is performed by interfacial activation, by direct contact with the support, and then other groups can be added, establishing new interactions with the biocatalysts and, thus, reducing the desorption rate (BARBOSA *et al.*, 2013; MELO *et al.*, 2017). Examples of this type of support are, with glutaraldehyde groups, octyl triethoxysilane, glycidoxypropyl trimethoxysilane and, with glyoxyl acyl groups octyl glyoxyl agarose. The results reported in the literature for these supports show stable and decreased desorption enzymes (CIPOLATTI *et al.*, 2017; MELO *et al.*, 2017; RODRIGUES *et al.*, 2019).

One of the more recent methodologies applied in the development of supports are miniemulsions. They can be defined as aqueous oil drop dispersions between 50-500 nm in diameter, prepared in a system containing oil, water, surfactant and a co-surfactant agent (CRESPY; LANDFESTER, 2010). However, these droplets are instable when saline buffers are employed at constant pH, and the product isolation is a complex step

(BIROLLI *et al.*, 2020). Although miniemulsions are not commonly used, this alternative may be promising and has advantages such as: simplicity in handling, non-excessive use of surfactants, colloidal stability, incorporation of hydrophobic compounds and relatively low cost. Miniemulsion polymerization is a tecnhnique that can be used to synthesize polymer nanoparticles, which can be employed as supports for enzyme immobilization (CRESPY; LANDFESTER, 2010; CIPOLATTI *et al.*, 2017). The method of miniemulsions is widely applied for the production of nanoparticles, whose preparation consists of three steps: heterogeneous two-phase preemulsion for the preparation of macroemulsions, homogenization for the formation of miniemulsions and, finally, the reaction that forms the nanoparticles, made possible by the miniemulsions. Nanoparticle formation usually occurs in a high-pressure homogenizer or with ultrasound. Polymeric nanoparticles can be used to encapsulate the material to be immobilized. Hydrophobic nanoparticles are formed in aqueous solution, while hydrophobic nanoparticles are formed in hydrophilic solvents. The most used hydrophobic solvents are cyclohexane, hexane, toluene and hexadecane (CRESPY; LANDFESTER, 2010; VALÉRIO *et al.*, 2015; QI *et al.*, 2014; CIPOLATTI *et al.*, 2017).

Another relevant methodology is the core-shell, which consists of a particle surrounded by a porous matrix (shell). Although few studies report the use of this method, the results presented are quite favorable. The core is commonly formed by polymeric particles nucleated by the suspension of the polymerization process and the shell by polymeric particles nucleated by the emulsion of the same process and coagulated over much larger suspension particles (MANOEL *et al.*, 2016; CIPOLATTI *et al.*, 2017).

A trend of recent years has been the co-immobilization, in which lipases are often employed. Several researches have shown interest in this method, because when using co-immobilization, the product released by one enzyme serves as a substrate for another enzyme, which is known as cascade reactions. Cascade reactions can involve many enzymes acting consecutively, in more or less complex mechanisms. The employment of this method has advantages, as the initial reaction rate can be accelerated, reducing or even eliminating the lag time produced when enzymes immobilized in different supports or free enzymes are used (CIPOLATTI *et al.*, 2017; RIOS *et al.*, 2019). In Table 1, different methods of lipase immobilization and their respective substrates are listed.

Table 1 - Lipase immobilization methods.

Methodology	Supports/ Immobilization agentes
Activation interfacial	Hidrophobic supports
Crosslinking	Glutaraldehyde, dextran aldehyde
Poli-ionic polymers	Polyethylenimine or dextran sulfate
Heterofunctional supports	Epoxy acyl with glutaraldehyde (octyl triethoxysilane, glycidoxypropyl trimethoxysilane) and the glyoxyl acyl groups (ocar-glyoxyl agarose) with glutaraldehyde
Miniemulsions	Magnetic nanoparticles
Core-shell	Core: Magnetic nanoparticles, polimetilmetacrilate, Shell: Polistirene; polidopamine, sílica, chitosan

Whole cells *vs* immobilized lipases

Biodiesel synthesis

Most reports applying whole cells as biocatalyst are related to biodiesel production. Some drawbacks of conventional catalysis industrially employed in the synthesis of biodiesel involves the production of large amount of alcaline wastewater, difficulty in catalyst recovery, high purity and high cost raw materials demands (VIEIRA *et al.*, 2018).

The requirement of oils with high purity makes the production of biodiesel by the alcaline transesterification reaction relatively expensive, representing about 70 to 80% of the overall process cost. The content of free fatty acids (FFA) greater than 0.5% and water above 0.25% in vegetable oils influence quanti- and qualitatively on biodiesel processing. The two-step chemical catalysis, that employs prior esterification to lower the concentration of FFA and subsequent alcaline transesterification, allows the use of cheaper raw materials

(VIEIRA *et al.*, 2018). Nevertheless, this esterification/transesterification process also presents some drawbacks such as the need for complete water removal from the medium, high energy consumption, poor selectivity, and low reaction rates. So, biocatalysis emerges as an alternative proposal to circumvent such problems, providing less complexity in downstream processes and the use of cheaper raw materials with high content of water and free fatty acids (AGUIEIRAS *et al.*, 2015). Lipases are powerful tools that catalyze hidrolysis of esters producing free fatty acids, di and monoglycerides and glycerol as sub-product.

However, the biocatalysts are still costly for industrial uses due to enzyme recovery, purification and immobilization steps. In addition, in biodiesel synthesis, productivity is still low because the biocatalyst stability and reusability are low and the reaction rates are lower than in the conventional route. Other drawbacks are mass transfer limitation and the inhibitory effect of alcohol to the enzyme activity (AGUIEIRAS *et al.*, 2015; BUŠIĆ *et al.*, 2018; THANGARAJ *et al.*, 2019).

Whole cells (WC) are a form of immobilization in which the cells themselves, usually grown on a support, are applied in the biotransformation process, containing the proteins of interest adhered to their surface, thus making use of extracellular enzymes. The great advantage of this method is its simplicity, being the enzyme immobilized, allowing easy separation, reuse and dispensing the purification step, generally conferring low total cost of the process. They are known as the cheapest form of catalyst formulation. As the enzymes are in their natural environment, cofactors are already supplied and cell wall components act in a protective way which confers great stability and enables its application in non-aqueous media or under harsh reaction conditions (LAM, 2009; GULDHE *et al.*, 2016; WACHTMEISTER; ROTHER, 2016; YU *et al.*, 2016; CIPOLATTI *et al.*, 2017).

Although whole cell technology has become a valuable tool for many biotransformation processes, there are some inherent drawnbacks associated that hinder its advance in industrial scale. Whole cells show lower selectivity compared to purified enzymes, which may unable its application for certain purposes due to the possibility of resulting undesired side products. Another disadvantage is that biochemical system of a microorganism is more complex than an isolated enzyme and both substrate and product can exert inhibition or toxicity to the microorganism resulting in a low productivity. In addition, there may be metabolites not related to the process that need to be removed in the downstream processes, but this problem can be circumvented by selective knockout of genes of host cells and overexpression techniques (LAM, 2009; WACHTMEISTER; ROTHER, 2016; LIN *et al.*, 2017).

Another well known disadvantage is the low mass transfer, which can be considered the most highlighted imposed by cell membrane. It may lead to low product concentration, making the downstream processes more complex and increasing the total manufacturing cost in the case of industrial applications. Alternatives to improve mass transference, by increasing the permeabilization of the celular wall and membrane, are procedures such as the addition of detergents, chelating agents or organic solvents and thermic shock. However, such procedures may damage the cells and also make the overall process more complex and costly, from the point of view of manufacturing and downstream processes (LAM, 2009; CARVALHO, 2011; WACHTMEISTER; ROTHER, 2016). It is known that permeability of the cell membrane is determined by the fatty acid composition and thus, by adding fatty acids in the culture media, it is possible to increase cell permeability (CARVALHO; CARAMUJO, 2018). This strategy was studied by Hama *et al.* (2004), where *R. oryzae* cell membranes were enriched mainly different fatty acids, wich the oleic and linoleic acids showed higher activity. The authors noted that cells with increased permeabilization lead to higher initial transesterification yields, and the more rigid ones lead to higher stability. The ratio of oleic and oleic + palmitic acids was optimized in order to obtain high stability and activity, with ratio of 0.67.

Purified lipases immobilized often have greater efficiency in the biotransformation process than whole cells, but the elimination of purification step confers higher cost relevance (GULDHE *et al.*, 2016; YU *et al.*, 2016; CIPOLATTI *et al.*, 2017). Tamalampudi *et al.* (2008) compared whole cells of *R. oryzae* lipase (ROL) immobilized onto biomass support particles (BSP) and free purified lipase (Novozym 435) for biodiesel production from Jatropha oil. In this case, the whole cell biocatalyst (WCB) showed higher lipolytic activity and conversion rate (maximum of 80.2%) than the free purified enzyme (maximum of 75.1%). Surprisingly, time to reach this maximum conversion rate was considerably lower for WCB (60 h) than for Novozym 435 (90 h). It was also observed that the commercial enzyme was more efficient in anhydrous conditions, which means that there is a step necessary to remove the water. For this specific research, all the parameters favored the WCB,

that is unexpected, based in other reports from the literature, in which WCBs show lower catalytic activities, yields and reaction time in comparison to purified enzymes (BALASUBRAMANIAM *et al.*, 2012). Catalytic activities, productivity and reaction time are some of the disadvantages of the WCBs in comparison to isolated enzymes that the scientific community has been trying to optimize (GOG *et al.*, 2012; AGUIEIRAS *et al.*, 2015).

On the other hand, the enzymes in WCB are more stable than purified enzymes. Such trend is because enzymes are found in their natural environment within cell. Thus, an advantage of the WCBs is that they have been more stable enzymatic sources; so, they can be reutilized more times. Tamalampudi *et al.* (2008) verified that both biocatalysts showed adequate residual activity, even after five cycles, suggesting that WCB is a promising biocatalyst for producing biodiesel.

In another similar study, Balasubramaniam *et al.* (2012) compared the lipolytic activity of WCB and purified lipase from *R. oryzae*, both immobilized on calcium alginate spheres, in the conversion of the sunflower waste cooking oil to biodiesel. In this study, the purified lipase showed higher conversion rates than the whole cell. The maximum yields were of 93% for the former and 84% for the latter, which was explained by inability of the acyl acceptor and oil to reach the enzyme and bind with the active sites as effectively as in purified enzyme. However, the yields obtained were comparable in terms of cost effectiveness (BALASUBRAMANIAM *et al.*, 2012).

Majewska *et al.* (2016) also performed one of the few studies that directly compare the efficiency of whole cell lipases and commercial lipases. They tested five different lipases and whole cells of eight different microorganisms for the hydrolysis of 2-butyryloxy-2-(ethoxyphenylphosphinyl) acetic acid and the whole cells presented the best conversion degrees, enantiomeric excess and enantioselectivity.

The optimization of the cultivation conditions, as pH, temperature, cultivation time, nature and concentration of the lipid inducers, is extremely important because they directly affect the ratio of the enzyme excreted to the medium and the enzyme immobilized on the surface of the mycelium, and consequently the catalytic activity of the enzyme in the whole-cell. The addition of lipid inducers may or not have relevance in lipase production, depending on the conditions and the microorganism used (RAJENDRAN *et al.*, 2009; COSTA *et al.*, 2017).

For whole cells of *R. oryzae* lipases, additional lipid sources such as fatty acids, triglycerides and oils confer higher yields, more stable and active enzymes than simple carbon sources. According to Hama *et al.* (2006), lipid inducers confer higher lipase retention in the cell membrane and, therefore, higher lipase activities are expected. The fatty acids present in the chosen inducer are responsible for the different effects obtained, being the enzyme production strongly affected by the amount of oleic acid. Olive oil, which contains 70% oleic acid in its composition, generally promotes the largest lipolytic activities for whole cells (CORTEZ *et al.*, 2017). Athalye *et al.* (2013) analyzed the effect of culture medium composition and cultive time on biodiesel production by *R. oryzae* WCB immobilized on polyethylene cubes. The highest lipolytic activity found, expressed by unity per biomass support particles (BSP), was 30.1 ± 4.21 U/BSP, with 1% Cottonseed oil and 1% glucose for 72 h of cultive time.

The WCB, as well as purified enzymes, can be employed in free or immobilized state. For cell immobilization, the methods widely used in enzyme immobilization technologies, such as adsorption, covalent binding, crosslinking, and encapsulation, are also used. However, specific criteria should be considered for the proper selection of support and immobilization method due to cell morphologycal characteristics. Studies have been shown that immobilization on a support provides better stability and higher usability for the system (CORTEZ *et al.*, 2017). Guldhe *et al.* (2016) compared the lipolytic activities of whole cells of *Aspergillus sp*. and *Candida sp.*, in a free state and immobilized in polystyrene cubes, on biodiesel production. The lipolytic activities, using free and immobilized *Aspergillus sp.* cells, were 177.5 U/g and 137.5 U/g, respectively, and for *Candida* sp. 132.5 U/g and 115.39 U/g, respectively. Although the highest catalytic activities presented were for free cells, the best properties and the highest biodiesel yield were obtained using cells immobilized within biomass support particles. Rakchai *et al.* (2016), working with *Aspergillus nomius*, obtained a maximum lipolytic activity of 0.65 U/mg for free suspension cells, under the ideal conditions established in 48 h of cultive. On the other hand, for whole cells of *Aspergillus nomius* immobilized in polyurethane foam cubes, an activity around 30% higher (0.87 U/mg) was reached.

Several supports can be used to cell immobilization such as cryogell beads obtained from polyvinyl alcohol (PVA) and chitosan beads added with olivinylpyrrolidone and polysphosphates, polyhydroxybutyrate (PHB) beads and diatomaceous earth (celite), vegetable bushing, calcium alginate and polytetrafluoroethylene (PTFE) membranes, being polyurethane foams the most used for the immobilization of filamentous fungi whole cell. The wide use of this material is due to its excellent properties such as high porosity, large surface area, low cost,

excellent mechanical properties and chemical stability (CORTEZ *et al.*, 2017). *R. oryzae* immobilized on polyurethane foams cubes is one of the most studied systems for whole cells biocatalysts (CORTEZ *et al.*, 2017). This tendency can be attributed to catalytic efficiency of the lipase of this filamentous fungus along with the simplicity immobilization methodology. However, some drawbacks inherent to the cell immobilization process exist and some researches have been carried out aiming to promote the efficiency catalytic and productivity increase (BAN *et al.*, 2001; HAMA *et al.*, 2006; HAMA *et al.*, 2007; WANG, *et al.*, 2010; ATHALYE *et al.*, 2013; ARUMUGAM; PONNUSAMI, 2014).

Concomitantly, in the last years, the use of magnetic nanoparticles (MNPs) as supports for catalysts has become theme of intense investigations. Magnetic nanoparticles constitute a new type of material used as carrier of immobilized lipases and can be obtained mainly by co-precipitation and hydrothermal methods, sonochemical technique, micro-emulsion, and flame spray synthesis route (ABU-DIEF; ABDEL-FATAH, 2018). Among the several MNPs, such as iron oxide-based (γ-Fe_2O_3 and Fe_3O_4), pure metal-based (Fe and Co), spinel-type ferromagnetic-based ($MgFe_2O_4$, $MnFe_2O_4$ and $CoFe_2O_4$) and alloy-based ($CoPt_3$ and FePt), MNPs, Fe_3O_4 NPs are the most used to enzymes immobilization, due to its unique properties involving low toxicity, larger surface area, stronger surface adsorption, better suspension property, supermagnetism, easy separation and recicle possibility and biodegradability (LIU *et al.*, 2018; MIAO *et al.*, 2018). Among its applications can be included photocatalytic degradation, dehydrogenation reactions, oxidation, alkylation, and C-C coupling (ABU-DIEF; ABDEL-FATAH, 2018). In literature, some studies with MNPs as the carrier of immobilization of lipases in biodiesel synthesis are found. It is shown that MNPs are excellent supports with potential environmentally friendly biocatalyst for biodiesel production (MIAO *et al.*, 2018; SEKOAI *et al.*, 2019).

Probably the most notorious properties presented by MNPs is that they can be dispersed in reactional medium, offering a large surface area, and the MNP supported catalysts can be easily recovered at the end of the reaction process by applying external magnetic field. In addition, the catalyst can be recycled several runs. Other characteristcs listed are better stability, activity and reusability of the enzymes (ABU-DIEF; ABDEL-FATAH, 2018; MIAO *et al.*, 2018). However, NPMs often exhibit high reactivity and are easily degraded, causing low stability of nanoparticles. Therefore, a surface functionalization is generally carried out through the presence of hydroxyl or amino groups, which still provides strong binding in the enzymes (ABU-DIEF; ABDEL-FATAH, 2018; BILAL *et al.*, 2018; LIU *et al.*, 2018; SEKOAI *et al.*, 2019). This type of support has shown to be promising and satisfactory results have been obtained by its use so far.

Chen *et al*. (2016) innovated when produced, for the first time, magnetic whole cell biocatalysts (MWCBs) by immobilizing *Pseudomonas mendocina* into Fe_3O_4-chitosan microspheres and applied for enzymatic biodiesel production. The results were compared with non immobilized whole cells. They encountered a highest enzyme activity of 2810 U/g after 8 h of cultivation, which is higher comparing to 710 U/g from *Aspergillus oryzae* immobilized on Celite (SILVA *et al.*, 2008), 1814 U/g from *Thermomyces lanuginosus* immobilized in tetraethoxysilane (BARÃO *et al.*, 2014), and 1785 U/g from recombinant *Pichia pastoris* applied as a whole cell biocatalyst (JIN *et al.*, 2013). The maximum yield obtained was 87.32% in 48 h and, after ten cycles, its yield was of 83.57% while for the Fe_3O_4-uncontained biocatalyst it was 74.06%. It can be inferred that immobilized whole cell lipases are more stable and thus they can be used more times in comparison to the non immobilized biocatalysts. Not many researches are found in the literature employing cells immobilized into magnetic particles. The idea in magnetizing the whole cells is to improve the mass transport during the reaction and facilitate the separation of the biocatalyst, reducing the reaction time and the operational complexity. Therefore, MWCBs can become a promising alternative for the industrial synthesis of various products, environmentally friendly and economically viable (ARSALAN; YOUNUS, 2018). Miao *et al.* (2018) investigated the use of lipase immobilized in amino-funcionalized Fe_3O_4 nanoparticles in the biodiesel synthesis. It was obtained 89,4% of maximal conversion, under ideal working conditions, and the conversion rate was maintained at 70% after five cycles, demonstrating the potential use of the biocatalyst. Karimi (2016) used silica coated NPMs for the synthesis of biodiesel from waste cooking oils obtaining 91% oil to biodiesel conversion and after five cycles its conversion was 54%, evidenciating again that the MWCBs can be reused efficiently, which is much desirable for industrial application especially in the economical point of view.

Short-chain esters synthesis

Short-chain esters constitute a group of highly relevant flavor and aroma compounds in the industry, with applications in food, beverages, cosmetics, and medicines, with an estimated market value exceeding US$ 22 billion per year. About food additives, flavor compounds represent 25% of the world market. These compounds can be used by natural or chemical sources, the first group being highly costly due to reduced concentrations in the materials to be extracted and low levels of use. Chemical sources, in turn, have greater economic viability. However, they use strong acids as catalysts (sulfuric acid and p-toluene sulfonic acid) and sometimes high temperatures and pressures, being considered harmful to the environment. In addition, chemical synthesis suffers from a lack of selectivity, generating unwanted products from parallel reactions, reducing efficiency, and raising the total cost of production. To overcome these disadvantages, different enzymes are reported as alternatives, such as oxidoreductases, lipoxygenases, hydroperoxide liases, alcohololoxidases and lipases. Lipases exhibit good catalytic potential and are the most popular enzymes to produce flavor esters by esterification and transesterification (BRAULT *et al.*, 2014; SOUZA *et al.*, 2017; OLIVEIRA *et al.*, 2019; VASILESCU *et al.*, 2019).

Most reports applying whole cell lipases are related to biodiesel production, thus not many data are available regarding other applications such as short-chain ester synthesis, and the most recent ones employ recombinant lipases which has been a trend in biocatalysis. Among the lipases used to the production of flavor esters and biodiesel, *Candida antarctica* lipase B (CALB) is one of the most researched, presenting some of the bests (if not the best) results in flavor esther synthesis. CALB has good specifity toward substrates, stereospecificity, can be applied under mild conditions in diferent reactions and has adequate stability and resistance to pH and temperature (SOUZA *et al.*, 2017; KUNDYS *et al.*, 2018). Like many other enzymes, this one is also limited for large scale application due to high cost of immobilized enzyme. Thus, whole cells would be a good alternative.

A method applied to improve the catalytic activity of these biocatalysts is the connection of a carrier protein to the surface of yeast cells, where this protein will make possible for exogenous lipases to be attached to the cell wall (JIN *et al.*, 2013; ZHANG *et al.*, 2015). *Pichia pastoris* is a methylotrophic yeast largely utilized, an expression host, for the production of industrial enzymes and biopharmaceuticals due to its great characteristics as growth to high cell densities, availability of strong and tightly regulated promoters and high recombinant protein production intra or extracellularly. In comparison to *Sacharomyces cerevisiae*, which is also another enzyme producer widely used as an expression host, *P. pastoris* can be fermented with cheaper carbon sources and grows to higher cell densities (AHMAD *et al.*, 2014; JIN *et al.*, 2013; PEÑA *et al.*, 2018). Su *et al.* (2010) studied the synthesis of flavor ester and developed two cell-surface display sistems in *P. pastoris*. CALB was displayed functionally on the cell surface of the *P. pastoris* using two archor proteins and it was obtained catalytic activities tenfold higher than that obtained with *S. cerevisae* (HAN *et al.*, 2009).

In flavor ester synthesis, heptane, cyclohexane and hexane are the solvents most utilized due to its highest conversion rates and low toxicity toward lipases. These non-polar solvents have shown better results, since polar solvents may interfere and distort the water layer around the enzyme (SÁ *et al.*, 2017; SOUZA *et al.*, 2017; VASILESCU *et al.*, 2019). High substrate loading is a characteristic much desired for the synthesis of flavor esters and some researches have been reported to optimize this parameter. Many studies report high conversion yields but low substrate loading, which would be a problem for applications in industrial scale (JIN *et al.*, 2012; YAN *et al.*, 2014). Jin *et al.* (2012) used whole cell CALB displayed in *P. pastoris* to produce flavor esters in a batch reactor apllying the carrier protein method with the agglutinin anchor (GS115/pKNS-CALB). Their optimal temperature for the reaction was 50 °C, which is considerably higher compared to non-recombinant lipases. The higher the temperature, the faster the reaction tends to be. The conversion yields and stabilty of the biocatalyst in their study was so great that at 70°C it was possible to obtain conversions higher than 90% after only 3 h. The reaction time provided by the biocatalysts in that study was remarkable, much lower than other researches (to the best of our knowledge, no other research reported better results in relation to time reaction). Another parameter that the study of Jin *et al.* (2012) excels is the high substrate loading capacity, which was of 0.6 M of acetic acid, being one of the highests ever reported for short chain fatty acids. Jin *et al.* (2012) also compared the efficiency of the whole cell with the comercial Novozyme 435 and even though the recombinant lipase had a synthetic activity of approximately 3 times lower than the comercial lipase, after only 4 h of reaction the whole cell biocatalyst converted 97% of the acetic acid while the Novozyme 435 was

only able to convert 63%. As it is desired of a good biocatalyst, reuse assays also showed good characteristics, with the relative activity remaining higher than 90% after 10 uses. Considering these characteristics, it can be concluded that whole cell CALB displayed in *P. pastoris* presents great potential for industrial application, and the study performed by Jin *et al.* (2012) is one with the best results in flavor ester synthesis.

In a more recent study (SOUZA *et al.*, 2017) that used CALB, this time immobilized in aminofunctionalized magnetic nanoparticles, optimal substrate load obtained was 0.4 M with ethanol and 0.5 M with methanol, with a conversion of butyric acid >97% and >93%, respectively in 8 h and heptane as solvent. This biocatalyst also retained more than 80% of residual activity after 10 cycles. Guillén *et al.* (2012) were able to convert >99% butyric acid in ethy butyrate with lipases immobilized on polymeric resin, but the reaction time was 3 times longer (24 h) than it was used for de Souza *et al.* (2017) and the reuse of residual activity was only 60% after just 6 cycles.

Something relevant to compare in these studies is the temperature, considering that the higher the temperature, the faster the reaction tends to be. Depending on the substrate employed, the ideal temperature may change due to the creation of protection effects on the termal denaturation of the enzymes. As for biodiesel production, in short chain ester synthesis it is also essential the presence of water for optimal lipolytic activity and enzyme flexibility (BRAULT *et al.*, 2014; BROGAN *et al.*, 2014). In systems with organic media, it is expected to have hydrolysis and organic synthesis occurring simultaneously according to the increase of water content, so the lower the water content the less hydrolysis will compete with the esterification (CHANDA; FOKIN, 2009; BRAULT *et al.*, 2014).

A possible explanation for the lower yields with water content increase is the insolubility of the fatty acids in hydrophilic phases. As most lipases are optimal at low water contents, procedures such as application of water adsorbing materials, prevaporation, gas stripping and continuous distillation can be employed (LONGO; SANROMÁN, 2006). In fixed volume vessels, cell loading is limited, thus, by using a fluidized bed reactor (FBR), this problem can be circumvented (BRAULT *et al.*, 2014). Brault *et al.* (2014) synthesized short-chain flavor ester by a heterologous biocatalyst, which was whole cell *E. coli* expressing LipIAF5-2. Like the other studies involving heterologous lipase expression, it aims to reach overexpression and, consequently, higher catalytic activities and product yields. LipIAF5-2 had its greatest activity at 40 °C, when glyceryl tributyrate was used as a substrate. They utilized membrane permeation techniques instead of cell immobilization as an alternative to circumvent mass transfer limitation of whol-cells. That was observed, in the study by Brault *et al.* (2014), that the optimum water content was 10%, however, synthetic activity of most lipases is optimal, typically, at water content below 1% (w/w) in organic systems; this may be disadvantageous considering that hydrolysis will be more active in comparison to most other lipases, but also interesting considering that it won´t be necessary *in situ* water removal during the reaction, which is applied for most researches in flavor ester biosynthesis. In this regard, the LipIAF5-2 can be interesting as such procedures won´t be necessary. Unlike other lipases, this presented highest activities for high acyl aceptor molar ratios, with the maximum being 10:1 (methanol: glyceyl tributyrate), charachterizing adequate alcohol inhibition resistance of mLipIAF5-2, a parameter very relevant in this study, considering that stepwise addition, which is observed in so many researches (specially for biodiesel production) to obtain higher yields by circumventing lipase alcoholic inhibition, can be avoided. For LipIAF5-2 methanol was the best acyl aceptor and it also had good substrate toleration (up to 1 M). The highest conversion yield obtained for glyceryl triacetate (transesterification) was (97.2 ± 3.3)% after 24 h at 35 °C. However, this lipase did not have good reusability conditions (55.360.3% after 5 cycles).

Among the few reports in literature employing non recombinant whole cell biocatalysts for the biosynthesis of flavor ester is the work perfomed by Xu *et al.* (2002), who utilized *R. chinesis* in non-aqueous phase. They were able to convert 96.5% of hexanoic acid at 30 °C with a substrate load of 0.6 M, the same as for Jin *et al.* (2012), but in 72 h. They were also able to convert more than 90% of acetic, butyric, valeric, heptanoic and octanoic acid for the same conditions, which shows good potential for ester synthesis.

Yan *et al.* (2014) also used non recombinant whole cell in the synthesis of short chain flavor esters. They studied the capacity of *A. oryzae* as a whole cell lipase non-immobilized using ciclohexane as solvent. The highest affinity of this enzyme was for the substrates hexanoic, heptanoic and octanoic acids. The heptanoic acid had the highest yield. The most interessant parameter in their study is that they tested high substrate loadings, which is very relevant production in industrial scale and most researches fail to have good results in these conditions, from 0.8 M to 3.0 M, and all of them were able to convert more than 80% in 48 h (up to 2 M), 72 h (2.5 M) and 120 h (3 M). These concentrations are much higher than the ones mentioned in the other studies.

Non-recombinant whole cell lipases are little explored in this area, heterologous whole cell biocatalysts have been more studied in the last years due to its higher catalytic activities and lower reaction times. Many reports also use magnetic nanoparticles, which is another trend very researched by the scientific community. Both these methods have shown great conversion rates for low reaction times.

Conclusions

The high enzyme cost, the longer reaction time and lipase inhibition by ethanol/methanol are the main obstacles for large-scale production and commercialization of enzymatic biodiesel. Whilst time of reaction, substrate load and conversion rate are among the parameters most in need for optimization in short chain esters synsthesis. Thus, the enzyme technology will only be able to compete with conventional chemical routes if economically attractive processes are further developed. Immobilized enzymes and whole cells biocatalysts have advantages and disavantages and, depending on the conditions employed, they may be economically comparable. Currently, the immobilized enzymes market is much more developed and thus they are much more commonly used. Besides, until now, MNPs and CALB have been the most successful methods in both applications embraced in this study. The integration of biotechnology with cost-effective industrial processes includes the production of cheaper and more stable lipases, the development of new catalysis technologies, and the exploration of cheaper potential feedstocks such as non-edible oils and fats as well as algae- and microorganism-based oils. The findings of various studies will provide specific information and the applications of enzyme technology will contribute to make enzymatic synthesis of biodiesel and flavor esters a sustainable perspective in the near future.

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