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ENZYME BIOCATALIZERS AS A SUSTAINABLE ALTERNATIVE IN GREEN BIODIESEL PRODUCTION

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Abstract: Biodiesel has provided an ecologically correct solution, being considered a renewable, biodegradable and non-polluting fuel. It can be easily produced through the transesterification of vegetable oils and animal fats, being used industrially due to its high rates of conversion and reaction through chemical catalysts. However, conventional chemical processes for the production of biodiesel consume a high amount of water and energy, and generate undesirable by-products, such as soaps, which make it difficult to separate and purify biodiesel. Thus, the enzymatic production of biodiesel appears as a potential alternative, presenting several advantages over conventional methods, in addition to generating a product of high purity and high yields, obtained at low reaction temperatures, in addition to bringing an easy recovery of glycerol. However, the biggest obstacles for the enzymatic production of biodiesel continue to be the cost of lipases, their relatively slower reaction rate when compared to chemical catalysts, and the inhibition of enzymes, caused by acyl acceptors and by glycerol itself, a by-product of the reaction. Therefore, there is a great interest in the creation of methods that aim to develop competitive biocatalysts for industrial applications, on a large scale, through the improvement of their catalytic properties such as: activity, stability and recyclability. Thus, obtaining a greener production of biodiesel, through a more efficient, clean and sustainable process.
Keywords: Biotransformation. lipase. Sustainability. enzymatic transesterification.

INTRODUCTION

The transition from fossil fuels to renewable energy systems has become indispensable for sustainable environmental and economic growth. Biodiesel has gained worldwide popularity as a renewable liquid fuel due to its clean combustion and renewability (CORACH; SORICHETTI; ROMANO, 2016). In addition to being biodegradable and non-toxic, biodiesel is also essentially free of sulfur and aromatics, producing lower and more oxygenated exhaust emissions than conventional petroleum-derived diesel, with similar properties in terms of fuel efficiency. Biodiesel is produced from natural oils and fats, and can be used as a substitute for petroleum-derived diesel without any modification to the existing diesel engine (MOFIJUR et al., 2013). It is commonly obtained from a chemical reaction called transesterification, by mixing vegetable oils or animal fats with a short-chain alcohol in the presence of a catalyst, with glycerol as a by-product of the reaction. It can also be obtained by the esterification process (NAGAO; KITO, 1990).

The use of biodiesel as a fuel has shown promising potential, due to its significant contribution to the environment, with the qualitative and quantitative reduction of levels of environmental pollution, and its use as a strategic source of renewable energy to replace diesel oil and other derivatives of Petroleum. However, the commercialization of biodiesel still presents some obstacles such as the price of the raw material and the operational costs. Such impasses can be overcome if the process of recovery and use of by-products (glycerin and catalyst) is optimized, leading, therefore, to a production of biodiesel at a competitive cost, in comparison with the commercial price of diesel oil (BOSLEY, 1997).

Through enzymatic technology, high quality products can be obtained in a clean way, meeting technological, market and environmental preservation needs. The enzymatic process can be applied to specific modifications of the chemical structure, carried out by biochemical catalysts, using enzymes contained in cells or isolated. The choice of biocatalyst occurs among living organisms, of animal, plant or microbial

origin. Biocatalysts are proteins that have a high catalytic power, have a high degree of specificity for their substrates, accelerate specific chemical reactions and, in some cases, can work both in aqueous solutions and in organic solvents. Thus, the application of enzymes as biocatalysts in industrial processes has gradually increased. The enzymes of greatest commercial interest are microbial enzymes, due to the short development period, the wide variety of metabolic processes and the infinity of microorganisms present in nature that can be tested, in addition to modifying and degrading complex organic molecules (SHAW et al, nineteen ninety).

Thus, this article aims to analyze enzymatic biofuels, which emerge as a potential alternative technology, more sustainable and efficient, with low environmental impacts, as well as economic benefits.

METHODOLOGICAL PROCEDURES

The article is a systematic review, classified as a structured literature review, with the objective of performing a critical analysis on the topic addressed, through a synthesis with the best evidence (ZUMSTEG; COOPER; NOON, 2012). The chosen database was Scopus, considered an important database of abstracts and citations of the literature with peer review. As a criterion for the search for articles related to the proposed theme, words such as: *enzimatic biodiesel* and *lipase-catalalysed transesterification*, to find topic related jobs. All search terms could occur in the title, abstract or keywords and only works in article format were selected. At this stage, 83 works were found. After reading the title and abstract of each article, and critical analysis of alignment with the objectives proposed by the present work, 30 articles were selected to compose this review.

THEORETICAL-INFORMATIVE FRAMEWORK

Biodiesel is defined as a mixture of monoalkyl esters of long-chain fatty acids, derived from renewable sources of lipids, such as vegetable oil or animal fat, that can be used in compression-ignition engines with little or no modification (Demirbas, 2002).

The most usual method to transform oil into biodiesel is transesterification, this reaction can occur in the presence of homogeneous or heterogeneous catalysts. Homogeneous characters have single-phase characters (single phase), and heterogeneous ones play a biphasic role (two or more phases) at the end of the reaction. Catalysts used for biodiesel production can be grouped into four categories: alkaline catalysts; acids; heterogeneous enzymatic and inorganic compounds (XU et al., 2018).

Homogeneous catalysis, although widely used industrially, has been losing ground to heterogeneous catalysis, which has great advantages such as: the possibility of reusing the catalyst, cleaner products, avoiding saponification routes and increasing yields. Enzyme catalysts are types of heterogeneous catalysts, an innovation whose main mechanism is the route performed by enzymes. Of these catalysts, lipases stand out, enzymes responsible for producing this catalysis in transesterification. Chemically, the transesterification reaction can be catalyzed by acid or alkali, which has many disadvantages, such as high energy consumption and the difficulty of transesterifying triglycerides with high free fatty acid content (MARCHETTI; MIGUEL; ERRAZU, 2007).

In addition, downstream processes such as glycerol recovery, removal of inorganic salts and product water, catalyst removal and alkaline effluent treatment are complex and incur additional costs. On the other

hand, the enzyme-catalyzed reaction using lipases provides a solution to the problems mentioned above, as it is more efficient, highly selective, involves less energy consumption and produces less waste. The recovery of glycerol is easier in the enzymatic process, in addition to the production of a high-grade glycerol, compared to the alkaline chemical process (BAJAJ et al., 2010).

RAW MATERIAL

The main raw materials used in the production of biodiesel are vegetable oils extracted from oilseeds. The cost of these materials represents about 70% of the total production costs. This means that the most suitable vegetable oils are those from crops with higher productivity per hectare, such as palm, canola, sunflower, soybean and microalgae species (BEHZADI; FARID, 2007).

Today, the high prices of petroleum-based fuels, the collapse of food-to-biodiesel initiatives, and concerns about rising levels of CO2 emissions into the atmosphere have created awareness of the need for alternative fuel solutions. In this context, microalgae emerged with optimism as one of the lowest cost raw materials for the production of biodiesel. However, the cost of producing high-grade algal oils is, and likely will be, an obstacle in the short term. Fats and oils can be characterized according to their physical properties, such as density, viscosity, melting point, refractive index or chemical properties, such as acidity, iodine index, peroxide index and saponification index, these parameters will influence the quality of the product. biodiesel. Currently, among the various vegetable oils available, those with a high content of oleic acid are the most indicated, due to the greater stability of their alkyl esters and their better characteristics as fuels (KNOTHE, 2005).

CONVENTIONAL PRODUCTION METHODS

The direct use of vegetable oils as biodiesel is only possible by mixing them with conventional diesel oil in an adequate proportion. The direct use of vegetable oils in diesel engines is technically not possible due to their high viscosity, low stability against oxidation (and subsequent polymerization reactions) and low volatility, which influences the formation of a relatively high amount of ash, due to incomplete combustion. Therefore, vegetable oils must be processed in order to acquire the necessary properties to be used directly in current diesel engines. Possible processes are pyrolysis (or cracking), microemulsion and transesterification. As the first two are cost-intensive processes, generating low quality biodiesel, the most common method to transform oil into biodiesel is transesterification. Transesterification consists of the reaction between triacylglycerols (contained in oils) and an acyl acceptor. The acyl group acceptors can be carboxylic acids (acidolysis), alcohols (alcolysis), or another ester (interesterification). Only alcoholysis and interesterification are of interest for the production of biodiesel. The starting esters in both are triacylglycerols (oils), and if the transformation is quantitative they produce a mixture of monoalkyl esters (biodiesel) and glycerol (alcolysis) or another triacylglycerol (interesterification) (MARCHETTI; MIGUEL; ERRAZU, 2007).

Alcohols are the most frequently used acyl acceptors, particularly methanol and, to a lesser extent, ethanol. Other alcohols can be used, eg propanol, butanol, isopropanol, branched alcohols and octanol, but the cost is much higher. As for the choice between methanol and ethanol, the former is cheaper, more reactive and fatty acid methyl esters are more volatile than fatty acid ethyl esters.

However, ethanol is less toxic and can be considered more renewable because it can be easily produced from renewable sources by fermentation. In contrast, methanol is mainly produced from non-renewable fossil sources such as natural gas.

The catalysts used in the transesterification reaction can be classified as: (1) alkaline catalyst (sodium hydroxide, NaOH; potassium hydroxide, KOH; sodium methoxide, NaOMe); (2) acid catalyst (sulfuric acid, phosphoric acid, hydrochloric acid); (3) enzyme catalyst (lipases); (4) inorganic heterogeneous catalyst (solid phase catalyst). The best known and most used process is the one that uses basic catalysts. However, if the initial vegetable oil contains a small amount of fatty acids (> 0.5%), as with the use of waste oils (frying oils), soaps form, which leads to a reduction in the yield and, above all, an increase in downstream processing of the produced biodiesel (ROBLES-MEDINA et al., 2009).

In addition, the need to treat the generated alkaline effluents and the high consumption of water during washing in the purification stages make the process that uses chemical catalysts not so ecologically correct. Vegetable oils with a high free fatty acid content are best esterified with the acid catalyst, no soap is formed, but a higher temperature and a higher substrate molar ratio is required (up to 30:1). However, acid catalysts are rarely used on an industrial scale because they are more corrosive and result in a slower reaction rate and lower yield than when (basic) hydroxide catalyst is used. Acid catalysts can be used together with basic catalysts (two-stage process). This two-step process allows for the use of low-cost raw materials, such as the residual oil with a high content of free fatty acids. Acid catalysts are also the best method when using oils extracted from microalgae biomass (ROBLES-MEDINA et al., 2009).

DISCUSSIONS

The enzymatic transesterification technology seems to be starting to be applied on an industrial scale. Recently, it has been claimed that this technology has been applied to industrialization with a capacity of 20,000 ton/year in China, which is the first industrial scale with lipase as a catalyst in the world until today. There are two main categories of enzyme biocatalysts: (1) extracellular lipases (i.e. the enzyme was previously recovered from the broth of life-producing microorganisms and then purified), the main producing microorganisms are: *Mucor miehei*, *Rhizopus oryzae*, *Candida antarctica* and *Pseudomonas cepacia*; (2) intracellular lipase (which still remains inside or on the producing walls of cells), in both cases, the enzyme is immobilized and used, which eliminates the subsequent operations of separation and recycling of the lipase (DU et al., 2008).

In general, lipases work under milder conditions (which implies less energy consumption), with oils from different sources, including residual oils with high acidity. This is because lipases catalyze the transesterification of both triacylglycerols and free fatty acids to produce monoalkyl esters. If the lipase is immobilized, it can be easily separated from the reaction mixture by filtration, or when the lipase is in a fixed bed, no separation is needed after transesterification and, in fact, the subsequent separation and purification of the biodiesel is easier. than with alkaline catalysts, in which the alkaline component needs to be eliminated, using large amounts of water and consequently being a less ecological process. Furthermore, lipases can be produced from genetically transformed microorganisms. For example, it is possible to improve the selectivity of lipases for some fatty acids to influence the composition of the alkyl ester produced or to increase its robustness against certain alcohols (AKOH et al., 2007). More recently,

a thermostable, short-chain alcohol-tolerant lipase suitable for biodiesel production has been cloned (KATO et al., 2007).

On the other hand, the main technical disadvantages of the enzymatic process are its slower reaction rates than the alkaline catalyst and, for example, the risk of enzyme inactivation due to methanol, and also the risk of glycerol inhibiting lipase. when covering it, due to its accumulation in the reaction mixture (these inhibition aspects will be mentioned later). Finally, the higher cost of enzymes could be greatly reduced if enzymatic processes were implemented industrially. In this sense, in 2005, Novozymes A/S, the main European enzyme producer, announced that the price of enzymes used in the production of bioethanol had dropped more than 30 times over the course of its creation. Similar reductions can also be predicted for lipases (VASUDEVAN; BRIGGS, 2008).

LIPASES

Lipases are enzymes (biocatalysts) that transport hydrolysis of triacylglycerol (TG) to glycerol and fatty acid, therefore, they are categorized in the class of hydrolases. These enzymes are present everywhere and, based on their origin, are classified as: plant, animal or microbial lipases. Vegetable lipases are not used commercially, while animal and microbial lipases are used extensively. The sources of animal lipases are the pancreas of cattle, sheep, pigs and pigs (SELLAPPAN; AKOH, 2005). Microbial lipases have gained great industrial importance and now share about 5% of the world enzyme market, after proteases and carbohydrases. The most commonly used biocatalyst for the production of biodiesel are microbial lipases, which are produced by a number of fungal, bacterial and yeast species. A large number of microbial strains have been used for lipase production, however, the most frequently reported enzyme sources are: *Candida* sp., *Pseudomonas* sp. and *Rhizopus* sp. (TREICHEL et al., 2010).

Lipase immobilization on solid supports is often used as a strategy to improve the catalytic properties of these enzymes. The immobilization process consists of physically attaching the enzymes to a solid support, so that the substrate passes over the enzymatic support and can be converted into the product. Immobilization has been increasingly used in industrial applications to facilitate the separation of biocatalysts from the process stream and therefore the recovery and purification of products (Spahn and Minteer, 2008). Immobilized biocatalysts have enzymes dispersed on the surface of the support, which increases the number of active sites available, due to the prevention of the formation of aggregates in an organic medium. Other advantages include greater thermal stability and solvent resistance, due to the greater structural rigidity of the enzyme obtained when several enzyme support bonds are formed (GARCIA-GALAN et al., 2010).

Furthermore, immobilized lipases are easy to recover by relatively simple operations such as filtration or centrifugation, making it possible to reuse them or carry out continuous processes while, at the same time, a high purity glycerol by-product can be easily recovered without the need for any process. complex separation. Currently, the most widely used immobilized fungal lipases for biodiesel production are Novozym 435, Lipozyme RM IM and Lipozyme TL IM (HERNANDEZ-MARTIN; OTERO, 2008).

MAIN FACTORS OF ENZYME INHIBITION

Ethanol and methanol have a stronger denaturing action compared to longer aliphatic alcohols. It is well known that if methanol is in a relatively high amount relative to oil, it can inhibit and deactivate a large proportion

of lipase. This effect has also been reported for ethanol, although to a lesser extent. In general, different lipases have different requirements regarding the amount of alcohol used for optimal biocatalysis (NELSON; FOGLIA; MARMER, 1996).

Glycerol, a by-product produced in the reaction, is also one of the main problems related to enzymatic transesterification reactions, causing the inactivation of the biocatalyst. Glycerol is hydrophilic and insoluble in oil, and can be easily adsorbed onto the surface of the immobilized enzyme, leading to a decrease in lipase activity and operational stability. This problem mainly occurs in Compact Bed Reactors (PBR), in which the accumulation of glycerol can also promote column clogging. Another hypothesis to explain the deleterious effects of glycerol is that it reduces the amount of water available for lipase activity. As the reaction proceeds, methanol is mixed with the free glycerol produced in the reaction and forms a second liquid phase, which is not completely miscible with the oil (DOSSAT; COMBES; MARTY, 1999).

INDUSTRIAL PRODUCTION AND ECONOMIC ASPECTS

Economic evaluation is the main driving force of process technology development, and it can be used to predict process plant cost, product manufacturing cost, compare product cost produced through different processes and conditions. The increase in global use of biodiesel has resulted in the current increase in raw material prices and also in the devaluation of the glycerol by-product. Therefore, biodiesel companies must adapt properly and remain economically viable.

An enzymatic biodiesel production plant can present economic advantages over the current chemical process plant, among them (YOU et al., 2008):

§ Acceptance of low quality and cheaper oils than the refined oils used in the traditional process (today a typical biodiesel plant spends about 80% of the production cost with the acquisition of oil);

§ Production of a cleaner glycerol (without contamination of the catalyst), which can be sold for a higher price;

§ Simpler and cheaper steps for pre-treatment of raw materials and post-reaction catalyst separation.

On the other hand, the high cost of enzymes in relation to the chemical catalyst is still the biggest disadvantage of this route, along with its longer reaction time. In this sense, much effort has been made to reduce the production costs of these biocatalysts and maximize their reuse. Although the economic analyzes show that currently the enzymatic processes for the production of biodiesel still have higher costs when compared to the conventional route, the increase in the useful life of the enzymes as well as the environmental factor related to the use of enzymatic technology indicate that biocatalysts represent a a truly attractive alternative for the production of biodiesel on an industrial scale (AGUIEIRAS; CAVALCANTI-OLIVEIRA; FREIRE, 2015).

To reduce operating costs, enzymatic biodiesel must be produced in continuously operating plants. Possible solutions tested on a laboratory scale could be PBRs, fluid beds, expansion bed, recirculation or membrane reactors. PBRs are very applicable for continuous production of biodiesel, but the main disadvantage is that the resulting glycerol remains at the bottom of the reactor and can deposit on the surface of the immobilized lipase, decreasing the catalytic efficiency. Recently, new methods for producing biodiesel at reduced costs have been reported in the literature. One of the methods employs the use of a whole cell biocatalyst obtained by

solid state fermentation (SSF). The advantages associated with using an SSF-generated biocatalyst are twofold: (1) microorganisms grow on low-cost substrates (agro-industrial waste) while maintaining a low moisture content; (2) The raw fermented solid material can be used directly as a biocatalyst, thus omitting three processing steps: lipase extraction, purification and immobilization. This leads to a significant reduction in lipase costs which translates into lower biodiesel production costs (KUMAR; KANWAR, 2012).

Protein engineering and targeted evolution can also improve lipase specificity, thermostability, activity and methanol tolerance (KOURIST; BRUNDIEK; BORNSCHEUER, 2010). A recent example of this effort is the high-level expression of a thermostable, methanol-tolerant lipase from Proteussp. in E. coli. The recombinant strain was able to produce biodiesel in the presence of high concentrations of water. Alternatively, recombinant E. coli was used as a whole cell biocatalyst. Directed evolution was subsequently employed to increase its thermostability and methanol tolerance, while retaining wild-type lipase activity at room temperature. One of the most recent developments of thermostable and methanol-tolerant lipases reports a newly constructed mutant of the lipase from P. mirabillis, known as Dieselzyme 4, which compared to the wild-type enzyme, has higher thermostability and better tolerance to methanol (KORMAN et al., 2013).

The existing challenges in the commercialization of enzymatic biodiesel refer, therefore, to the regression in the use of existing conventional processes, and mainly, to the scaling up of technologies based on whole cell enzyme catalysts. Further advances in the development of innovative and inexpensive enzyme immobilization techniques with low reaction time, greater stability and enzyme activity must be developed for the global economic acceptance of large-scale enzyme biodiesel production.

FINAL CONSIDERATIONS

Due to the high conversion rates and, mainly, its lower operating cost, the process of producing biodiesel with chemical catalysts is currently the dominant method on a large scale from virgin oils. However, they have several disadvantages inherent to their process, including difficulties in glycerol separation and purification, catalyst recovery and recycling, saponification problems and the need for consecutive washings to remove impurities from biodiesel. This makes the process more energy-intensive and generates an excessive amount of wastewater.

Therefore, enzymatic catalysis comes as an alternative, offering a series of environmental and economic advantages over the chemical method, such as: reaction conditions at room temperature, enzyme reuse, high substrate specificity, lower alcohol to oil ratio, prevention of side reactions, minimization of impurities, easier product separation and recovery, biodegradability and environmental acceptability. Its disadvantages, when compared to conventional chemical catalysis, can be overcome with the use of recombinant DNA technologies, enzymatic improvement through protein engineering and other evolution methods, and consequently, increasing lipase stability, substrate specificity and catalytic efficiency, which will facilitate overall process cost reduction.

This way, greater exploration and integration of these technologies would promote large-scale industrial production, making the process economically viable, in addition to the sustainable production of biodiesel through a green process.

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