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Research review paper

Has the time finally come for green oleochemicals and biodiesel production using large-scale enzyme technologies? Current status and new developments

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ABSTRACT

With the growth of the chemical industry over the last decade, the need for cheaper (and more environmentally friendly) alternatives to petrochemicals of ever-increasing cost has grown steadily. Oleochemicals and biodiesel (OC/BD) are considered as green alternatives to petroleum derivatives, because they come from renewable oils and fats. OC/BD are currently produced by the traditional energy intensive chemical catalyzed methods, which have several economic and environmental drawbacks. For these reasons, the enzymatic production of OC/BD has attracted a growing attention for their greener pathway with respect to the chemically catalyzed processes. Lipase-catalyzed processes have a low energy requirement, since reactions are performed under atmospheric pressure and mild temperature and without the creation of side reactions. Furthermore, utilization of enzyme catalysts offers many advantages such as reducing the initial capital investment due to simplified downstream processing steps. Despite all the previous advantages, however, the high cost of lipases restricted their large-scale utilization. In the past decade, efforts have been made to reduce the cost of the enzymatic-catalyzed synthesis of OC/BD. However, most previous studies have studied only the technical feasibility of the lipase-catalyzed reactions and overlocked the economic viability. This review critically discusses the factors affecting the promotion of the economic feasibility of the enzymatic processes from the lab to large scale. These include reactor configuration, type of feedstock, conditions optimization, immobilization, lipase-producing microorganisms, and

Abbreviations: OC/BD, Oleochemicals/Biodiesel; ENZ, Enzymatic Process; CHEM, Chemical Process; FFA, Free Fatty Acids; TAG, Triacylglycerides; FAMEs, fatty acid methyl esters.

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substrate diversification. In addition, this review reports the recent advances in lipase-catalyzed production of fatty acids, fatty esters, monoglycerides, and biodiesel in the lab as well as in the large-scales. To the best of authors' knowledge, this is the first review article reports the recent global progress achieved in both lab- and large-scale for the enzymatic production of OC/BD.

1. Introduction

Oleochemicals and biodiesel (OC/BD) are biodegradable chemicals that are derived from fats and oils (Abdelmoez and Mustafa, 2014; Hasan et al., 2023; Sreeharsha et al., 2023; Su et al., 2022), that are considered as carbon neutral alternatives to many petrochemicals. They are produced by well-established chemical technologies that have been commercialized worldwide (Abdelmoez et al., 2013; Bashir et al., 2022; Khan et al., 2022). However, such methods consume a considerable amount of energy and pose a threat to the environment for the use of hazardous chemical catalysts. In addition, they produce many untargeted byproducts due to the lack of reaction selectivity (Hosney and Mustafa, 2020). Recently, commercial utilization of enzymes technology has grabbed much attention due to its cleaner and sustainable nature. Enzyme technology offers many advantages when they are used as biocatalyst replacing the chemical catalysts such as lowering the reaction temperature, and producing clean products without side reactions (Mustafa et al., 2016). In addition, the investment in the plant-based enzyme technology is much cheaper than that of using chemical technologies because of the lower processes steps required for the enzymatic process (Hosney et al., 2020a). Fig. 1 shows a comparison between enzymatic- and chemical-catalyzed processes in terms of number of processes steps and temperatures used for producing glycerin esters (Mustafa et al., 2016).

However, the use of enzymatic method in commercial production is constrained by high costs of enzymes. (Hosney et al., 2020b). In the last ten years, significant advancements in laboratory, pilot, and industrial settings have been made to lower the price of enzyme synthesis and utilization. The enzymatic process is now economically competitive as compared to the conventional method due to the significant reduction in production costs that have occurred under specified configurations and conditions (Aprile et al., 2023; Mustafa, 2021; Zhong et al., 2019).

This review reports the advances achieved in enzymatic OC/BD commercialization and related research, including: i) the reduction of enzyme production cost, ii) the optimization of enzyme performance in a given process, iii) the use of low-quality raw materials and iv) the evaluation of different reactor designs. It highlights current advancements in lipase research and development as one of the key elements in creating a method that is cost-competitive for OC/BD. It also offers an update on the present difficulties and chances for OC/BD commercialization from a techno-economic standpoint. Additionally, relevant enzymatic OC/BD producers, markets, difficulties, and commercialization potential are critically explored. Environmental factors are also taken into account. It is worth noting that most previous reviews reported only the enzymatic advances of OC/BD based on a lab scale without discussing the recently successful implemented large-scale enzymatic plants, globally. To the authors' knowledge, this is the first review that reports the progress regarding the currently available large-scale enzymatic-based plants for OC/BD production. Such discussion bridges the gap between the lab and commercial scales and provides a benchmark regarding how challenges associated with enzymatic process implementation can be converted into opportunities.

2. Advances in oleochemicals enzymatic production

Production of oleochemicals such fatty acids, fatty esters, and monoglycerides using enzymes have recently received much attention. The advantages of enzymatic catalyzed process (ENZ) over the chemical process (CHEM) have been highlighted and more scaleup studies are urgently needed. Fig. 2 shows a review about ENZ and CHEM processes in terms of process advantages and selectivity.

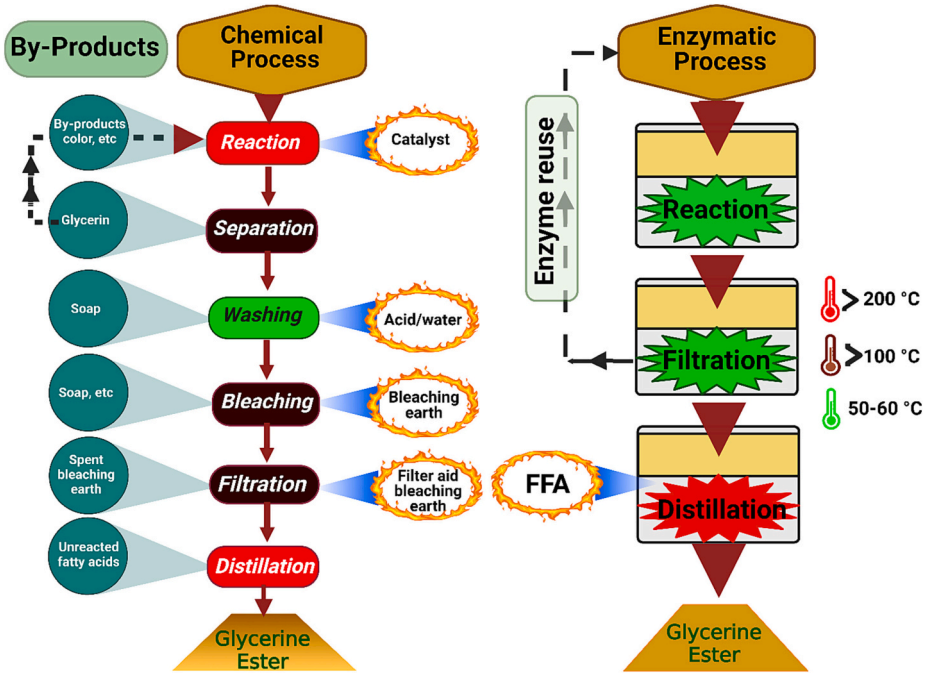

Fig. 1. Comparison between processes steps required for producing glycerin laurate using the enzymatic and chemical methods. FFA refers to free fatty acids.

2.1. Free fatty acids

Free fatty acids (FFAs) are the main substance of a many products such as fatty alcohols, soaps, cosmetics, food and pharmaceuticals. The conventional commercial method for the synthesis of FFAs is based on the hydrolysis of animal fats and vegetable oils at high pressures (50–60 bar) and temperatures (250–330 °C) (Abdelmoez et al., 2013; Baena et al., 2022; Satyarthi et al., 2011). Triglycerides can be splitted with steam/water to produce 1 mol of glycerol and as 3 mol of FFAs as shown in Fig. 2. However, such process requires a remarkable high investment associated to the use of large reactor resistant to the reaction conditions adopted and to its high energy requirement. In addition, due to these extreme conditions, polymerization of oils and fats take place resulting in a dark and colored final products with a reduction of reaction yield (Mustafa et al., 2023b). Therefore, additional purification steps are required to purify the glycerol and fatty acids such as distillation, escalating the economy of the total manufacturing process. Both hydrolysis and subsequent distillation generates considerable emissions that affect negatively to the environment (Haider Ali et al., 2015). For fragile unsaturated oils such as castor oil, the conventional fat hydrolysis process yields high order of by-products that alternative processes are required (Puthli et al., 2006).

For these reasons, the enzymatic hydrolysis of oils and fats is gaining much attention as a more sustainable technology. Novozymes A/S R&D efforts have resulted in an optimized process that achieved a castor oil to free fatty conversion of 98% a laboratory set-up (Holm et al., 2018). For the same process, using soybean oil as a starting feedstock a splitting conversion of 98% was obtained when a two-step countercurrent process was developed. The typical process parameters were adopted as follows: temperature of 40–65 °C, reaction time of 48–92 h, water to oil molar ratio of 1:1 and the mineral acidity was neutralized by adding from 5 to 100 ppm of NaOH (Holm et al., 2018). Enzymatic hydrolysis is often more time-consuming than chemical reactions, which reduces the overall productivity when the process is compared to the conventional methods. To reach the same productivity, larger reactor volumes are needed in case of enzymatic process. However, many purification steps are carried out in the chemical process, which consumes time and cost. Therefore, the time and cost required for extra reaction vessels in case of enzymatic hydrolysis are clearly outweighed by the yield gains and improved product quality. It should be remembered that enzyme reactors are more straightforward than those used for chemical processes (Mustafa et al., 2023a).

Oleon also studied the oils and fats splitting process using enzymatic technology in a project funded by the EU Horizon 2020 program, namely, LIPES project (Life Integrated Process for Enzymatic Splitting of triglycerides) (Rusli et al., 2020). LIPES is granted to develop an alternative green route to the existing traditional splitting method of triglycerides to fatty acids and glycerin. They have achieved a result that the proposed enzymatic approach would results in a more resource-efficient process, saving at least 80% energy and 45% water compared to current methods. Moreover, the suggested enzymatic system has the capability to generate specific fatty acids with industrial potential at a reduced overall variable expense compared to current methods, while also offering their utility as intermediate substances for a wider range of applications. Lipase catalyzed lipid splitting is still at the research stage, with early industrial trials concentrating on soft oils like castor oil where

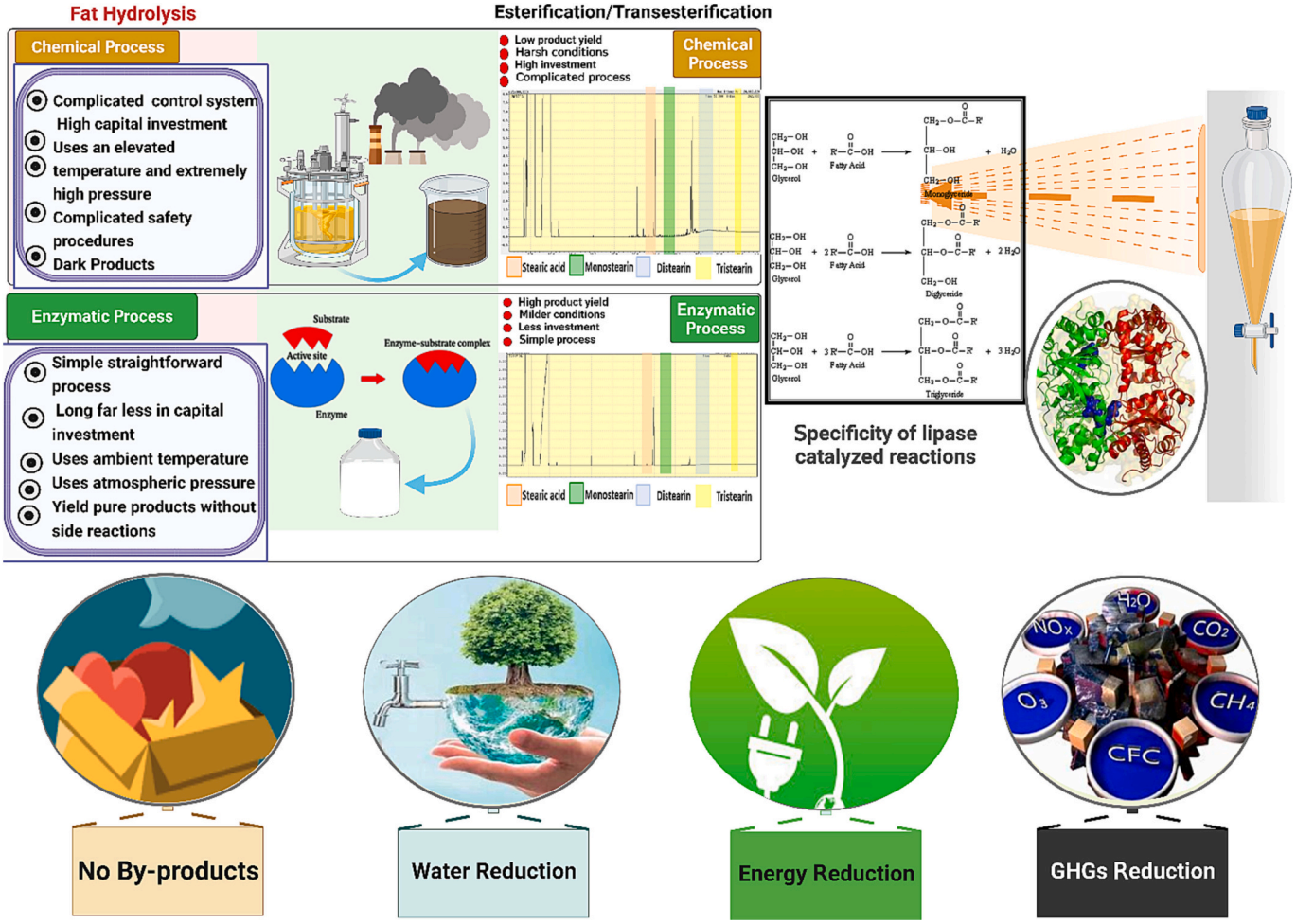

Fig. 2. Advances on lipase catalyzed production of oleochemicals and biodiesel.

conventional techniques have significant difficulties.

2.2. Fatty esters

In literature, several papers are available about the enzymatic production of fatty esters, such as the enzymatic production of monoglycerides (Mustafa et al., 2023b), isopropyl esters (Mustafa and Niikura, 2022), fatty acid sugar esters (Siebenhaller et al., 2018), 2-ethylhexyl esters (Hosney and Mustafa, 2020). The selectivity and mild processing conditions of the lipase catalyzed reactions guarantee both the high process yields and production of high-purity molecules (Mustafa, 2023; Mustafa and Niikura, 2022). Evonik Industries is the first global manufacturer of biotechnologically-produced emollient esters. The company has been making myristyl myristate, decyl cocoate, cetyl ricinoleate and isocetyl palmitate using enzymatic technology since 2008. Evonik emphasized the effectiveness and profitability of using this green technology in practical large-scale applications. These products are highly priced with high profit margins, meaning the cost of enzymes do not negatively affect profits. In addition, Evonik has dealt with the challenge of the high cost of enzymes by using a packed-bed reactor with a circulation loop.

Cetyl ricinolate (a cosmetic wax) enzymatic production is carried out for the first time in an industrial scale by Evonik Industries AG, Germany. This product appears in the market with the commercial name of Tegosoft CR. In the past, the synthesis of this product was carried out at high temperature of 240 °C by using tin oxalate as a catalyst (Nisar et al., 2021). Such harsh conditions led to the polymerization of ricinoleic acid, consequently resulting in a low product yield of only 61%. On the other hand, the use of lipase allows to operate at milder conditions typically from 30 to 50 °C, by obtaining a high yield of 93% (Holm et al., 2018). Evonik has reported that is also possible to achieve a positive effect on reducing global warming by 67% of CO_2 footprint compared to the use of traditional chemical emollients (Evonik Industries).

Oleon – together with Belgian research organization VITO and six other European partners from Belgium, France, Germany and Italy – was granted EU Horizon 2020 research funding of €13.3 M over four years (September 2019–August 2023). Their INCITE (Innovative Chemo-enzymatic InTEgrated processes) project aims to widen implementation of enzymatic processes technologies in the commodity, fine and specialty chemical industry in Europe. The ultimate objective is to establish a working industrial demo unit focused on enzymatic catalysis at Oleon's Oelegem location, close to Antwerp. Oleon has effectively manufactured isopropyl palmitate using enzymatic methods in a 150 kg pilot facility at its production site, employing a lipase-driven, solvent-free approach to create oleochemical esters

2.3. Monoglycerides

A monoglyceride is an amphiphilic lipid that has an acyl group of fatty acids and 2 hydroxyl groups. Thus, it possesses lipophilic and

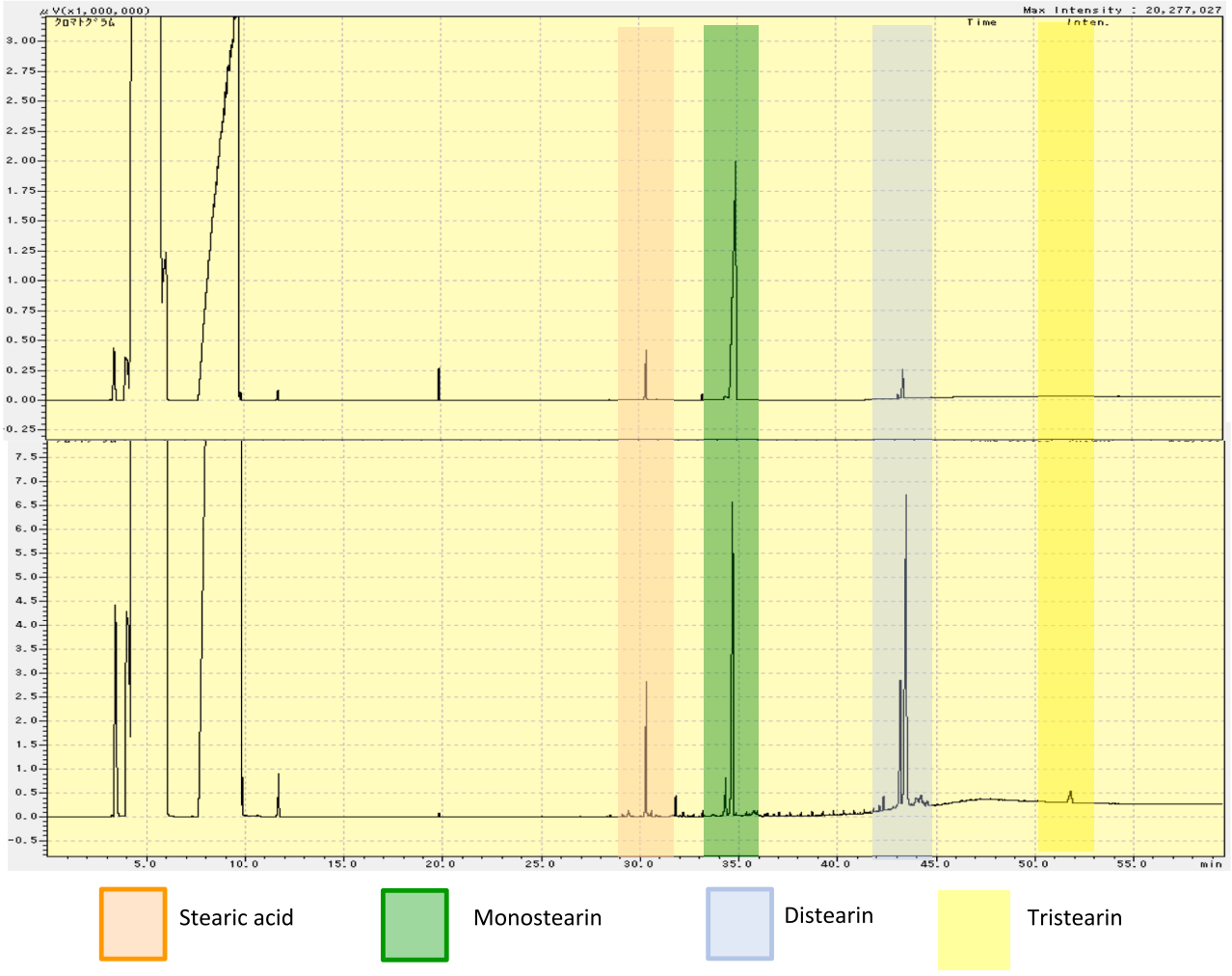

Fig. 3. GC Chromatograms of a selective synthesis of glyceryl monostearate from stearic acid and glycerol at 60 °C in the presence of *tert* butanol using Novozym 435 (top) against the traditional chemical method (down). (Mustafa et al., 2022b), License Number: 5553231128948xxx.

hydrophilic characteristics. The acyl group (R-C=O) is located on the glycerol backbone providing 1-monoglyceride (α-monoglyceride) and 2-monoglyceride (β-monoglyceride), as denoted in Fig. 3. Currently, monoglycerides are recognized as promising lipid antibacterial agents due to their amphiphilic properties (Nitbani et al., 2022b; Yoon et al., 2018). The intended antimicrobial activities include antibacterial, antifungal and antiviral properties. As a result of the interactions between the lipophilic and hydrophilic components of monoglycerides with the cell walls of microbial pathogens such as bacteria and fungi, cell membrane can be destabilized causing cell death. According to several references, monoglycerides of fatty acids such as monocaprin, monocaprilin, monolaurin, monomiristin, monoolein and monolinolein show excellent antimicrobial and antioxidant properties (Wang et al., 2013). In order to produce monoglycerides that are non-toxic, economically viable, and environmentally friendly, organic synthesis researchers must develop various possible reactions. There have been numerous attempts to synthesize monoglycerides using lipase catalysts. Lipase showed excellent catalytic activity on any lipid substrate such as fatty acids, fatty acid methyl esters and fatty acid ethyl esters. Also, it is used in many reactions such as transesterification, esterification, and ethanolysis. It reacts to triglycerides with either glycerol or protected glycerol in ethanol to produce 1-monoglycerides and 2-monoglycerides.

2.3.1. Esterification reaction

Various types of lipases serve as catalysts in the esterification process that combines lauric acid and myristic acid with glycerol, resulting in the formation of monolaurin and monomiristin, respectively. Lipozyme IM-20 (Carla et al., 2004), CALB lipase (Freitas et al., 2007), lipase G (Freitas et al., 2010), Lipozyme RM IM (Mustafa et al., 2016) and Novozym 435 (Lozano et al., 2017) are the major lipases used. For example, Novozym 435 (Lozano et al., 2017) was used as catalyst in the esterification reaction of lauric acid with glycerol. For the same process, Lipozyme IM-20 (Carla et al., 2004) showed a good catalytic activity in the esterification reaction with a yield of 45.1%. In the adopted reaction conditions (55 °C, 6 h ~~hours~~), the ratio of lauric acid to glycerol of 1:1 produces 26.8% dilaurin as a side product. CALB lipase (*Candida antarctica*) is a type of lipase immobilized on polysiloxane-polyvinyl alcohol particles (POS-PVA). This enzyme successfully catalyzed the esterification reaction of lauric acid with glycerol at a molar ratio of (1:3) with a yield of 36% at 60 °C after 6 h ~~hours~~ of reaction (Freitas et al., 2007). Lipase G from the fungi *Penicillium camemberti is* immobilized on an epoxy SiO2-PVA composite. It produced alpha monolaurin (59.4%) and alpha monomiristin (47.9%) with selectivity >50%. This reaction was carried out at a ratio glycerol: reactant of 1:8, 60 °C, time reaction of 6 h, stirring at 200 rpm and 5% (*w*/w) of enzyme. Despite the absence of a reaction solvent, lipase G proved to be very effective in order to catalyze the esterification reaction of lauric and myristic acid with glycerol, respectively. However, it was still observed the formation of side products such as di-and triglycerides. As shown in the lipase G treatment, Lipozyme RM IM demonstrated effective activity in the conversion of lauric acid and glycerol to monolaurin. This was achieved with yields of 50% and 34.6% for monolaurin and dilaurin, respectively (Mustafa et al., 2016). Mustafa et al. (2023b) could produce almost 90% pure alpha glycerin monostearate from an enzymatic esterification reaction between glycerol and stearic acid compared to only 22.5% using the chemical method as shown in Fig. 3.

Among all the lipases previously mentioned, Novozym 435 is the most excellent lipase for converting both lauric and myristic acid to their monoglycerides with 100% of yield and selectivity. The optimum yield was achieved at a mole ratio of glycerol to lauric acid of 4, temperature of 60 °C, 8 h, Novozym 435 load of 60 mg per mmol of lauric acid, and in ionic liquid [C12mim][BF4]. In a recent study conducted by Miao and Li (2021), monolaurin was synthesized using Novozym 435 in a microreactor at 58 °C with a conversion rate of 87.0% in 20 min compared to a batch method. Furthermore, t-BuOH/tert-amyl alcohol (1:1, *v*/v) was found to enhance the selectivity of lauric acid conversion to monolaurin by 90.6%. The conversion of capric acid to monocaprin was performed using Lipase from the fungi *Candida antarctica* (CAL), resulting in a high yield of 96.9% (Xia et al., 2003). Wang et al. (2013) described the creation of 1-monoolein via a two-step process. The first stage involved the esterification of oleic acid with 1,2-acetonide glycerol to yield 1,2-acetonide-3-oleoylglycerol, using Novozym 435 as the catalyst. The second stage entailed the removal of the protective acetonide group from 1, 2-acetonide-3-oleoylglycerol in a methanol environment, resulting in the formation of 1-monoolein. 1,2-acetonide-3-oleoyl glycerol was obtained with 93.4% yield when combined with dichloromethane, Novozym 435 at 8%, 60 °C for 8 h ~~hours~~. Next, deprotection of 1, 2-acetonide-3-oleoyl glycerol in methanol has resulted in 1-monoolein with 76.5% yield and 96.2% purity.

2.3.2. Transesterification reaction

The use of lipase in transesterification reactions to produce monolaurin was successfully performed. Novozym 435, a *Candida antartica* lipase immobilized on a macroporous acrylic polymer resin, proved to be an excellent catalyst in the catalytic reaction of methyl laurate and glycerol to monolaurin. Under solvent-free conditions with a reactant mole ratio of 1, the conversion rate of monolaurin was low (<50%), with a monolaurin yield of 47.6% (Jamlus et al., 2016). In contrast, increasing the molar ratio of methyl laurate and glycerol to 6 in tert-butanol/isopropanol (20:80 *w*/w) it is possible to obtain a yield of monolaurin of 82.5%. Novozym 435 was also used to the glycerolysis of methyl laurate to monolaurin with a yield of 47.6%. The reaction conditions proceed for 24 h with 5% of enzyme concentration (Chen et al., 2019). In addition, Satyawali et al. (2021) reported that monolaurin and monocaprilin had successfully been synthesized with a conversion rate of >90% under solvent-free conditions using Novozym 435. Monolaurin and monocaprilin were produced with 70% selectivity in a packed bed reactor, and enzyme reuse was possible. Various references indicate that Novozym 435 is the most suitable type of enzyme for catalyzing the glycerolysis reaction (Abdelmoez and Mustafa, 2014). This reaction produces monoglycerides in high yields. Despite this, the conversion rate and selectivity of monoglycerides have not reached 100% due to glycerol found as a side product. Glycerol, a hygroscopic material, allows for disturbances in the equilibrium of the esterification reaction. In addition, the increased water content affects the equilibrium which is shifted towards the reactant (fatty acid) and the product (monoglyceride), consequently, the yield of product is not favored. In the absence of protection, glycerol has 3 hydroxyl groups that can react with fatty acids to form diglycerides and even triglycerides. As the latest breakthrough, (Nitbani et al., 2022a) have synthesized 1-monolaurin (α-glycerol monolaurate) from the reaction of methyl laurate and protected glycerol (1,2-acetonide glycerol) using Lipozym TL IM as a catalyst. The purity and yield of monolaurin are 100% and 74.6%, respectively. This reaction involves 1,2-acetonide-3-lauryl glycerol as an intermediate substance. Lipozym TL IM proved effective to catalyze the reaction of methyl laurate and 1,2-acetonide glycerol to obtain 1,2-acetonide-3-lauryl glycerol with 92% purity and 82.1% yield. Finally, 1,2-acetonide-3-lauryl glycerol must be deprotected to form monolaurin. The use of protected glycerol base and lipozyme catalyst TL IM was helpful to obtain monolaurin of excellent quality. Here it appears that lipozyme TL IM, one of the cheapest lipases produced by Novozymes A/S, is able to provide significant catalytic activity in the synthesis of monolaurin from lipid substrate of methyl laurate.

2.3.3. Ethanolysis process

2-Monoglyceride is a compound characterized by the location of the acyl group bound to C2 of the glycerol backbone. To produce 2-monoglycerides, a regioselective sn-1,3 lipase catalyst is needed. Lipozym TL IM is one of the major sn-1,3 lipases. In order to produce 2-monoglyceride compounds, the use of triglycerides containing identical acyl groups enhances yield. Trilaurin (Nitbani et al., 2021), trimiristin and tripalmitin (Nurmala et al., 2018) can be synthesized from the

esterification reaction of lauric and myristic acid. The whole hydroxyl group in glycerol is completely esterified when molar ratio of fatty acid to glycerol is 10:1 for the reaction under acidic conditions. The excess of unreacted fatty acids and acid catalysts at the end of the process can be neutralized by adding a weak base such as Na_2CO_3. Lipozym TL, a regioselective (sn-1,3) catalyst, can break the acyl groups of trilaurine, trimirstin and tripalmitin with ethanol at positions 1 and 3 to produce 2-monolaurine (Nitbani et al., 2021), 2-monomyristine and 2-monopalmitine (Nurmala et al., 2018), respectively. 2-monoglycerides can be isolated from the reaction mixture using a hydroalcoholic solution and the side product was removed with n-hexane. The compound 2-monolaurin a white solid was synthesized with 77.5% yield and 97% purity. The product 2-monomiristin a yellow viscous liquid was synthesized with 18% yield, while 2-monopalmitin is a yellow solid with 8% yield. Despite the massive available literature about enzymatic monoglycerides production, the enzyme technologies are yet applied on laboratory scale due to the challenges associated with enzyme economy. The available useful literature concerning the commercial scale of enzymatic glycolysis is still limited.

2.4. Factors influencing the enzyme activity

In aqueous environments, factors such as temperature, pH, and substrate availability are pivotal in maintaining enzyme stability. Similarly, when enzymes operate in oils and fats, temperature and pH remain essential elements for optimizing both their activity and stability. However, other important factors that can directly affect the performance of an enzyme in a reaction, such as reaction time, physicochemical parameters and the presence of salts, must be considered. Strong acids or peroxides can also shorten the life of immobilized enzymes used in the oil processing process. Tropical plant oil, such as palm oil, is typically bleached utilizing acid-activated bleaching earth and/or degummed with phosphoric acid. The internal pH of the enzyme will decrease and its half-life will be shortened as a result of migration of citric acid and acid residues to the enzyme granulate due to oil acidity. When the enzyme pH decreases, both the enzyme's stability and activity are being affected. Although the pH of the oil cannot be determined, the pH can possibly be measured in the water extract of the oil and determine whether water-soluble organic acids and inorganics are present by comparing the results (Holm et al., 2018). The peroxide value, a measure that signifies the existence of oxidizing agents, adversely affects the stability of enzymes (Hernandez and Fernandez-Lafuente, 2011). Additionally, because the immobilized enzyme functions as a filter bed, components like phosphorus (incomplete degumming) and nickel soaps might result in column clogging (Villalba et al., 2016). As a result, these polymeric contaminants must be kept at low concentrations. It is possible to employ a number of ways to deliver oils of the proper grade and determine their expected impact on enzyme stability. Sodium hydroxide with a tiny amount can neutralise any oil residual acidity when a transesterification reaction is started from triglycerides, and normally the enzyme productivity is largely boosted (Holm et al., 2018).

3. A valid enzymatic process

In order to obtain a feasible enzymatic process, both lipase production and its use as a biocatalyst should be optimized. Several factors must be considered when it comes to lipase production, such as: i) the producing microorganisms, ii) immobilization and iii) substrates diversification. As per the enzymatic catalyzed process, factors such as optimizing the reaction conditions, feedstock source, and reactor configuration are of fundamental importance. Fig. 4 shows the representation of the aforementioned factors. The following is reported a critical discussion about of the role of these variables in lipase activities.

3.1. Focus on lipase production/utilization

3.1.1. Lipases immobilization

The techniques of immobilization were explored for almost 107

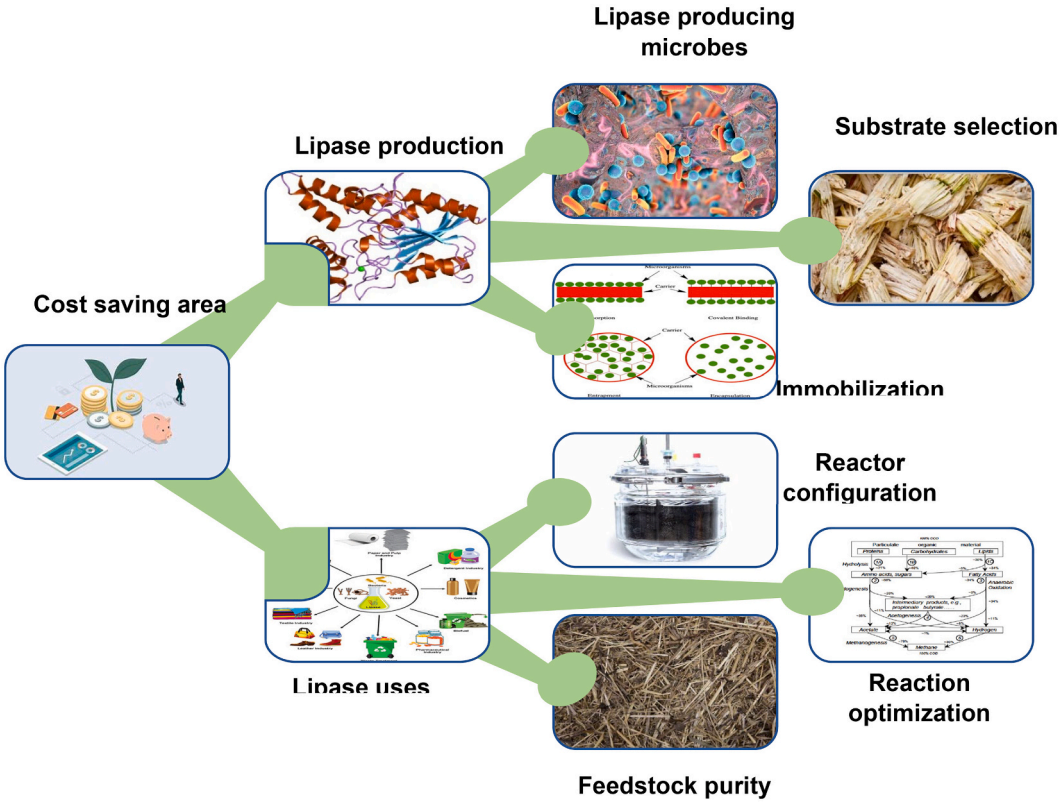

Fig. 4. Stages where a cost competitive enzymatic reaction can be developed

years and new approaches were published. Immobilization processes are required mainly to improve the stability of the enzymes, enabling their application at an industrial level, in reactors and in less mild conditions, such as the existence of solvents, high temperatures, and different pHs. Actually for some bioprocess, the immobilization process of enzymes turn out to be commercially feasible (Romero-Fernández and Paradisi, 2020), linked to the probability of reusing the enzymes. The recoverability of the enzyme has a direct impact on process costs, which can be a strong ally in the quest of making enzymatic processes economically viable (Cipolatti et al., 2014; Rodrigues et al., 2021).

The selection of a substrate for enzyme immobilization ought to consider various attributes, including its chemical and thermal resilience, potential for reusability, broad accessibility, cost-effectiveness, lack of solubility in the reaction environment, strong affinity for the enzyme, biocompatibility, mechanical robustness, and the existence of reactive functional groups. In addition, understanding the best way for the enzyme to interact with the chosen support will be the basis for building an efficient biocatalyst. A hydrophobic character, for example, makes it possible to form strong interactions that fixate lipases onto the support (Peng et al., 2020). In other cases, the carrier matrix might contain functional groups such as amines, epoxides, aldehydes, carboxylic bases, and thiols that bind with amino acid residues of the enzyme (Vafaei et al., 2020).

Several materials have been studied for the immobilization of lipases, such as: polymers, natural and synthetic, inorganic particles, magnetic and ceramic (Cipolatti et al., 2014; Mulinari et al., 2020; Pinto et al., 2020). Although the immobilization of enzymes can have a several drawbacks as decrease of activity during the immobilization process, high cost of carrier matrix and mass transfer resistance (Sheldon, 2007), some newly innovative materials have been used to solve these problems and improve the stabilization of protein after immobilization (An et al., 2020; Bilal and Iqbal, 2019; Singh et al., 2020).

A great deal of work has been dedicated to the immobilization of lipases, mainly due to the many possibilities of application of such enzymes. Different techniques have been adopted, like i) adsorption, ii) covalent bonding, iii) crosslinking, and iv) confinement. Each methodology has advantages and disadvantages that must be taken into account (Table 1). Immobilization by adsorption is the most used method, mainly due to its relative simplicity and low cost (da Silva Dutra et al., 2022; Fernandez-Lafuente et al., 1998; Fernandez-Lorente et al., 2007). The immobilization takes place in the most energetically favorable way with the possibility of reuse of the support after enzyme inactivation. The method of immobilizing substances through adsorption onto water-repellent surfaces is widely employed. This approach specifically focuses on securing the lipase enzyme in its most reactive state, commonly referred to as the “open form.” In this state, the protein sequence adjacent to the enzyme's active site, known as the “lid,” shifts position, fully exposing the active site to the surrounding reaction environment. When lipases are in a water-based medium, they exist in a dynamic equilibrium between their open (active) and closed (inactive) forms. In the inactive or closed form, the lid obstructs the active site, preventing interaction with the reaction medium. However, when a hydrophobic surface is introduced, this equilibrium tilts in favor of the open, active form of the enzyme. As a result, the lipase becomes immobilized in its active state through a specialized process known as “interfacial activation.” (Manoel et al., 2015; Palomo et al., 2002).

Immobilization can also be performed by covalent binding to the matrix, which leads to rigidity of the enzyme structure. It is considered one of the most effective methods in relation to the stabilization of the enzyme against changes in temperature, pH and organic solvents. It requires a previous stage of activation of the support and there is no possibility of desorption of the enzyme after its use (Cipolatti et al., 2014; Rodrigues et al., 2021). Several approaches can be adopted when it comes to cross-linking enzymes: cross-linked enzyme (CLE), cross-linked enzyme crystals (CLEC), cross-linked enzyme aggregates (CLEA) and cross-linked spray-dried enzyme (CSDE) are the most widely used. In these methodologies, the biocatalysts are obtained through crosslinking with some agents, resulting in the formation of enzymatic crosslinks. The formation of reticulates allows them to become insoluble and be reused for several cycles of reaction. Some studies point out that enzymes immobilized by this methodology exhibit high activity and stability with the reduction of steric impediments since a solid support is not used (Ahmad and Sardar, 2015; Sena et al., 2021; Shah et al., 2006).

Another methodology considered is the confinement. This methodology promotes the protection of the enzyme from direct contact with the reaction medium, minimizing or preventing its inactivation. It is a relatively simple method that does not promote structural alterations and can be used to immobilize one or more enzymes without a preliminary purification step. However, the application of this methodology can have several disadvantages because of the mass transfer and the interaction with the substrate. Furthermore, the control of pore size turns out to be difficult (Ge et al., 2012; Lee et al., 2009).

Besides that, other strategies can be used for the immobilization of lipases. The enzyme immobilization process is complex and there is no single immobilization method or a support applicable to all enzymes. The physicochemical characteristics of the enzyme and the reaction conditions must be taken into account in order to choose the most suitable support (Cipolatti et al., 2017; Rodrigues et al., 2021).

Table 1
Advantages and disadvantages of enzyme immobilization methodologies.

Methodology	Advantages	Disadvantages	Examples of supports	Reference
Adsorption	• relative simplicity • no support activation required • low cost • possibility of reuse of the support after enzyme inactivation • little change in the enzyme's conformational structure	• randomness of the enzyme-support interaction • possibility of desorption of the enzyme in the reaction medium	Polystyrene, polymethylmethacrylate	(Cipolatti et al., 2018; Cunha et al., 2014)
Covalent bonding	• considered the most effective in terms of thermal and operational stabilization of enzymes • the enzyme is not desorbed from the support	• possibility of partial inactivation and/or reduction of the catalytic activity of the enzyme due to alteration in its native conformation • impossibility of reuse of support	Polyacrylonitrile nanofiber membrane	(Gupta et al., 2013)
Crosslinking	• high catalytic activity • high stability • no solid support required	• some crosslinking processes require prior crystallization of the enzyme	Whole cell stabilized with polyethylene glycol	(Sena et al., 2021)
Confinement	• protection of the enzyme from direct contact with the reaction medium • the enzyme does not bind with the support • no prior purification required	• mass transfer limitations • limitation of substrate diffusion through the pores of the matrix • many components used in the synthesis of the matrices can inactivate the enzymes	Polyurethane foam	(Nicoletti et al., 2015)

Some studies have been dedicated to the immobilization of more enzymes on the same matrix (co-immobilization or multi-enzyme systems) (Hwang and Lee, 2019; Zhang and Hess, 2017). Co-immobilization is a potential alternative for reactions that required the simultaneous presence of more enzymes characterized by different properties. Co-immobilization can be an interesting alternative to immobilization technology, which must be carefully evaluated. Cascade reactions can benefit from this technique. An example is the starch liquefaction and saccharification steps, where starch is first hydrolyzed into soluble oligosaccharides and to be converted into glucose in the sequence. The food industry has benefited from this technique, but many articles cite the advantages of using co-immobilized enzymes for application in biodiesel. Blends of lipases can greatly increase the overall reaction rate. The article by Arana-Peña et al. (2020) reported that the strategy of using co-immobilized enzymes does not make sense if there is a need for high selectivity or specificity. However, they stated that its use is interesting for the complete use of heterogeneous matrices. Rodrigues and Ayub (2011) study showed that a biocatalyst composed of a mixture of lipases from *Candida antarctica* (CALB) and *Rhizomucor miehie* (RML) was more efficient than lipase from *Thermomyces lanuginosus* (TLL) in the transesterification of soybean oil, even though TLL was more active when evaluated individually, which was attributed to a possible synergistic effect. Therefore, the use of co-immobilization can be applied in order to utilize this beneficial synergistic effect.

In co-immobilization, enzymes used can be from the same class or from different classes, depending on the reaction that will be used. One of the challenges is the preservation of the activity of both enzymes, when compared with the activities of both individually in solution. It should be considered whether an enzyme catalyses the product of the previous enzyme, and therefore requires more careful planning of how the immobilization process will be carried out. The type of support and the type of binding should also be carefully evaluated in order to avoid possible randomness so that one enzyme does not predominantly occupy the support spaces to the detriment of the other, or one causes steric hindrance in their respective active sites.

The use of these biocatalysts presents some advantages as the improvement of reaction yields, acceleration of the speed reactions, less intermediate products; reduction of steps, contributing to lower production cost etc. (Arana-Peña et al., 2021; Metzner et al., 2015; Szita et al., 2018). Although the multi- enzymatic systems are interesting, it must be carried out with caution. The use of enzymes in cascade may have enzyme inactivating effects (Jiao et al., 2020). The balance between advantages and problems must be evaluated before starting a biocatalysts design and some requirements can be highlighted: a) studies of different sizes of each protein must be done; b) enzyme immobilization rate needs to be studied; c) the same strategy need be applied for all enzymes involved in the immobilization; d) study of loading capacity of the support is crucial because it is naturally reduced, with the presence of different enzymes in the system; e) higher difference in the stabilities between each enzyme can happen (Arana-Peña et al., 2021).

More recently, the use of nanoflowers has received attention (Zhang et al., 2019; Zhang et al., 2021). In this reticulation technique, hybrid organic-inorganic structures are formed by obtaining flower-shaped nanostructures (da Costa et al., 2022). The use of these biocatalysts presents a series of benefits in term of high surface area, greater stability and excellent catalytic activity. In addition, the application has been encouraged in other areas such as biomedicine and the development of biosensors (da Costa et al., 2022; Perwez et al., 2023; Xu et al., 2023).

The combination of several methodologies, including uses and modifications of conventional techniques, novel materials and compound associations has contributed significantly to the improvement of the area. For example, the immobilization of lipase from *Candida antarctica* type B and *Thermomyces lanuginosus* (co-immobilization) in nanoflower structures (Costa, 2023). Size modification and the research for nanostructures (Cipolatti et al., 2014; Zahirinejad et al., 2021), improves the enzyme's interaction with the substrate. More porous supports, which allow the immobilization of a greater amount of enzyme (Hernandez et al., 2011; Rios et al., 2019). More/less hydrophobic materials will have different results in interfacial lipase activation (Bernal et al., 2014; Manoel et al., 2015; Pinto et al., 2020). Much has already been done in this regard, but by carefully reviewing the literature, we can see how important these improvements are. This means that the area of enzyme immobilization continues to attract attention in terms of innovation and importance. Table 1 presents some advantages and disadvantages attributed to the different methods of immobilization. Despite many materials can be used as supports, the table presents just one example among the many possibilities.

3.1.2. Application of microorganisms in lipases production

Microorganisms have been the main source of obtaining lipases, although these enzymes can also be produced by plants and animals (Jaeger and Reetz, 1998; Treichel et al., 2010). Microbial lipases represent the most widely used class of enzymes in biotechnological applications and organic synthesis due to their production capability in a controlled environment (bioreactor), which makes them independent of seasonal environmental changes. In addition, they are characterized by higher catalytic activity, ease of genetic manipulation, large-scale production and the possibility of using cheaper alternative means (Javed et al., 2018; Salgado et al., 2022; Salihu et al., 2012).

Eukaryotes and prokaryotes are the major lipase producers and their characteristics can range according to the microorganism used. Lipases from extremophile microorganisms (psychrophiles and thermophiles) are widely used for high yields compared to mesophilic microorganisms and also generate fewer by-products and side reactions (Rabbani et al., 2022; Verma et al., 2021).

Many microorganisms can produce lipases, but only a limited number of microbial lipases are used industrially (da Silva Dutra et al., 2022; Verma et al., 2021). The main microbial lipases have been produced by submerged cultivation. The development and innovations in the field of engineering will strongly contribute to the advancement of submerged processes on a large-scale. Much has also been accomplished in the production of lipases using solid state fermentation, where solid substrates with low percentages of water are used (Ávila et al., 2019; do Nascimento et al., 2021). Such substrates, in addition to acting as a source of nutrients, also serve as a physiological support. Solid state processes are usually cheaper considering the use of agro-industrial coproducts, the simplicity of the reactors and low risk of contamination since it uses low humidity. However, in general, it does not show agitation, although much progress has been made in this direction, which leads to non-homogeneity of the medium and limitations of heat and mass transfer, in addition to the difficulty of controlling operational parameters (Mitchell et al., 2019; Singh and Mukhopadhyay, 2012).

Filamentous fungi are widely used in solid state cultivation, mainly to secrete a high amount of extracellular lipases, which facilitates their use. Among the main fungi used for the production of lipases are the genera *Aspergillus* sp., *Fusarium* sp., *Mucor* sp., *Rhizomucor* sp., *Rhizopus* sp., *Penicillium* sp. and *Thermomyces* sp. (Chandra et al., 2020; da Silva Dutra et al., 2022; Treichel et al., 2010). Different species will require different growing conditions, such as aeration conditions, sources of carbon, nitrogen and other nutrients, pH, temperature and agitation. Fungi did diversify throughout the evolutionary process, using wide cultivation conditions and presenting few nutritional requirements, which simplifies their use in various residual matrices (Salihu et al., 2012). Regarding yeasts, some examples of lipase producers include *Candida rugosa, Candida antarctica, Candida tropicalis, Yarrowia lipolytica, Rhodotorula glutinis, Pichia Bispora, Trichosporon asteroids, Saccharomycopsis crataegenesis*, among others (Treichel et al., 2010). Among those cited, *C. rugosa* (Cipolatti et al., 2021; Guimarães et al., 2022; Patel et al., 2015; Sarno and Iuliano, 2020) and *C. antartica* (Chandra et al., 2020; Cipolatti et al., 2018; Cipolatti et al., 2021; da Silva Corrêa et al., 2020) have been widely used in recent years, in different applications. Bacteria can also be good lipase producers, such as *Pseudomonas*

aeruginosa, *Burholderia multivorans*, *Burkholderia cepacia*, *Staphylococcus caseolyticus*, *Bacillus subtilis*, *Bacillus licheniformis*, among others (Chandra et al., 2020; Contesini et al., 2020; Nimkande and Bafana, 2022; Treichel et al., 2010). Many works have focused on bacterial sources of lipases, mainly because they consider it capable of withstanding the industrial environment (Javed et al., 2018).

Over the last decade, it was observed the great efforts were done by biotech industries to improve enzymatic productivities and in the development of new approaches in order to satisfy current market demands to increase the lifespan of biocatalysts (Gonçalves et al., 2019). Several engineering methods have been used to improve the properties of lipases, such as site-directed mutagenesis of the lid domain, rational design, point mutation, epPCR and random mutagenesis (Bornscheuer, 2008; Bornscheuer and Pohl, 2001; Javed et al., 2018). Improved stability at high temperatures and the ability to tolerate solvents has revolutionized and expanded the application of enzymes in industrial processes. Other properties can also be improved, such as activity and stability at different pHs, increase or modification of enantioselectivity and alteration of specificity for a substrate (Adrio and Demain, 2014; Zorn et al., 2016).

The production of lipase by microorganisms is associated with their growth and, consequently, with variations in the composition and cultivation conditions. Many studies are dedicated to optimizing these conditions to improve the lipase activity obtained, as exemplified in Table 2.

The metagenomics technique appears as the best strategy to obtain new lipase sequences (Fig. 5). Different methods can be used in metagenomics with success depending on the vectors and expression systems used. The use of DNA sequencing of clones that allows the functional screening of specific molecules as enzymes and antibiotics, appears to be a great industrial opportunity.

Considering the commercialization of lipase-catalyzed technology, it is necessary to assess the intended application, the choice of substrate, the reactor design, the enzyme immobilization technique, the support of immobilization, and the economic viability.

3.1.3. Substrate diversification for industrial demands

The dissemination of the use of enzymes comes up against the high cost of obtaining them, which often makes their industrial application unfeasible. Therefore, several works have been dedicated to obtain low-cost culture media and development of suitable bioprocesses in order to enable greater productivity and throughput. Agricultural processes generate a high amount of waste annually. Such residues are rich of evaluable compounds (such as simple sugars, carbohydrates) which can

Table 2
Lipase producing microorganisms: exploring growth-associated factors and cultivation conditions.

Groups	Source of lipase	Substrate	Growing conditions	Lipase production	Reactor	Reference
Bacteria	*Bacillus amyloliquefaciens*	Wheat bran, oat bran, Sunflower meal, rice bran, soya extract and bagasse	72 h, 40 °C, pH 7.0	80.6 U/mL	Tray bioreactor	(Mazhar et al., 2023)
	Bacillus aryabhattai	Olive mill wastewater	pH 8, 27 °C	28.3 U/mL	STR	(Paz et al., 2023)
	Metarhizium anisopliae	Babassu residues			Tray bioreactor	(da Silva et al., 2022)
	E. coli BL21 (DE3), transformed with pET51b-lipase 42	Banana waste juice	10 h, 30 °C, pH was not adjusted	200 U/mL	Shake flask	(Chai et al., 2018)
	Stenotrophomonas maltophilia	Groundnut cake, rice bran, wheat bran, neem cake, and coconut cake	28 °C, pH 6 (groundnut cake, neem cake and coconut cake), pH 7 (rice bran and wheat bran)	groundnut cake 74.1 U/mL; coconut cake 61.9 U/mL; neem cake 58.7 U/mL; wheat bran 49.6 U/mL; rice bran 28.2 U/mL	Erlenmeyer flasks	(Neethu et al., 2015)
Yeast	*Candida cylindracea*	Palm oil mill effluent	36 h, 30 °C, 1.0 vvm, 400 rpm	41.5 U/mL	Stirred tank bioreactor	(Salihu et al., 2011)
	Yarrowia lipolytica	Soybean hulls	35 °C, pH 7.0	32 U/g (when supplemented with concentrated YPO medium)	Tray and insulated packed-bed bioreactors	(do Nascimento et al., 2021)
	Yarrowia lipolytica	Mango wastes (peel, tegument and kernel)	27.9 °C, pH 5.0, 187 rpm, inoculum concentration of 0.96 g/L	3500 U/L of extracellular lipase/ 68.03 U/(g of residue)	Erlenmeyer flasks	(Pereira et al., 2019)
Fungi	*Aspergillus niger*	Cotton seed, red gram husk, *Prosopis juliflora*	35 °C, pH 7, 70% initial moisture content (*v/w*), 72 h	269.87 U/gds	Tray bioreactor	(Mandari et al., 2020)
	Aspergillus niger	Rice bran and *Jathropa* seed cake	5 days, anaerobic conditions, room temperature	282 U/mL	Tray bioreactor	(Putri et al., 2020)
	Aspergillus niger	Rice bran and Jatropha seed cake			Tray bioreactor	(Khootama et al., 2018)
	Aspergillus niger	Sheanut cake	30 °C, pH 7,0, 7 days, 180 rpm	45.6 U/mL	Conical flasks	(Salihu et al., 2016)
	Aspergillus oryzae, Aspergillus japonicas	Castor de-oiled cake	30 °C, 6 days, pH 7.0, moisture content 100%	25 U/g	Erlenmeyer flasks	(Jain and Naik, 2018)
	Penicillium simplicissimum	Babassu cake	72 h, aeration rate of 11.6 L/min, 30 rpm	49.4 U/g of dried mass	Tray-type reactor	(Gutarrra et al., 2009)
	Penicillium citrinum	Ground-nut oil refinery residue	28–30 °C, 100 rpm, 24 h	5786 U/L	Erlenmeyer flasks	(Miranda et al., 1999)
	Rhizomucor miehei	Cottonseed meal	30 °C and 90 wt% of moisture, 96 h	93 U/g	Tray bioreactor	(Aguieiras et al., 2019)
	Rhizomucor miehei	Babassu cake	35°C, 1.0L air/min, 5wt% water, 48 h	30 U/g	Fixed-bed bioreactor	(Ávila et al., 2019)
	Rhizopus arrhizus	Waste cooking oil (WCO)	presence of 10 g/L WCO	520 U/g	Shake flask	(Çağatay and Aksu, 2021)
	Garbage enzyme	Pomegranate, orange and pineapple peels	33 °C, pH 6, 210 rpm, 4 days	57.4 U/mL	STR	(Selvakumar and Sivashanmugam, 2017)

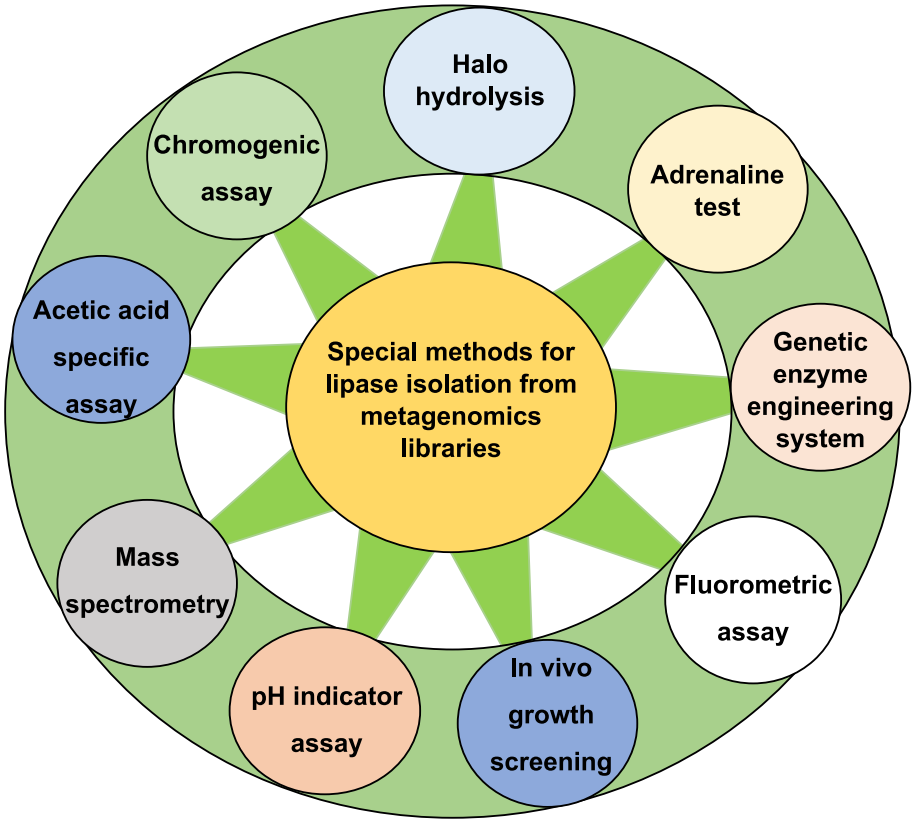

Fig. 5. Functional screening methods for the isolation of lipases from metagenomic libraries.

be useful as nutrients for biotechnological processes of enzyme production. In fact, if we think about the use of these matrices, the term "residue" can be excluded, and we can use "coproduct", for example. Among these coproducts, it can be mentioned leaves, stems, straw, seeds, bark, etc. (Sakina et al., 2023). The use of food processing coproducts, such as agro-industrial residues from oil extraction, is an

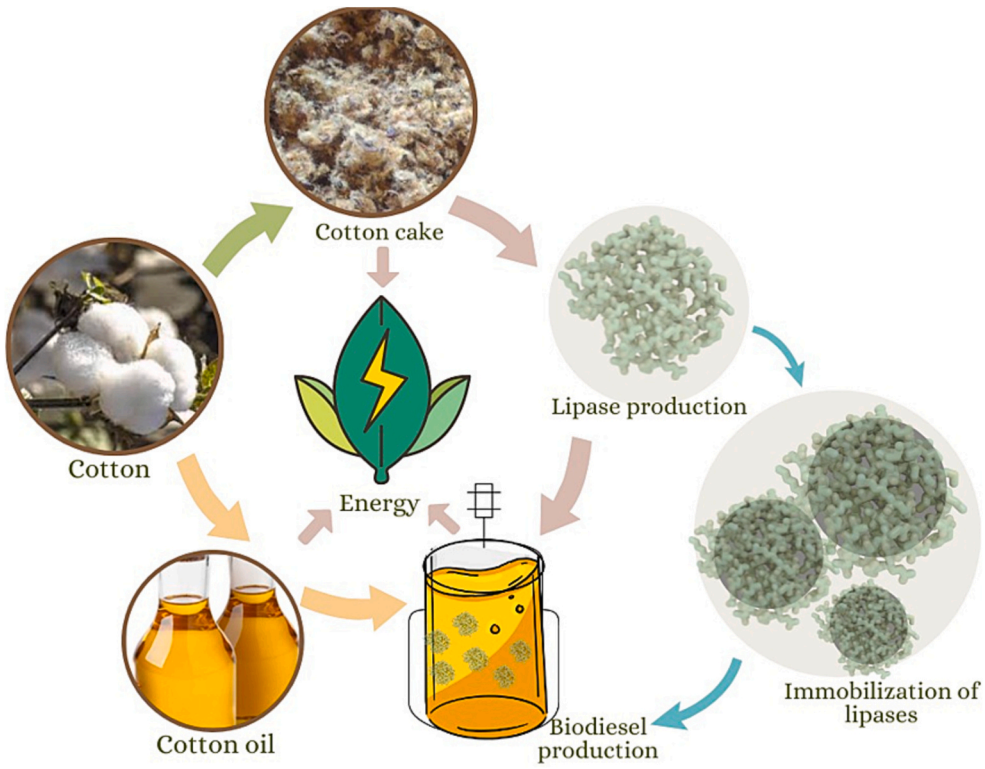

Fig. 6. Application of cotton crop in the concept of process circularity.

interesting alternative in the production of lipases (Abdelmoez et al., 2013). Therefore, adding value to this material can contribute positively to the environmental problem, in addition to significantly reducing the costs of the process (Salihu et al., 2012).

The concept of process circularity has been demonstrated in several papers (Bakan et al., 2022; Ibrahim and Mustafa, 2022; Kumar and Verma, 2021). Fig. 6 exemplifies, in a very simple way, the concept of circularity of processes that include the production of lipases. In the example shown, the cotton crop is being explored. At the end of the extraction process, a "cotton cake" was obtained. This residue, which contains traces of oil, can be used as a substrate for the production of lipases. In addition, it can be useful as a support for the immobilization of the lipase produced. After extracting the enzyme, the cellulosic residue can be used to produce energy for the process itself. Finally, the biocatalyst obtained can be applied to cottonseed oil to enzymatic production of biodiesel, closing a recovery cycle.

There are a variety of feedstocks that can be used as substrates for the production of lipases, as reported in Table 3. Residual biomass serve as a support for the cultivation and the process can take place in tray-type reactors (da Silva et al., 2022). In the search for alternative substrates for the production of lipases, Brazil stands out for its rich biodiversity. Many papers using agro-industrial residues/co-products for lipase production come from Brazilian research. Matrices such as babassu (*Attalea speciosa*) (Gutarrra et al., 2009), palm (*Elaeis guineenses*) (Collaço et al., 2020; Oliveira et al., 2018), castor bean (*Ricinus communis* L.) (Godoy et al., 2009), macauba (*Acrocomia aculeata*) (Ávila et al., 2019), açaí (*Euterpe oleracea* Mart) (Mares et al., 2021), cassava (*Manihot esculenta*), pinhão-manso (*Jatropha curcas* L.) are abundant in the country and can be a low-cost source of lipases.

Table 3
Main residual biomass used in the production of lipases.

Source of lipase	Substrate	Reactor	Reference
Bacillus amyloliquefaciens	Wheat bran, oat bran, Sunflower meal, rice bran, soya extract and bagasse	Tray bioreactor	(Mazhar et al., 2023)
Bacillus aryabhattai	Olive mill wastewater	STR	(Paz et al., 2023)
Metarhizium anisopliae	Babassu residues	Tray bioreactor	(da Silva et al., 2022)
Rhizopus arrhizus	Waste cooking oil	Shake flask	(Çağatay and Aksu, 2021)
Yarrowia lipolytica	Soybean hulls	Tray and insulated packed-bed bioreactors	(do Nascimento et al., 2021)
Aspergillus niger	Cotton seed, red gram husk, *Prosopis juliflora*	Tray bioreactor	(Mandari et al., 2020)
Aspergillus niger	Rice bran and *Jathropa* seed cake	Tray bioreactor	(Putri et al., 2020)
Rhizomucor miehei	Cottonseed meal	Tray bioreactor	(Aguieiras et al., 2019)
Rhizomucor miehei	Babassu cake	Fixed-bed bioreactor	(Ávila et al., 2019)
Yarrowia lipolytica	Mango wastes (peel, tegument and kernel)	Erlenmeyer flasks	(Pereira et al., 2019)
Aspergillus oryzae, Aspergillus japonicas	Castor de-oiled cake	Erlenmeyer flasks	(Jain and Naik, 2018)
E. coli BL21 (DE3), transformed with pET51b-lipase 42	Banana waste juice	Shake flask	(Chai et al., 2018)
Aspergillus niger	Rice bran and Jatropha seed cake	Tray bioreactor	(Khootama et al., 2018)
Garbage enzyme	Pomegranate, orange and pineapple peels	STR	(Selvakumar and Sivashanmugam, 2017)
Aspergillus niger	Sheanut cake	Conical flasks	(Salihu et al., 2016)
Stenotrophomonas maltophilia	Groundnut cake, rice bran, wheat bran, neem cake, and coconut cake	Erlenmeyer flasks	(Neethu et al., 2015)
Candida cylindracea	Palm oil mill effluent	Stirred tank bioreactor	(Salihu et al., 2011)
Penicillium simplicissimum	Babassu cake	Tray-type reactor	(Gutarrra et al., 2009)
Penicillium citrinum	Ground-nut oil refinery residue	Erlenmeyer flasks	(Miranda et al., 1999)

A promising strategy is the use of associated residual biomass, as in the work of (Amenaghawon et al., 2022), that used a mixture of banana peels, pineapple peels, and coconut pulp waste to produce lipase. The authors also tested the effect of different inducers, such as castor oil, jatropha oil and olive oil, which led to a 30.6% increase in the of lipases productivity.

Studies of production and simultaneous immobilization of lipases are excellent alternatives for reducing process costs. Normally, the produced enzyme needs steps of extraction and purification to be able to be applied. Using an enzyme in the matrix in which it was produced can be an alternative for application in processes that do not require a high degree of purity of the biocatalyst, such as in the synthesis of biodiesel. Some researchers add inert supports to the cultivation, such as polypropylene, intending to improve the characteristics of the biocatalyst, especially with regard to its reuse. This support can be added in both solid-state and submerged cultivation (Greco-Duarte et al., 2023). Many studies point out that integrated processes reduce costs with transport and waste generation The use of shared infrastructure can maximize capital savings (Brêda et al., 2022). Although they still lack studies and implementation, everything points towards the emergence of new and increasingly efficient, integrated processes.

3.2. Focus on lipase catalyzed processes

3.2.1. Reactor design

Although the main reactors used are STRs (Stirred Tank Reactors), PBRs (Packed Bed Reactors) and FBRs (Fluidized Bed Reactors) (Remonatto et al., 2022), the great advantage of the use of reactor is the possibility of separate configurations and operating methods. The flexibility of the biotechnology industries is unquestionable, however, as mentioned before, it is necessary to evaluate the intended application for the smartest choice of each reactor in relation to the biocatalyst used. STRs are widely used to heterogenous systems employing immobilized lipases. It can be operated in batch, fed-batch and continuous mode. This one can be used on laboratory and pilot scale with possible control of the variables of the system and due to the high operational stability (Remonatto et al., 2022).

Due to mixture stirring level afforded by the mechanical agitations (or impellers), the mass transference problems are negligible. STRs can use distinct impellers that can be chosen in relation to the biocatalyst immobilized on a support. If there is a shear stress, it is necessary to choose other mechanical agitation pattern or it will be necessary to evaluate the leading to the leaching of protein of the support (Manoel et al., 2013; Otari et al., 2020; Palma et al., 2021). In this case, minimizing loss of the catalyst is crucial for non-decrease of the lipase activity. Studies aiming to investigate the support resistance generally use batch reactors due to practicality, flexibility, higher dispersion of the substrate, simple equipment, and easy reaction control (Hama and Kondo, 2013).

After STRs, the PBRs appear as the most used bioreactors with immobilized lipases. In this system, the biocatalyst is packed inside a column with the possibility to control the amount of enzyme loaded. (Zhong et al., 2020). The use of lipase immobilized on PBRs is interesting due to the high productivity and at lack of shear forces that are uncommon in this system. However, the mass transference is limited. The compaction of immobilized lipases inside of the column, pressure

difference and the size of immobilized lipase, must be evaluated because it can cause preferential paths within the column (Fernandes, 2010; Manoel et al., 2013). In this case, STRs can be the better option. It should also be emphasized that when STRs are used, a substantial amount of lipases are lost during catalyst removal step by filtration (after the reaction), which requires new fresh enzymes to be added to the system to compensate for the losses. This challenge could be avoided when PBRs are used. Practically (on a commercial scale), the most successful enzymatic reactor configuration for biodiesel production is the STR, where liquid lipase is used. Most commonly, the free enzyme is recovered by centrifugation. In contrast, the most used reactor configuration for Oleochemicals production is the PBR, where immobilized lipases are used. In both configurations, the economic feasibility has been proved on commercial scales (Tan et al., 2010).

The use of FBR is efficient in the mixture stirring level, however, longer reactions times are required for biotransformation with a consequent reduction of productivity. This bioreactor is characterized by the fluid injected from the bottom and flows upwards encountering the immobilized lipase, that is kept by a fluid through the bed (Remonatto et al., 2022). Although less common, others reactors can be used with immobilized lipases as biocatalytic membrane reactors (BMRs) (Stankiewicz and Moulijn, 2000), bubble column reactors (BCRs) (Lee et al., 2009), vortex flow reactors (VRFs) (Ibáñez-González and Cooney, 2007) Fig. 7 shows the most common reactor configurations are currently in use for producing OC/BD.

When it comes to the large-scale application, the mechanically stirred tank reactor configuration can't be seen as a favorable solution due to many reasons. Some enzyme particles are lost in such batch method during loading and emptying the reactor. In addition, the shear stresses due to the mechanical agitation can denature the enzyme. Due to these reasons, the fixed bed reactor configuration received considerable attention (Holm et al., 2018). In September 2001, Karlshamns (now AAK) in Sweden by the cooperation with Novozymes, was the first company in the world that has produced an enzymatically prepared vegetable fats using Lipozyme TL IM (Nielsen, 2002). The results of these production trials in Sweden provided a crucial foundation for additional study and development. Despite the numerous difficulties over the past two to three years' worth of work faced and the uncertainties regarding the significant influence of oil quality on enzyme productivity. The trials in Sweden proved beyond a shadow of a doubt that a very acceptable production economy for esters was feasible. Although the early large-scale research employing the single reactor concept showed that ester production was feasible, the practical operation did not function well in an industrial setting that was always in operation. During use, oil flows down the fixed bed of enzyme, progressively lowering the reactor's enzyme activity (Husum et al., 2004). What are interesting in their configuration with operating many columns in series, it is no longer necessary to have complete conversion in the first column, however, the complete conversion is achieved at the end of the serial reactor.

Additionally, the total economy is improved because the first column in the series can now be adjusted down to practically zero activity. Since the remaining columns can still be used, it is simple to replace the exhausted enzyme in the first column without halting production. The exhausted material is then replaced with a new charge of enzyme, and this column is connected via the pipe system to the end of the conversion line. Currently, the factory runs at a flow of 2 kg of oil for every kg of enzyme per hour. Once the conversion drops to the acceptable threshold level, the oldest column is recharged; this normally happens once every two to three weeks (Hosney and Mustafa, 2020). The fat conversion is measured at the outlet of the final reactor. The most practical way to get rid of used enzyme catalyst is by using the same procedure as for bleaching earth. Since its introduction in 2002, this method of operation has been used.

3.2.2. *Assessing cost-effective low-quality feedstocks for biodiesel production*

One of the main benefits of the lipase catalyzed method is that simple pre-treatment is not necessary when using inexpensive feedstocks with a high percentage of water and free fatty acids (FFAs). Today, refined edible oil, such as soybean, refined rapeseed, and palm oil are the raw materials used for the manufacturing of biodiesel in the majority of

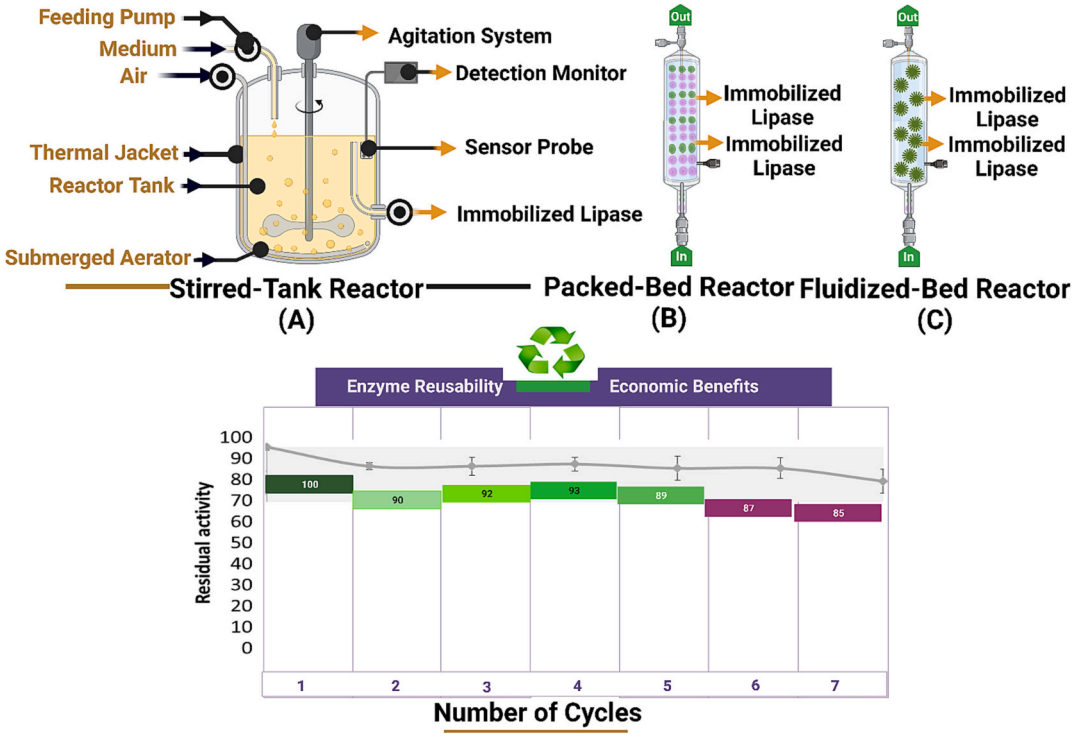

Fig. 7. Schematic representation of the main types of reactor used for reactions with immobilized lipases.

countries (Encinar et al., 2011; Hosseinzadeh-Bandbafha et al., 2023; Li et al., 2023). >85% of the overall cost of production goes towards the price of this oil feedstock. Microbial lipid is a promising source that is being studied extensively nowadays (Tan et al., 2023). It has important economic aspect to increase the availability of low-quality oil feedstock at a reduced cost for the manufacturing of biodiesel. Low-quality raw materials for the production of biodiesel can be obtained from a variety of sources, such as used cooking oil, animal fats, brown grease, sewage sludge, and unprocessed oils like grassland oils or crude palm oil and other plant (di Bitonto et al., 2016; di Bitonto et al., 2020; Lou et al., 2008). A high relative concentration of FFAs and water in low-quality raw materials has a number of negative effects on the manufacture of alkali-catalyzed biodiesel. FFAs will produce soaps when they react with the alkaline catalysts, making it difficult to separate the final product and resulting in poorer conversion yields and greater catalyst use. In addition, triglycerides present in the system can be hydrolyzed with the formation of other FFAs.

Typically for the alkaline-catalyzed process, the level of water and FFAs in the feedstock should be lower than 0.1% (*w*/w), and 1.0% (w/w) respectively (Amoah et al., 2016; Pastore et al., 2014). For the alkaline-catalyzed method, a challenging pre-treatment is typically required. Prior to the alkaline transesterification of triacylglycerides (TAG) to fatty acid methyl esters (FAMEs), it is necessary to esterify the FFA and lower their amount to <1% (w/w) using sulfuric acid in the presence of methanol. Chemical biodiesel has a number of disadvantages, one of which being the two-step acid-base process, which raises the cost of manufacturing. Lipases are widely used because they may transesterify TAG and FFA in a single process. Other downsides of acid catalysis include reactor corrosion and a higher reaction temperature. Generally, catalysts for heterogeneous acids were created (Abdellatief et al., 2023), such as acid-activated montmorillonite (Chen et al., 2023), carbon-based catalysts and molybdenum oxide catalysts supported by Al_2O_3 (Ibrahim and Halim, 2021). However, due to stability and cost difficulties, no heterogeneous acid catalyst has been used in actual manufacturing.

The enzymatic method is advantageous for processing lower-grade raw materials, as it doesn't necessitate specific levels of free fatty acids or water content, unlike chemical reactions driven by alkaline or acidic catalysts (Hama and Kondo, 2013). The absence of soap formation in the enzymatic approach leads to a greater conversion yield. Furthermore, the quality of glycerol produced is superior because the process doesn't involve the use of either alkaline or acidic substances. Low-quality feedstocks can be utilized without the need for extensive pre-treatment (Elgharbawy et al., 2021). For example, crude palm oil can be directly utilized in the enzymatic method, whereas the chemical approach mandates that refined palm oil undergo degumming and bleaching processes (Crabbe et al., 2001).

4. Available companies for large-scale enzymatic biodiesel production

Among oleochemicals, fatty acids methyl esters (biodiesel) are the largest produced material enzymatically in a commercial-scale at many countries. The move towards enzymatic biodiesel production started in 2014. However, the total number of enzymatic biodiesel producers is still limited. U.S. has many large-scale producers of enzymatic biodiesel, however, the common American biodiesel producer Aemetis, Inc. has established the largest 50 million gallons per year (about 189 million liters) enzymatic biodiesel plant in Kakinada, India at 2017. In addition, Aemetis Inc. used their own patent to convert the waste oil feedstocks to biodiesel using enzymes. In 2015, Viesel fuel LLC has conducted a 11 million gallons per year enzymatic biodiesel facility using liquid lipase formulations from Eversa Transform, (*A. oryzae*). The producing facility was established after great cooperation between Viesel, Novozymes A/S and academia on converting waste cooking oil and brown grease containing high free fatty acids to biodiesel using enzymatic technology (Holm et al., 2018). Buster Biofuels has established its 5 million gallons per year in San Diego after partnership with Novozymes A/S, Viesel fuel LLC and Tactical Fabrication LLC. In order to develop the technology, Novozymes partnered with Piedmont Biofuels several years ago. Since then, the enzyme manufacturer has collaborated with Viesel Fuel, Blue Sun Biodiesel, and WB Services (Green Energy Products and Adkins Energy). Novozymes A/s has also a considerable contribution as enzyme manufacture with the global equipment supplier Desmet Ballestra in creating state of art design for enzymatic biodiesel production in a concrete commercial manner. In addition, SRS International Corp. and Biodiesel Experts International LLC have teamed up to offer turnkey biodiesel refineries all over the world. By combining the enzymatic technical process expertise of Biodiesel Experts with 'SRS' proven biodiesel technology for conventional chemical processes, SRS was able to provide turnkey enzymatic biodiesel production facilities. In the past seven years, all of the research mentioned above has exclusively been done in the US.

TransBiodiesel is another enzymes manufacturer which was founded in 2007. It is the inventor (6 US patents) of the Enzymatic Game-Changer Technology which could transform economics of the biodiesel production around the world. TransBiodiesel sells the immobilized enzyme " TransZyme A" to Biodiesel producers and is willing to partner with biodiesel producers, waste vegetable oil collectors and waste water treators to make Biodiesel. TransBiodiesel gives a " Written Guarantee " that each one ton of "TransZyme A" the Immobilized Enzyme produces >4000 Tons of biodiesel. TransBiodiesel has some relationships with U. S. oil and biodiesel producers. They have an ongoing collaboration with U.S. customers. There are many implemented projects in US based on TransBiodiesel technology, however, the names of these companies are not published. In South Korea, the M-energy company uses TransZyme A technology to produce 30,000 tons annually of enzymatic biodiesel using brown grease extracted from the grease trap.

China has also many enzymatic biodiesel producers, Hunan Rivers Bioengineering Co. Ltd. uses the common enzyme Lipozyme 435 for producing 20,000 tons of enzymatic biodiesel annually. Table 4 lists the current companies that produce biodiesel using the enzymatic technology globally. As a conclusion, enzymes have proved their selves as a robust and effective catalysts for enzymatic biodiesel production due to its technical and economic benefits. More future investments are expected to come.

Brown grease and used cooking oil, both second-generation feedstocks, are the principal feedstocks used in the commercial manufacture of enzymatic biodiesel. Waste cooking/frying oils often include 20–60% FFA by weight because water and heat are capable to enhance the splitting of TAG into FFA. Brown grease, on the other hand, is defined as grease that contains >15% weight-per-weight of FFA. Table 4 further indicates that the preferred biocatalyst for the commercial process is commercial lipases, which are used in both the free diffusing and immobilized phases.

5. Economic feasibility of lipase catalyzed methods

Although the enzymatic process has been used for oleochemicals production for thirty years, a real commercial enzymatic process still needs to be implemented. The main significant barrier restricting the widespread adoption of oleochemicals production using enzymes is the need to recycle the enzyme. de Sousa et al. (2023) have analyzed the literature based on web off science data. The authors showed that the reported papers ignored / discussed the economic feasibilities in a general way, without in depth analysis. In addition, there are almost no reported studies about scale up. Surprisingly, such lack in the economic feasibilities study is less intense when it comes to enzymatic biodiesel. As in the discussion in section 4, unlike oleochemicals, there are many enzymatic biodiesel companies. It should be mentioned, that economic studies related to lipase catalyzed process is crucial in order to create a robust and valid industrial process. Fig. 8 shows a review about lipase

Table 4
Commercial enzymatic biodiesel production: feedstock, annual production of biodiesel, and supply of lipase.

Name of Company	Country	Source of Lipase	Feedstock	Production/year	Reference
Blue Sun Energy		Callera® Trans L lipase from Thermomyces Lanuginosus	Palm fatty acid distillate, used cooking oil, corn oil	30 million gallons	(Kotrba, 2014)
Buster Biofuels	USA		Brown grease, used restaurant oil, fish oil	5 million gallons	(Kotrba, 2014)
SRS International Co.		Immobilized lipase		5 million gallons	
Viesel Fuel LLC		Eversa Transform® from *A. oryzae*	brown grease, Waste cooking oil,	11 million gallons	(Hobden)
TransBiodiesel Ltd.	Israel	TransZyme A	Used cooking oil, acid oil, animal fat,brown grease	50,000 tons	(Zambare et al., 2021)
EnzymeCore			Low-quality fats and oils with polar lipid content and high free fatty acid	1500 tons	
M-Energy	South Korea		Grease trap, Brown grease extracted from fat, oil, grease	30,000 tons	(Tafesh, 2015)
Olfar	Brazil	Immobilized Callera® Trans L lipase from *Thermomyces Lanuginosus*	Recovered vegetable oil, animal fat, soybean oil	378 million liters	(Andreaus et al., 2019)
Hunan Rivers Bioengineering Co. Ltd.	China	Immobilized Novozym 435® (lipase B from *Candida antarctica*)	Beef tallow, soybean oil	20,000 tons	(Toldrá-Reig et al., 2020)
Lvming and Environmental Protection Technology Co. Ltd.		*Candida* sp. *99–125* Lipase	Waste cooking oil	10,000 tons	(Toldrá-Reig et al., 2020)
Aemetis Biorefinery, Inc.	India		Low grade used cooking oils, Brown grease, palm fatty acid distillate and other plant oil waste feedstocks	50 million gallons	(Aemetis-Inc)

catalyzed synthesis (scientific literature evolution reported from 2011 to 2022):

Even though most of the current publications addressed only the technical feasibility of the enzymatic technology and overlocked the economic viability's importance, there are few considerably related studies that strived the reduction of the overall production cost of the enzymatic processes. Nevertheless, such studies faced two main obstacles: the risk of cross-contamination between the successive batches and the risk of losing the enzyme's activity during processing. Therefore, the current research mainly focuses on finding new strategies to maintain enzyme activity throughout prolonged use. For example, immobilization used to be an effective solution to main enzyme activity; immobilized lipases can be used several times without losing much activity, which favors the overall process economy. Meanwhile, the efficient use of liquid enzymes is receiving much attention due to their positive economic validity in the large-scale production of biodiesel. For this reason, lessons should be taken from these positive success stories and transferred to enzymatic oleochemicals production.

It is quite important that cost of lipase should not be compared directly to the cost of the conventional chemical catalyst; however, the overall process economy should be compared. First, the capital cost of investing in the enzymatic-based business is much cheaper than the chemical technology due to the more straightforward process and less of equipment. Second, lower energy consumption in the enzymatic process makes it more economically attractive (Holm et al., 2018; Mustafa et al., 2023a). Besides all the above, the more environmentally advantageous ENZ method should not be covered. The clean nature of the enzymatically produced products has become a marketing tool as the consumers become more educated and the green-produced products have increased interest. The analysis hinges on various factors that influence the cost of the biocatalyst. It is crucial to meticulously assess the “lipase production cost” (whether through submerged or solid-state cultivation), the “lipase reaction cost” (which considers the substrate and final product), and the “type of support” employed when utilizing immobilized lipase, influencing the chosen immobilization mechanism.

Mustafa et al. (2022a) has developed an ENZ process for monostearin production with a manufacturing cost higher that of the corresponding CHEM process by 10%. However, after including the plant capital costs calculations, the obtained return on investment and net presented value are positive. This emphasizes that the ENZ process can be strongly competitive to the CHEM process. In some instances, not all favorable factors may be available for the analysis. Nevertheless, it is essential to prioritize the most influential factor for the most accurate economic evaluation. For instance, according to Andrade et al. (2019), a pivotal threshold is that the enzyme must be reusable at least 300 times for the process to be economically feasible. Conversely, in other cases, the emphasis might be on reducing the cost of the biocatalyst for immobilization, as exemplified by Budžaki et al. (2017). It's worth noting that the cost of the support material can sometimes represent nearly 90% of the total biocatalyst price. Therefore, optimizing this aspect can be an excellent strategy for the overall process efficiency.

The main recommendations that can be taken out from this article should be linked by the main title of this review. Many efforts should be put in place to encourage the manufacturers investing in ENZ for oleochemicals/biodiesel production. Examples of such are that enzymes provider should not only supply enzyme but also should provide a

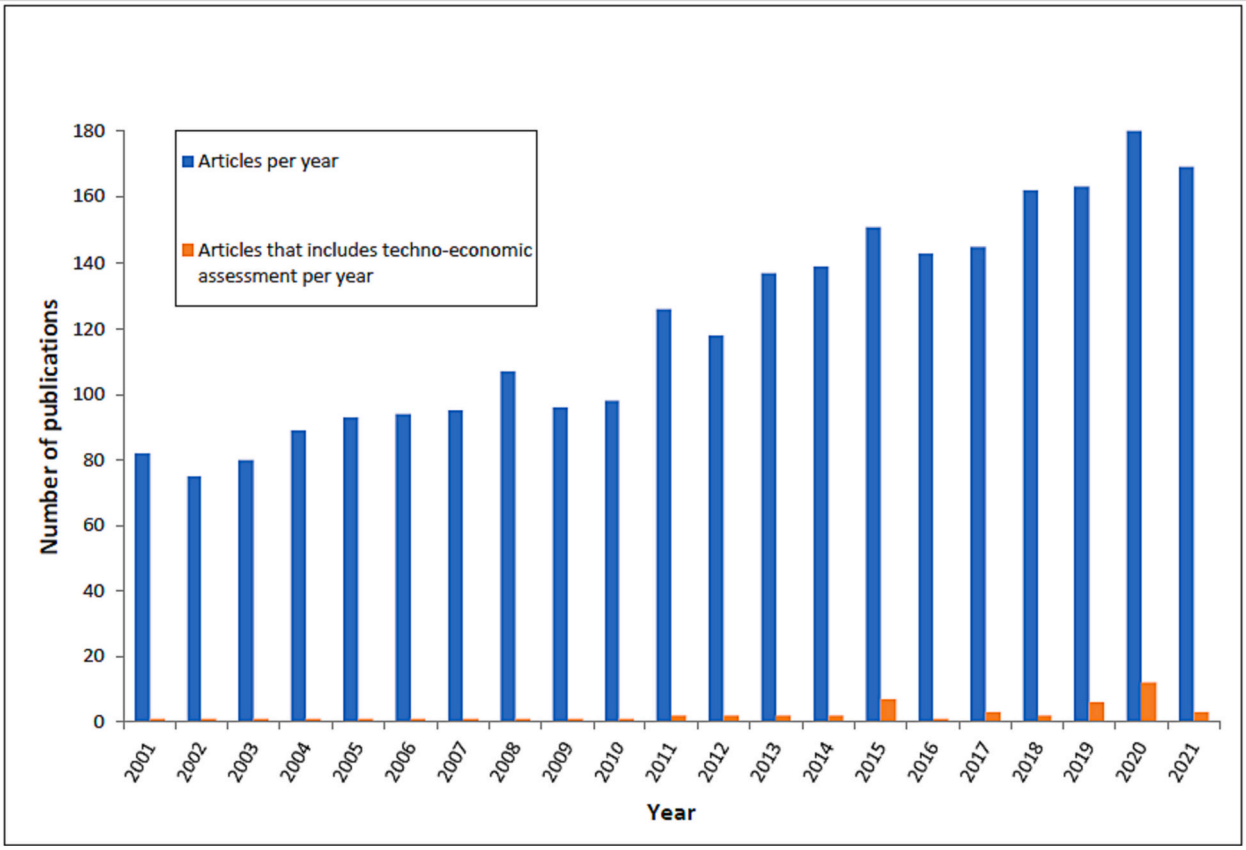

Fig. 8. Lipase catalyzed synthesis (scientific literature evolution reported from 2011 to 2021). Blue bars indicate studies considered only technical feasibility, while orange indicates studies considered the economic feasibility. The survey was obtained from Web of Science database (de Sousa et al., 2023) (MDPI publication, open access). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solution to help and encourage customers to sustain their business. Moreover, the engineering companies that provide the production equipment should align very well with the enzymes producers to offer a well-established and reliable esterification/hydrolysis ENZ technology. The companies interested with applying ENZ technologies should possess qualified staff to properly optimize the ENZ process and therefore to maximize the profit. Finally, the very interesting ease of business due to the cheap investment of the enzymatic process remain the main reason that would continue attracting people.

6. Related governmental policies

Governmental policies for enzymatic BD/OC typically focus on promoting the production and use of BD/OC derived from enzymatic processes. Enzymatic BD/OC production is considered an environmentally friendly and efficient alternative to traditional BD/OC production methods, such as transesterification and esterification. These policies aim to support the growth of the enzymatic biodiesel industry and address various environmental and economic objectives (Borin et al., 2019).

Here are some key aspects of these policies; (1) Incentives and Subsidies: To encourage the adoption of enzymatic products, governments may provide financial incentives and subsidies to OC/BD producers who use enzymatic processes. These incentives involve low-interest loans, tax credits, or grants to help offset the higher initial costs associated with enzymatic production (Demirbas, 2009); (2) Regulatory Framework: Governments establish regulations and standards to ensure the quality and safety of enzymatic OC/BD. These regulations often include guidelines for enzyme usage, product quality, and environmental impact. Compliance with these regulations is necessary for producers to access incentives and sell their products in the market (Janaun and Ellis, 2010); (3) Renewable Energy Mandates: Many governments set renewable energy targets or mandates, requiring a certain percentage of transportation fuels to come from renewable sources, including biodiesel. Enzymatic biodiesel can contribute to meeting these mandates, and producers may receive incentives for complying (Morone et al., 2023); (4) Environmental Initiatives: Enzymatic biodiesel is often promoted as a more environmentally friendly alternative to conventional biodiesel production methods due to reduced energy consumption and fewer chemical byproducts. Government policies may prioritize the use of enzymatic biodiesel in public transportation or government fleets to reduce greenhouse gas emissions (Ng et al., 2010); (5) Education and Outreach: Governments may invest in educational programs and outreach efforts to inform the public, farmers, and OC/BD producers about the benefits of enzymatic biodiesel production. These programs aim to raise awareness and promote the adoption of this technology. (6) Trade and Export Opportunities: Governments may support the export of enzymatic BD/OC and related technologies to promote economic growth and international collaboration in the renewable energy sector (Amigun et al., 2011); (7) Research Partnerships: Governments may foster partnerships between industry stakeholders, research institutions, and universities to accelerate the development and commercialization of enzymatic biodiesel technologies. Overall governmental policies for enzymatic OC/BD aim to create a favorable environment for its production, reduce greenhouse gas emissions, and contribute to energy security and sustainability goals. These policies often combine financial incentives, regulatory frameworks, and public awareness campaigns to support the growth of the enzymatic biodiesel industry.

7. Conclusions

The facility applying the enzymatic process must have a well-established research and development team in order to well optimize the process technically and economically. It is important to compare the costs of the entire process in order to assess the effectiveness of an enzymatic process compared to conventional chemical processes. The yield of the enzyme method is often higher and less purification is required. Overall, enzyme procedures are therefore more affordable than conventional chemical ones. An analysis of the complete process showed that, despite the comparatively high cost of the enzymes in comparison to the chemical catalyst, savings on product purification and isolation, lower energy costs, and increased yields made the enzymatic method economically viable. Currently, the time for implementing the enzymatic processes seems suitable as there are considerable simulations efforts that are being done from all parties, including enzyme manufacturers, researchers, oleochemicals/biodiesel processors, and marketing analysts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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