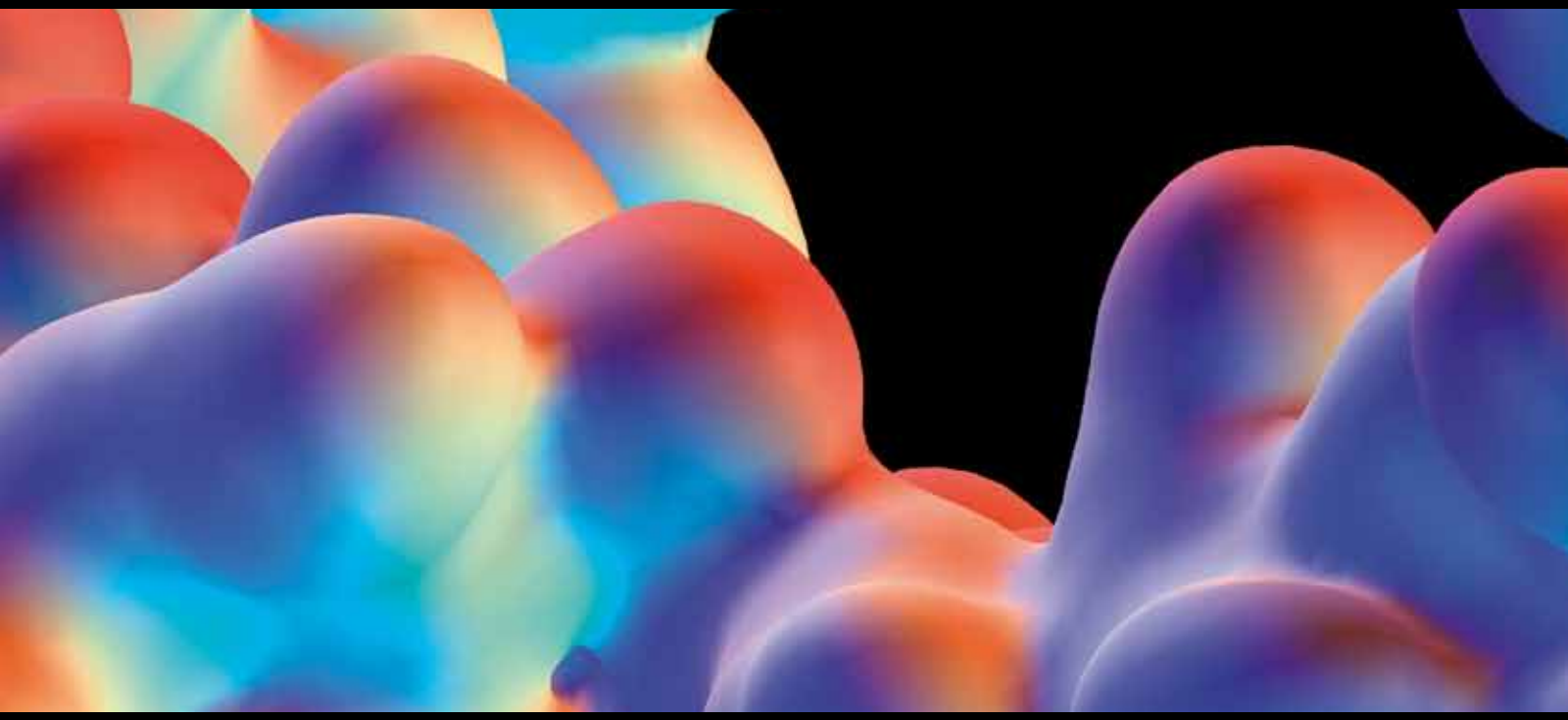
Enzyme Research

Selected Papers from ENZITEC 2010

Guest Editors: Denise M. G. Freire, Elba P. S. Bon, Gustavo Viniegra-Gonzalez, Francisco Gírio, and Robert F. H. Dekker



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Editorial Board

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Editorial

Selected Papers from ENZITEC 2010

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The fundamental need for environmental preservation has been calling, around the world, for the development and deployment of friendly industrial processes. Moreover, presently, development has to take into account sustainability. Within this context, enzyme technology is of outmost importance, having a major role for the achievement of the goals of sustainable development. This technology is of particular significance to Brazil due to the need to preserve Brazil's singular ecosystems, biodiversity, and quality and availability of the country's water resources. Furthermore, Brazil has an unrivalled availability of natural wealth, in both diversity and quantity, to be processed, via biocatalysis, into a wide range of diverse and innovative products as well as into biofules. Enzymes are the logical tool to process renewable resources as both of them have naturally evolved to match each other. It is now, therefore, time to develop dexterity and expand the use of enzymes in a fully efficient manner. As biochemical processes are cleaner than its chemical counterparts and generate higher-quality products, this shift would benefit the country's socioeconomic development.

However, going from traditional chemistry into enzymatic processes is more than a technical and economical challenge, it is a change in the way we think technology—scientific and technical knowledge— and thoughtfulness are much needed.

The Brazilian Seminar on Enzyme Technology (ENZITEC) has been taking place every two years since 2003. Its main objective has been to further the knowledge on the wide range of industrial, technical, and specialty enzymes alongside promoting its economic viability. The event encompasses the worldwide interest in industrial enzyme research and uses *vis-à-vis* the necessary move toward biocatalysis. Eight papers, which were selected from a wealth of submitted manuscripts, were accepted for publication in this special issue of Enzyme Research. They relate to fundamental areas of enzyme research and technology such as microbial screening, enzymes production and characterization, fine chemistry, biorefinery, and biofuels.

Denise M. G. Freire
Elba P. S. Bon
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Research Article

Chemistry Based on Renewable Raw Materials: Perspectives for a Sugar Cane-Based Biorefinery

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Carbohydrates are nowadays a very competitive feedstock for the chemical industry because their availability is compatible with world-scale chemical production and their price, based on the carbon content, is comparable to that of petrochemicals. At the same time, demand is rising for biobased products. Brazilian sugar cane is a competitive feedstock source that is opening the door to a wide range of bio-based products. This essay begins with the importance of the feedstock for the chemical industry and discusses developments in sugar cane processing that lead to low cost feedstocks. Thus, sugar cane enables a new chemical industry, as it delivers a competitive raw material and a source of energy. As a result, sugar mills are being transformed into sustainable biorefineries that fully exploit the potential of sugar cane.

1. Introduction

In a market economy, corporations aim to maximize profit. Governments or society in general impose constraints on and regulate the maximization of profit. Product quality, operational safety, and respect for the environment all have to be fulfilled. To maximize profit, the chemical industry always seeks the most competitive feedstock. When competitive feedstock is available, opportunities for new processes and technologies arise. Technology for converting carbon dioxide into a feedstock source has been developed and used in Brazil since the inception of Pro-álcool, the Brazilian national alcohol program, in the 1970s [1]. Given the legislation, the market demand for sustainable products, and the progress being made in biotechnology and catalysis, developers are working on new processes, which are giving rise to a wide range of biobased products. The old sugar mills (*engenhos*) have become efficient industrial facilities (*usinas*) that produce sweeteners, ethanol, and bioenergy. Recent developments involve transforming sugar mills into biorefineries. Besides sweeteners, biofuel, and bioenergy, the biorefineries will be able to produce bioplastics and other chemicals by using the whole cane via alcohol chemistry and fermentative routes. This essay analyzes the industry's historical response to new developments stemming from the rise of competitive feedstock and the opportunities that it creates. It focuses on sugar cane, its availability as a feedstock source, and the technologies that can turn sugar cane into an important starting point for chemistry of the future. Based on recent examples of processes and market developments, a new arrangement of integrated agroindustry, processing sugar cane and yielding higher value products, is foreseen—a sugar cane biorefinery.

2. The Chemical Industry and the Quest for Feedstock

2.1. Competitive Feedstock. The history of the chemical industry is one of developing new processes for using the most competitive feedstock. Competitive feedstocks make it possible to reach the lowest process cost and are available in

quantities compatible with world-scale production. In early times, chemistry was based on natural products. The most important technology was the extraction and purification of a target compound. Production was characterized by a small production scale. This was because access to feedstock was restricted, often in foreign lands [2]. Discoveries made new natural feedstocks available, thus giving rise to new products. In the 19th century, during the Industrial Revolution, demand was high for coal, which was used to fire the steam engine. As a result, coal exploration was widespread, making coal abundant at low prices. The chemical industry recognized the opportunity. Chemistry based on coal grew as a discipline. Transformation yielded many products, such as organic dyes and pigments. It was not by chance that the chemical industry was established in regions where demand for industrial coal was high, such as Germany. However, production scale still reflected only regional demand and transformation was expensive, not to mention the expensive mining operations. Coal-based chemistry dominated until World War II [3]. In the second half of the 20th century, large-scale automobile consumption led to a high demand for fossil-based fuels. As a consequence, crude oil production improved enormously and huge refineries went on stream in order to provide fuel for individual mobility [4]. Again, the chemical industry recognized the opportunity in the new feedstock. Using by-products from the refineries made it possible to use inexpensive production processes. Petrochemistry was thus born. It is today's most important source of raw materials for the chemical industry. Its characteristics are world-scale production and highly integrated processes. Limitations include costly investment in equipment [4]. Moved by the awareness that it is consuming finite resources and climate change, society is searching for alternative feedstocks. One possibility is the use of carbohydrates.

Nowadays, carbohydrates are largely available worldwide. Improvements in agricultural technology, precision agriculture and plant biotechnology enable high-scale production of carbohydrate at lower cost. New agricultural frontiers and further technological development point to further production growth. Thus, it will be possible to use carbohydrates for both food and chemistry. As an example, Brazilian grain production increased at the same time as bioethanol production [5–7].

Besides their wide availability, carbohydrates are also renewable, in contrast to finite fossil feedstock. All carbon comes from atmospheric carbon dioxide under fixation during photosynthesis. In this process, carbon dioxide and water are transformed into biomass and oxygen, consuming light energy. If a biorenewable feedstock is used as the source of carbon, fossil carbon is not released into the atmosphere. Thus, there is no direct contribution to green house gas production [8].

Table 1 shows the comparison of petrochemicals and biorenewables based on carbon content. The opportunity for the chemical industry offered by carbohydrates can be seen.

The first relevant information is the comparative availability of petrochemicals and sugar cane-based products. This is important in view of the impact of a new process on the price of the feedstock. The second information is the comparative price for carbon in petrochemicals and biorenewables. To analyze the opportunity for the chemical industry, one needs a comparison based on the price for carbon, since this is the actual base of chemistry.

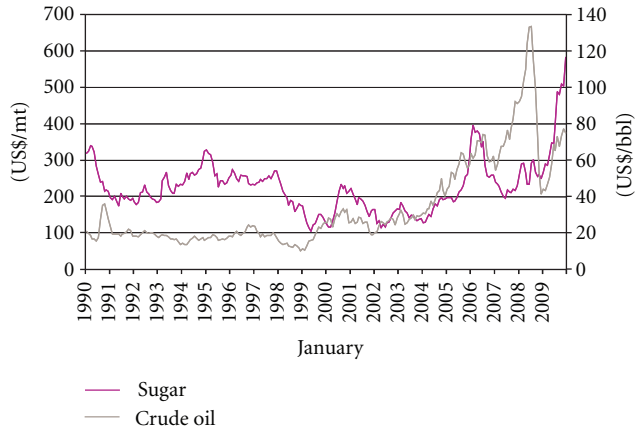


Figure 1: Sugar price (US$/mt) compared to crude oil price (US$/bbl) evolution, source: NYBOT.

Not only the present price is important in evaluating prices, but also the long-term perspective. Crude oil reached a record-high price of US$ 140 in the second half of 2008, and prices are increasing again after the economical crisis. The engine driving this development is the situation known as "peak oil." Starting from zero, production grows over time until it peaks. Then, production irreversibly declines at the same rate at which it grew [9, 10]. Such a development can lead to constant high prices. A projection by the International Energy Agency (IEA) suggests that the production of conventional crude oil reached its peak in 2006, at about 70 million barrels a day [11]. The Energy Information Agency (EIA) estimates that the crude oil barrel price will lie between US$80 and US$100 on the next years [12].

Prices for biorenewables fluctuate due to several factors, including weather. Every time, however, the price is high, there is a trend to overproduction, limiting the price peak to some ranges. Figure 1 shows price evolution of sugar and crude oil from 1990 until 2009.

Not only the abundance and the price make a feedstock competitive. It is also necessary to have the technology to transform it to the desired product under economic conditions. Sugar cane can be converted into chemicals via biotechnological (fermentative) or chemical (alcohol-based catalysis) processes. Both offer a new paradigm for the economy of scale. The development and implementation of new processes, however, are very expensive. Perhaps we are reaching the time when expenses are overcompensated by the better economic performance of biorenewables.

2.2. Bio-Based Products and Sugar Cane. Competitive feedstock and transformation technology are necessary, but not sufficient for a product to be successful. Market acceptance is fundamental to a product. This deals with price, performance, and image.

Table 1: Feedstock comparison based on carbon content.

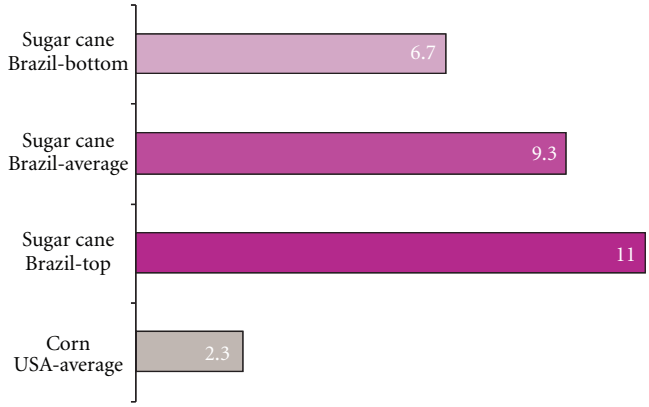
	Formula	Production (million·mt/a)	Price (€/t)	C (%)	H (%)	O (%)	Carbon (€/mt C)
Petrochemicals							
Ethylene	C_2H_4	110	875	86	14		1017
Propylene	C_3H_6	75	778	86	14		905
Benzene	C_6H_6	45	576	92	8		626
Naphta	C_nH_{2n}	362	416	86	14		484
Biorenewables							
Sucrose	$C_{12}H_{22}O_{11}$	172	326	42	6	52	476
Bioethanol	C_2H_6O	39	326	52	13	35	627

Prices as of September, 2009, €1 = US$1.42.
References: *Naphtha* Europe, *Propylene* Europe, *Ethylene* Europe, *Benzene* Europe, *Sugar* Contract number 11, *Ethanol* (Hydrous) Brazil.

Recent studies suggest that biobased products already enjoy market demand. For instance, over a third of Americans and Europeans can be considered "green thinkers" who actively seek and pay more for green products [13]. Opinion research in Brazil leads in the same direction [14]. Nevertheless, a biobased product must perform comparably to other products of the same price. Thus, Brazilian cars are filled with ethanol if it does not exceed 70% of gasoline price [15, 16].

Among the established carbohydrate sources, sugar cane is currently the one showing the best performance. The first reason is attributable to the efficient biochemical photosynthesis pathway, the C_4 route [17]. Secondly, it grows in a tropical environment with sun and water, which are abundant throughout the year. Further, its soluble carbohydrate (sucrose) is ready to use [1]. Over the last 30 years, the development of biofuel technology for production enabled a mature technology for a sugar cane-based economy. Another fundamental aspect is the use of the whole crop, which amounts to a very positive energy balance and the lowest carbon footprint [18]. Besides the sugar, the energy for the sugar mill is obtained from bagasse, the fiber residue from sugar cane after the milling process. On top of that, its energy efficiency is much higher than that of corn and wheat [19], as shown in Figure 2. For these reasons, sugar cane can be considered the green crude, and is capable of supplying the carbon needed by the market at competitive prices and with the sustainability demanded by the society.

2.3. Sugar Cane Processing Development. Although a very significant development in sugar cane technology was noted with Pro-álcool in 1975, the beginning of the development dates back to the 16th century [1]. In 1532, Martim Afonso de Souza introduced sugar cane in Brazil. The Portuguese conqueror brought the technology from Madeira and Azores Islands. From the 16th century until 1930s, sugar cane agriculture focused on sweeteners production, although some spirits were also distilled for consumption in the colony. The sugar mills used man or animal power to extract sugar juice, and production was characterized by low technology [1]. In 1930, the sugar mills were transformed into *usinas* when Brazilian president Getúlio Vargas determined the addition



Source: [18], USDA 2010

Figure 2: Energy efficiency of sugar cane ethanol (bottom), sugar cane ethanol (average), sugar cane ethanol (top), and corn ethanol (USA average) productions: energy efficiency expressed as ratio BOE output/BOE input, BOE = barrel of oil equivalent (Source: [18], [20]).

of 5% (v/v) of ethanol to gasoline, leading to a mix of products: sugar and ethanol. From 1930s until 1990s the traditional *usinas* produced sugar, ethanol from molasses, and used the bagasse to produce steam [21].

In the 1990s the Brazilian energy market was liberalized [22], opening a new outlet opportunity for sugar cane processors. The bagasse surplus present in the *usina* could be converted into bioelectricity in high-pressure boilers [8]. The energy could be sold to the grid. Besides, at the same time, the sugar sector in Brazil was deregulated. As a consequence, innovation turned to be the best way for *usinas* to increase profitability. Since then, the modern *usinas* produce sweeteners, biofuel and bioenergy. The simplified flowchart of a modern *usina* is shown in Figure 3.

3. Routes for Chemicals from Sugar Cane

The production of chemicals based on sugar cane is achieved by two different routes: alcohol chemistry and fermentative routes. Both will be discussed in detail.

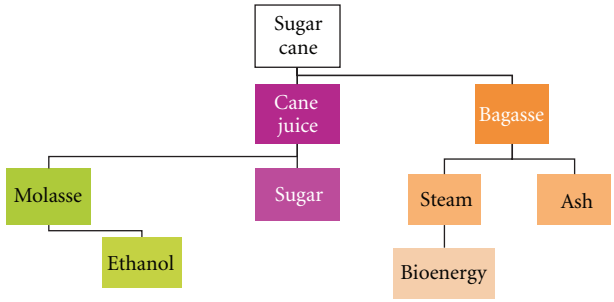


Figure 3: Flowchart of a modern *usina*.

3.1. The Alcohol Chemistry Route. The alcohol chemistry route combines two successful technologies: alcohol fermentation and catalytic processes. Alcohol fermentation has been known since 7,000 B.C.; its efficiency suppressed the petrochemical route of ethanol production. The catalytic processes are the heart of modern chemical industry. High yields are possible at high temperatures and pressure and with expensive investment to drive reaction in the desired direction. A comparison of alcohol chemistry and petrochemical routes shows lower sensitivity to economy of scale for the former.

Many biobased products can be obtained from ethanol via the alcohol chemistry route. The ethylene production from alcohol opens the door to bioplastics production. Salgema produced 100 kilotons per year ethylene based on alcohol in the 1980s for the dichloroethene/polyvinyl chloride (DCE/PVC) production. The production was abandoned due to low crude oil prices in the 1980s, which inhibited the competitiveness of this technology [23]. Ethylene is a precursor of several plastics (e.g., PE, PVC, PVA, polystyrene), and its world market was 128.0 million metric tons in 2008 [24]. Recently, *Braskem, DOW* and *SOLVAY INDUPA* renewed the world's attention to the production of bioethylene [25]. *Braskem S.A.* started production of biopolyethylene in Triunfo-RS, Brazil, in 2010. *DOW Brasil S.A.* announced that it would continue its plan for fully integrated production of polyethylene starting from sugar cane in Minas Gerais, Brazil, and *Solvay Indupa S.A.I.C.* is going to produce PVC from ethanol in its site in Santo André-SP, Brazil [26–28]. The total announced bioplastic production will require 2 million tons of sugar.

Tires can also be made from sugar cane. Synthetic rubber for tire production is made from butadiene, which can be obtained through the catalytic conversion of alcohol. This process was developed in Russia in the early 20th century. The Lebedev process was modified by Carbide and Carbon Chem Corp (USA) in the 1940s. The aim of production was to supply the U.S. Army with tires during World War II [29]. The butadiene production from ethanol was also carried out in Brazil (by *Coperbo*) from 1967 to 1986 with 27.5 kilotons per year capacity. The yield of the process—approximately 70%—and the comparatively high ethanol price in the 1980s limited the process's economic attractiveness [30, 31]. Approximately 350 kilotons of synthetic rubber containing butadiene were produced in Brazil in 2007 [32]. This rubber is made of oil because natural latex is much more expensive. These tires can be produced once again from biorenewable feedstock. Currently there is favorable demand for biomaterials in the automotive industry (market pull). At the same time, improvements in the catalytic reaction selectivity (technology push) can enhance economic attractiveness again.

Further alcohol chemical routes start with the oxidation of ethanol. It yields acetaldehyde, which is an important chemical intermediate for the production of several chemicals, such as acetic acid, peroxiacetic acid, acetic anhydrid, butanol, keten-deketen, crotonaldehyde, pentaerythritol, chloral, pyridine, and acetic esters. This way, ethanol reaches through chemistry different markets in many applications: agricultural, food and packaging, construction, coatings and inks, cosmetics and pharmaceuticals. The oxidation of ethanol and the production of alcohol-based solvents are carried out by *Rhodia Brasil Ltda.* in Paulinia-SP, Brazil [33].

Ethylic ethers are a further class of products derived from ethanol. Ethyl *tert*-butyl ether (ETBE) is an oxygenate gasoline additive that can replace methyl *tert*-butyl ether (MTBE) [34]. It can be a renewable portion of fuel, which conforms with European legislation and is preferred by the mineral oil industry to other biorenewable fuels, such as the direct addition of ethanol to gasoline. As its production requires isobutylene, it is unlikely to take place at the *usina*. *Braskem S.A.* produces in Triunfo-RS, Brazil, 212 kilotons per year of ETBE for the export market [35]. Moreover, diethyl ether can also be obtained from ethanol. It is an important solvent for the chemical industry, used in the production of cellulose plastics, for example, cellulose acetate.

3.2. The Fermentative Route. The fermentative route is based on the conversion of carbohydrate (sugar) by a microorganism to the desired compound. The classical example is ethanol fermentation. The fermentative route is characterized by completely different conditions from the traditional chemical production, thus requiring a different equipment setup. For example, the fermentation is traditionally conducted between 30°C and 37°C and atmospheric pressure. Although in many cases the equipment must be designed to support sterilization, these are mild conditions compared to petrochemical processes. Therefore, the value of necessary equipment investment is considerably lower. A further important difference is related to the purification steps. In the fermentative route, many transformation steps are carried out by one single microorganism and in one single reaction vessel. Consequently, fewer purification steps are required. The process setup is also flexible regarding biological improvement; that is, continuous improvement of the microorganism is possible without any necessary modification to the equipment during production life time. On the other hand, the development of a microorganism exacts a high price for R&D before commercial production may start. The differences to the classical equipment setup allow a paradigm change as regards the economy of scale for fermentative processes.

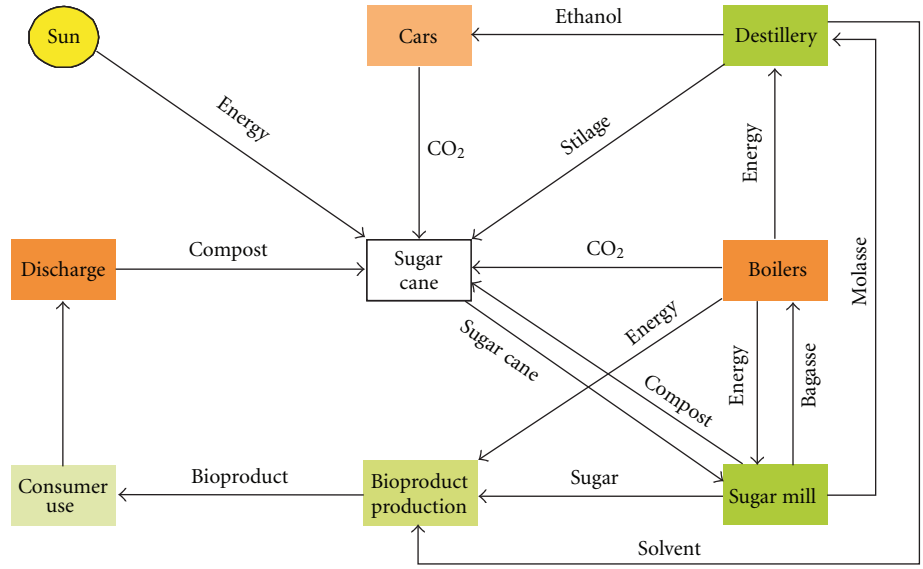


Figure 4: Biorefinery concept: in an efficient biorefinery, mass and energy flow are integrated and recycled in order to provide the maximum efficiency and the lowest cost; the integration into agriculture enables waste streams to be reused in cane plantation and CO_2 to be recycled via photosynthesis.

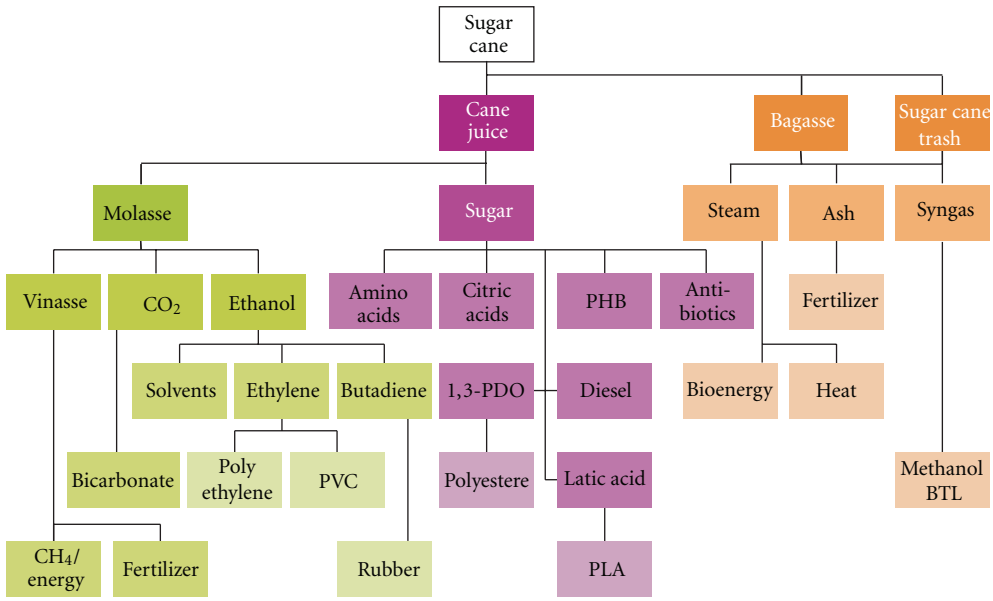


Figure 5: Example of biorefinery flowchart.

The fermentative route has profited from recent developments in biotechnology, such as metabolic engineering, proteomics, and metabolomics. These developments enable debottlenecking or even the construction of new pathways in microorganisms. As a consequence, process performance can be significantly enhanced. Some examples of chemicals from fermentative production will be presented briefly.

Biodegradable plastics can be produced via the fermentative route. Poly(hydroxy-butyrate) (PHB) is a biopolymer obtained from sugar cane through a fully renewable concept. Sugar is the substrate for fermentation, a process that enables the microorganism to accumulate the polymer. The cells are harvested at the end of fermentation and the polymer is recovered from biomass. The production of copolymers is also possible. PHB biodegradability enables special applications, for example, tubes for culture of seedlings, which degrade after the seedling is planted. *Biocycle—PHB Industrial SA* has a pilot plant of PHB production fully integrated in a *usina* using the bagasse as energy [36].

Another example of fermentative production of chemicals is done on an industrial scale by *DuPont* (*E. I. du Pont de Nemours and Company*). At this company, fermentation products are used as a chemical platform. Bio-PDO (1,3-propanediol) is produced via a synthetic biochemical route.

In a subsequent step, the diol undergoes a reaction for polyester formation, yielding a high performance polymer that performs in the same way as the polymer produced via the traditional chemical route [37]. The production facility is located in the USA, where corn is used as source of carbohydrate. Nevertheless, sugar from sugar cane could be used instead of corn glucose.

Amino acids are also produced via the fermentative route. They are constituent of protein. Some aminoacids are therefore important feed additive. As protein constituents, amino acids are used by the pharmaceutical industry in the preparation of parenteral nutrition. Other amino acids have important applications in the food industry [38, 39]. In Brazil, 200 kilotons per year of the amino acid lysine were produced in 2008 for animal nutrition [40]. The amino acid production is an example of how genetic engineering increases the competitiveness of the fermentative route. Thus, *Evonik Degussa GmbH* succeeded in producing amino acids on a commercial scale by fermentation. In the past, the amino acids were obtained by the hydrolysis of protein containing animal products [41, 42].

Solvents can also be obtained by fermentation. The fermentation of wild type *Clostridium* yields a mixture of solvents containing acetone, butanol and ethanol (ABE). ABE industrial technology was developed by Chaim Weizmann in 1916 for the acetone production. The ABE fermentation process was used in Eastern Europe, South Africa and China on an industrial scale until the 1990s [43]. The products find application as chemical building block, solvents and biofuel. The process has natural limitations like the products mixture, the maximum final yield and concentration, combined with high energy demand for purifying the products. Nevertheless, ongoing industrial development (*Butamax Advanced Biofuels LLC* as joint-venture from *DuPont & BP*) is opening up new perspectives for this process. Several patents have been applied for the exclusive production of Butanol to be used as renewable fuel additive [44, 45].

3.3. Further Developments. Besides existing processes, we can observe the intense development of new processes. This fact is coherent, given the assumption that the competitiveness of biorenewable feedstock is increasing. One important example is the company *Amyris*. *Amyris Biotechnologies Inc.* developed a pathway to obtain renewable diesel and jet fuel from sugar cane by fermentation. The substance called "farnesene" can now be produced by genetically engineered yeast. A pilot-scale production facility is located in Campinas-SP, Brazil, while an industrial scale production *usina* is under construction in Pradopolis-SP, Brazil [46, 47].

Another new development is the gasification of bagasse and sugar cane trash (includes cane tops and stalk leaves) that yields biomass-to-liquid (BTL). Bagasse and sugar cane trash are abundant at the *usinas*, and its gasification leads to syngas. From syngas it is possible to obtain liquid fuels, methanol, hydrogen and other substances via catalytic reactions [48]. This technology replaces fossil fuel by renewable biomass. However, the industrial application is limited by the economics.

Beyond the described activities, many other companies and partnerships participate in this technological development, for example, *DSM/Roquette, Novozymes/Cargill, Shell/Virent, Genencor/DuPont and Perstorp* [49–53]. As confidentiality is necessary to enable advantages, the announcements represent only a small fraction of research activities, so much more has not been disclosed.

4. Sustainability and Production Facilities

One of the major drivers for the market acceptance of biobased products is consumers' awareness of climate change. Therefore, it is fundamental for biobased products to feature their ability to reduce the carbon footprint. Moreover, the present development anticipates future environmental protecting legislation. Thus, proactive action will provide a competitive advantage.

The biobased production, when integrated in a *usina* to form a biorefinery, enables highly sustainable processes. On one hand, sugar cane provides a highly efficiently exploited source of carbon for the chemical industry. Sugar cane agriculture has been deemed more sustainable than other comparable feedstock sources such as corn [19]. On the other hand, the use of bagasse and sugar cane trash as source of energy for the process, which avoids the use of fossil fuels, has a further positive impact on the carbon footprint. Figure 4 shows the concept of a biorefinery.

One example is the production of bioplastics from sugar cane in a biorefinery. The industry average carbon dioxide emission is 1.8 kg of CO_2 eq. per kg of polyethylene produced from conventional resources [54], while for polyethylene from sugar cane it is 2.1 kg of CO_2 eq. per kg of polyethylene [55], because when sugar cane grows, it captures carbon dioxide. This way, the total avoided emission is 3.9 kg of CO_2 eq. per kg of polyethylene.

5. Conclusion and Outlook

We can see the production of chemicals based on carbohydrates, especially on sugar cane, accelerating. Old processes are being operated anew, new processes are going on-stream and R&D is taking on an impressive dimension. Thus, we can assume that in the future, sugar cane will be used to produce a wide range of products: sweeteners, biofuel, bioenergy, bioplastics and other chemicals. At the same time, the vision of the *usinas* being established at sites where many sugar cane-based processes are integrated is becoming a reality. This is possible, as the production integration into a biorefinery enhances the competitiveness of bioprocesses. The possibilities of inclusion in a biorefinery flowchart are shown in Figure 5. As there is a wide range of processes and products, the combination of routes is possible, which allows the biorefinery to be very flexible.

The enormous perspectives for biobased production and biorefineries correlate with corresponding challenges. An important question to be answered is: who are going to be the players able to manage all the relevant issues? According to a study published by McKinsey, successful players will

be companies with a strategic fit to the biobased chemical business, with a competitive advantage through Intellectual Property, fermentation know-how, market intelligence, with competence in access to feedstock, metabolic and genetic engineering, fermentation stream know-how, downstream processing, processing capacity, product integration, marketing and distribution, and with conditions for high R&D investments [56]. We should remember that the development may take 10 years to reach the market. To cope with all challenges, partnering along the value chain is an option that should be used frequently.

At present, we can observe that R&D centers are often located in the USA and Europe, but there are increasing activities in Brazil, too. For production, Brazil is the most economically attractive choice due to its superior sugar cane economics, well-developed infrastructure, timing and availability of greenfield opportunities, but there are also opportunities in China, some countries of Southeast Asia and of Africa [56].

It is undeniable that sugar cane has its own chemistry. Sugar cane delivers a competitive raw material and energy to the chemical industry. *Usina* production integration allows its transformation into a biorefinery that can use all the sugar cane potential. There is already an existing market for sugar cane-based chemistry. Alcohol chemistry products, amino acids, bioplastics, solvents and many other products are enjoying growing market demand, favored by the growing number of "green thinker" consumers, who are willing to consume biobased products made, for example, from sugar cane. New options regarding the economy of scale and new technologies make biobased products economically attractive, as proved by many investments on the sugar cane value chain in recent time. Brazil has an outstanding position, where the development of the technology and processes for sugar cane-based products is already taking place.

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Research Article

Chiral Pharmaceutical Intermediaries Obtained by Reduction of 2-Halo-1-(4-substituted phenyl)-ethanones Mediated by *Geotrichum candidum* CCT 1205 and *Rhodotorula glutinis* CCT 2182

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Enantioselective reductions of *p*-R_1-$C_6H_4C(O)CH_2R_2$ (R_1 = Cl, Br, CH_3, OCH_3, NO_2 and R_2 = Br, Cl) mediated by *Geotrichum candidum* CCT 1205 and *Rhodotorula glutinis* CCT 2182 afforded the corresponding halohydrins with complementary *R* and *S* configurations, respectively, in excellent yield and enantiomeric excesses. The obtained (*R*)- or (*S*)-halohydrins are important building blocks in chemical and pharmaceutical industries.

1. Introduction

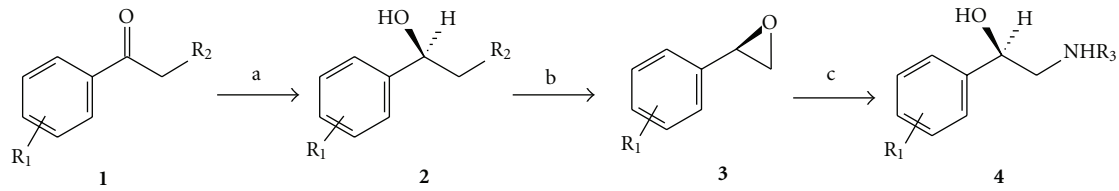
Chiral halohydrins are important and valuable intermediates in the synthesis of fine chemicals and pharmaceuticals as optically active 1,2-aminoalcohols. The halohydrin (*R*)-1-aryl-2-haloethanol may be used for the preparation of (*R*)-1-aryl-2-aminoethanols that are used as α- and β-adrenergic drugs.

An interesting chemoenzymatic synthetic route to obtain optically active 1-aryl-2-ethanolamines is from the enantioselective reduction of the correspondent α-haloacetophenones giving halohydrins that are transformed into an epoxy that reacts with the appropriate amine (Scheme 1) [1, 2].

An enormous potential of the use of microorganisms and enzymes for the transformation of synthetic chemicals with high chemo-, regio-, and enantioselectivity has been increasing in the pharmaceutical industry [3]. The dehydrogenases in the form of whole cells for the production of chiral styrene oxides have been used on a pilot-plant scale [4]. Therefore, a large number of papers have appeared reporting the enantiomeric reduction of α-bromoacetophenone [5–10] and α-chloroacetophenone [4, 6, 7, 11–17] by whole cells of microorganism and also by isolated enzyme [18] giving halohydrins in high enantiomeric excesses (ee).

There are few examples of biocatalytic reduction of α-haloacetophenone having suitable substituted group attached to the aromatic ring for enantioselective preparation of some target 1-aryl-2-ethanolamines [2, 19]. It is known that some examples of biocatalytic reductions of α-haloacetophenone that have substituted groups like 3-chloro [20, 21], 4-nitro [10, 22], and 3,4-methylenedioxy [23–25] were mediated by a number of microorganisms. Also, isolated enzymes have been used to reduce α-haloacetophenone having various kinds of substituted groups [26, 27].

The performances of *Rhodotorula glutinis* CCT 2182 and *Geotrichum candidum* CCT 1205 in bioreduction of α-haloacetophenone have been calling our attention due to the efficiency and complementary enantioselectivity of these microorganisms giving the corresponding (*R*)- and (*S*)-halohydrins in high ee, respectively [8]. Also, those microorganisms show the same efficiency in the reduction of α-azido-*para*-substituted acetophenones [28]. In this work, we use those two microorganisms for reduction of α-bromo- and α-chloroacetophenones having *para*-substituted groups to produce separately both enantiomers of halohydrins that can be used as chiral building blocks for preparations of the corresponding 1,2-aminoalcohols.



R_1 = substituent group; R_2 = Cl or Br; R_3 = H, alkyl or aryl group

Scheme 1: (a) reduction using chiral catalytic reagent or biocatalytic process; (b) base; (c) amine.

2. Materials and Methods

IR spectra were recorded on a Bomem MB Series spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 spectrometer in $CDCl_3$. Gas chromatographic analyses were performed using a Shimadzu GC/MS Class 5000, with helium as carrier gas. The fused silica capillary columns used were either a Supelco Simplicity ITM (30 m × 0.25 mm × 0.25 μm) and a chiral GC-column CHIRASILDEX (30 m × 0.25 mm × 0.25 μm). Optical rotation was measured with a J-720, VRDM306 JASCO, 589.3 nm (25°C) spectropolarimeter. The melting points were obtained in MQAPF-301-MicroQuímica equipment.

The 2-bromo-1-(4-substituted phenyl)-1-ethanones **1a-e** were *obtained* with brominating 4-substituted acetophenones in CH_2Cl_2 at 0°C, and 2-chloro-1-(4-substituted phenyl)-1-ethanones **1f-j** were prepared applying the Wyman and Kaufman methodology [29] by chlorination of corresponding 4-substituted acetophenones with sulfuryl chloride in CH_2Cl_2 at 0°C. All other reagents and solvents were reagent grade.

The racemic 2-halo-1-(4-substituted phenyl)-ethanols **2a-j**, used as reference for the determination of ee in a GC provided with a chiral column, were obtained by reacting the corresponding **1a-j** with $NaBH_4$ in water/methanol at rt. All other solvents and reagents were reagent grade.

2.1. Growth Conditions for Microorganisms Culture. The microorganisms *Geotrichum candidum* CCT 1205 (isolated from industrial waste water treatment—Preston, United Kington) and *Rhodotorula glutinis* CCT 2182 (isolated from *Psidium guajava*—Atlantic Rainforest, Brazil) were stored at "André Tosello" Research Foundation (Campinas, Brazil) [30]. *G. candidum* was cultivated in 400 mL of nutrient broth **1** (10 g/L malt extract, 5 g/L peptone, 10 g/L glucose, 3.12 g/L K_2HPO_4, and 11.18 g/L KH_2PO_4) at 28°C, and *R. glutinis* was cultivated in 400 mL of nutrient broth **2** (3 g/L Yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L glucose) at 30°C. Both yeasts were incubated for 2 days on an orbital shaker (200 rpm) before use. All materials and medium were sterilized in an autoclave at 121°C before use and the yeasts were manipulated in a laminar flow cabinet.

2.2. General Procedure for Bioreduction of 2-Halo-1-(4-substituted phenyl)-ethanones. The yeasts were incubated for two days (400 mL nutrient broth in Erlenmeyer of 1 L). After that, the ketone **1** (2 mmol) dissolved in 1.5 mL of ethanol was added directly to the suspension where the yeasts grew. The resulting suspension was stirred in an orbital shaker (200 rpm) at 28°C for *G. candidum* and at 30°C for *R. glutinis* until the full conversion of **1** (18 h). The product was extracted with CH_2Cl_2 and purified by flash silica gel column chromatography using hexane/ethyl acetate (7 : 3).

*2.3. (S)-(+)-2-Bromo-1-(4-bromophenyl)ethanol (S)-**2a**.* The bioreduction of ketone **1a** (0.556 g, 2 mmol) by *Geotrichum candidum* CCT 1205 furnished (*S*)-**2a** (0.540 g with 96.4%) as colorless solid, m.p. 72°C; $[\alpha]_D^{25}$ +40.0° (*c* 1, $CHCl_3$) [lit. −31.0°, *c* 2.9, $CHCl_3$ for *R* isomer, 94% ee] [31], giving an optical purity of >99% determined by GC using a chiral column; IR (KBr): 3402, 3086, 3064, 3049, 3026, 2958, 2922, 2852, 1593, 1488, 1420, 1402, 1218, 1192, 1071, 1010, 828, 722, 680, 613 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.74 (s, 1H, OH), 3.46 (dd, 1 H, J = 8.4 Hz and 11.3 Hz, CH_2), 3.59 (dd, 1 H, J = 3.6 Hz and 11.3 Hz, CH_2), 4.87 (dd, 1H, J = 3.6 Hz and 8.4 Hz, CH), 7.24–7.31 (m, 2H, Ph), 7.48–7.51 (m, 2H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 39.72, 72.97, 122.12, 127.43, 131.55, 138.99; MS *m/z* (rel. int. %): 188 (5), 187 (71), 186 (7), 185 (79), 183 (4), 182 (2), 171 (2), 169 (2), 159 (13), 158 (2), 157 (17), 155 (4), 120 (4), 119 (2), 106 (3), 105 (6), 103 (4), 102 (9), 91 (14), 90 (6), 89 (7), 79 (6), 77 (100), 75 (18), 78 (46), 76 (16), 50 (62), 51 (57), 43 (39).

*2.4. (R)-(−)-2-Bromo-1-(4-bromophenyl)ethanol (R)-**2a**.* The bioreduction of ketone **1a** (0.556 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 furnished (*R*)-**2a** (0.554 g, 99.0% yield) as colorless solid, m.p. 72°C; $[\alpha]_D^{25}$ −40.4° (*c* 1, $CHCl_3$) [lit. −31.0°, *c* 2.9, $CHCl_3$ for *R* isomer, 94% ee] [31], giving an optical purity of >99% determined by GC using a chiral column; ^{1}H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

*2.5. (S)-(+)-2-Bromo-1-(4-chlorophenyl)ethanol (S)-**2b**.* The bioreduction of ketone **1b** (0.467 g, 2 mmol) by *Geotrichum candidum* CCT 1205 furnished (*S*)-**2b** (0.448 g, 95.1% yield) as colorless oil; $[\alpha]_D^{25}$ +38.7° (*c* 1, $CHCl_3$) [lit. 38.6°, *c* 1.15, $CHCl_3$ for *S* isomer, 91% ee] [32], giving an optical purity of >99% determined by GC using a chiral column; IR (film): 3392, 3088, 3051, 3030, 3003, 2957, 2896, 1596, 1492, 1428, 1408, 1338, 1310, 1256, 1310, 1256, 1199, 1173, 1089, 1072, 1013, 973, 944, 897, 834, 778, 752, 704, 674 cm^{-1}; ^{1}H NMR

(300 MHz, $CDCl_3$) δ 2.74 (sl, 1H, OH), 3.48 (dd, 1H, J = 7.0 Hz and 11.3 Hz, CH_2), 3.61 (dd, 1H, J = 5.8 Hz and 11.3 Hz, CH_2), 4.87 (dd, 1H, J = 5.8 Hz and 7.0 Hz, CH), 7.24–7.28 (m, 2H, Ph), 7.31–7.51 (m, 2H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$) δ 39.72, 72.97, 122.12, 127.43, 131.56, 139.00; MS *m/z* (rel. int. %): 143 (29), 142 (11), 141 (100), 139 (4), 138 (2), 121 (7), 115 (6), 113 (16), 112 (6), 111 (4), 108 (7), 107 (5), 105 (4), 104 (2), 103 (2), 102 (2), 91 (2), 89 (1), 79 (11), 78 (9), 77 (70), 76 (2), 75 (15), 74 (9), 70 (6), 63 (8), 51 (28), 50 (30), 49 (3), 44 (22), 43 (20), 40 (32).

*2.6. (R)-(−)-2-Bromo-1-(4-chlorophenyl)ethanol (R)-**2b**.* The bioreduction of ketone **1b** (0.467 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 furnished (*R*)-**2b** (0.457 g, 97.0% yield) as colorless oil; $[\alpha]_D^{25}$ −38.7 (*c* 1, $CHCl_3$) [lit. 38.6, *c* 1.15, $CHCl_3$ for *S* isomer, 91% ee] [32], giving an optical purity of >99% determined by GC using a chiral column; ^{1}H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

*2.7. (S)-(+)-2-Bromo-1-(4-methylphenyl)ethanol (S)-**2c**.* The bioreduction of ketone **1c** (0.426 g, 2 mmol) by *Geotrichum candidum* CCT 1205 furnished (*S*)-**2c** (0.413 g, 96.0% yield) as colorless oil; $[\alpha]_D^{25}$ +48.3° (*c* 1, $CHCl_3$) [lit. +41.8°, *c* 1.0, $CHCl_3$ for *S* isomer, 95% ee] [32, 33], giving an optical purity of >99% determined by GC using a chiral column; IR (film): 3378, 3064, 3044, 2971, 2931, 2907, 2836, 1612, 1585, 1511, 1458, 1443, 1368, 1300, 1243, 1205, 1174, 1115, 1087, 1069, 1034, 1004, 898, 830, 807 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.32 (s, 3H, CH_3), 2.46 (sl, 1H, OH), 3.50 (dd, 1H, J = 8.7 Hz and 10.4 Hz, CH_2), 3.62 (dd, 1H, J = 3.4 Hz and 10.4 Hz, CH_2), 4.87 (dd, 1H, J = 3.4 Hz and 8.7 Hz, CH), 7.18–7.26 (d, 2H, J = 8 Hz, Ph), 7.30 (d, 2H, J = 8.0 Hz, Ph); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.16, 40.12, 73.67, 125.91, 129.32, 137.32, 138.16; MS *m/z* (rel. int. %): 217–215 (M^+, 2-2), 202 (2), 200 (2), 138 (7), 137 (93), 136 (16), 135 (13), 134 (45), 123 (1), 122 (2), 121 (2), 120 (3), 119 (5), 118 (5), 117 (8), 115 (4), 110 (4), 109 (49), 108 (4), 107 (4), 105 (4), 104 (2), 103 (4), 102 (2), 95 (3), 94 (34), 93 (43), 92 (9), 91 (38), 90 (2), 89 (5), 81 (2), 79 (9), 78 (8), 77 (30), 76 (4), 75 (3), 74 (4), 68 (5), 67 (3), 66 (13), 65 (20), 64 (11), 63 (21), 62 (8), 61 (3), 55 (4), 54 (1), 53 (10), 52 (8), 51 (30), 50 (19), 49 (1), 45 (8), 44 (6), 43 (100), 41 (12), 40 (10).

*2.8. (R)-(−)-2-Bromo-1-(4-methylphenyl)ethanol (R)-**2c**.* The bioreduction of ketone **1c** (0.426 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 furnished (*R*)-**2c** (0.410 g, 95.3% yield) as colorless oil; $[\alpha]_D^{25}$ −48.3 (*c* 1, $CHCl_3$) [lit. +41.8°, *c* 1, $CHCl_3$ for *S* isomer] [32], giving an optical purity of >99% determined by GC using a chiral column; ^{1}H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

*2.9. (S)-(+)-2-Bromo-1-(4-methoxyphenyl)ethanol (S)-**2d**.* The bioreduction of ketone **1d** (0.458 g, 2 mmol) by *Geotrichum candidum* CCT 1205 gave (*S*)-**2d** (0.453 g, 98.0% yield) as colorless oil; $[\alpha]_D^{25}$ +19.8° (*c* 1, $CHCl_3$) [lit. −37.7°, *c* 1.0, $CHCl_3$ for *R* isomer, 87% ee] [31, 34], IR (film): 3371, 3062, 3030, 2973, 2928, 2907, 2878, 1616, 1581, 1511, 1458, 1442, 1368, 1300, 1240, 1205, 1174, 1112, 1081, 1069, 1024, 1001, 892, 830, 804 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.72 (sl, 1H, OH), 3.61 (dd, 1H, J = 8.7 Hz and 11.2 Hz, CH_2), 3.70 (dd, J = 3.9 Hz and 11.2 Hz, CH_2), 3.79 (s, 3H, CH_3), 4.86 (dd, 1H, J = 3.9 Hz and 8.7 Hz, CH), 6.89 (d, 2H, J = 8.8 Hz, Ph), 7.31 (d, 2H, J = 8.8 Hz, Ph); ^{13}C NMR (75 MHz, $CDCl_3$) δ 42.12, 56.28, 78.95, 114.94, 127.71, 133.40, 160.03; MS *m/z* (rel. int. %): 233–231 (M^+, 1-1), 218 (1), 215 (1), 214 (1), 202 (2), 200 (2), 153 (2), 152 (4), 151 (2), 138 (6), 137 (100), 135 (11), 134 (9), 122 (2), 121 (2), 120 (2), 119 (20), 110 (3), 109 (16), 108 (3), 107 (2), 105 (2), 104 (1); 103 (4), 102 (2), 95 (3), 94 (16), 93 (2), 92 (7), 91(18), 90 (2), 89 (4), 81 (2), 79 (6), 78 (5), 77 (21), 76 (2), 75 (3), 68 (2), 67 (2), 66 (5), 65 (12), 64 (9), 63 (6), 55 (1), 54 (1), 53 (8), 52 (4), 51 (12), 50 (14), 45 (3), 44 (2), 43 (79), 41 (10), 40 (8).

*2.10. (R)-(−)-2-Bromo-1-(4-methoxyphenyl)ethanol (R)-**2d**.* The bioreduction of ketone **1d** (0.458 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 gave (*R*)-**2d** (0.452 g, 97.8% yield) as colorless oil; $[\alpha]_D^{25}$ −19.7 (*c* 1, $CHCl_3$) [lit. −37.7°, *c* 1.0, $CHCl_3$ for *R* isomer, 87% ee] [31], ^{1}H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

*2.11. (S)-(+)-2-Bromo-1-(4-nitrophenyl)ethanol (S)-**2e**.* The bioreduction of ketone **1e** (0.488 g, 2 mmol) by *Geotrichum candidum* CCT 1205 gave (*S*)-**2e** (0.480 g, 97.6% yield) a light yellow solid, mp 98°C; $[\alpha]_D^{25}$ +25.0° (*c* 1, $CHCl_3$) [lit. +32.1°, *c* 1, $CHCl_3$ for *S* isomer, 91% ee] [33, 35], giving an optical purity of >99% determined by GC using a chiral column. IR (KBr): 3455, 3109, 3079, 2947, 2924, 2889, 2851, 1601, 1520, 1347, 1291, 1203, 1074, 1012, 855, 760, 730 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.83 (sl, 1H, OH), 3.53 (dd, 1H, J = 8.4 Hz and 10.6 Hz, CH_2), 3.68 (dd, 1H, J = 3.5 Hz and 10.6 Hz, CH_2), 5.03–5.08 (m, 1H, CH), 7.45 (d, 2H, J = 8.8 Hz, Ph), 8.22 (d, 2H, J = 8.8 Hz, Ph);^{13}C NMR (75 MHz, $CDCl_3$): δ 39.32, 72.52, 123.60, 126.65, 146.10, 146.90; MS *m/z* (rel. int. %): 153 (8), 152 (100), 149 (2), 141 (1), 139 (1), 136 (2), 127 (1), 125 (2), 122 (5), 106 (10), 105 (9), 102 (4), 95 (5), 94 (11), 91 (8), 78 (13), 77 (17), 66 (6), 51 (17), 50 (13), 43 (20).

*2.12. (R)-(−)-2-Bromo-1-(4-nitrophenyl)ethanol (R)-**2e**.* The bioreduction of ketone **1e** (0.488 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 gave (*R*)-**2e** (0.483 g, 98.0% yield) a light yellow solid, mp 98°C; $[\alpha]_D^{25}$ −25.0° (*c* 1, $CHCl_3$) [lit. +32.1°, *c* 1, $CHCl_3$ for *S* isomer, 91% ee] [33, 36], giving an optical purity of >99% determined by GC using a chiral column; ^{1}H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

*2.13. (S)-(+)-2-Chloro-1-(4-bromophenyl)ethanol (S)-**2f**.* The bioreduction of ketone **1f** (0.467 g, 2 mmol) by *Geotrichum candidum* CCT 1205 gave (*S*)-**2f** (0.468 g, 99.4% yield) as colorless oil; $[\alpha]_D^{25}$ +35.0° (*c* 1, $CHCl_3$) [lit. −35.87°, *c* 1.1072, $CHCl_3$ for *R* isomer, 99% ee] [14], giving an optical purity of >99% determined by GC using a chiral column; IR (film):

3421; 3106; 3087; 3064; 3031; 3006; 2956; 2895; 1593, 1575, 1494; 1453; 1426; 1387; 1336; 1300; 1295; 1248; 1200; 1085; 1064; 1074; 1030; 1012; 972; 944; 917; 869; 824; 768; 724; 698 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2.41 (sl, 1H, OH), 3.61 (dd, 1H, J = 8.7 Hz and 11.3 Hz, CH_2), 3.73 (dd, 1H, J = 3.4 Hz and 11.3 Hz, CH_2), 4.88 (dd, 1H, J = 3.4 Hz and 8.7 Hz, CH), 7.28 (d, 2H, J = 8.4 Hz, Ph), 7.51 (d, 2H, J = 8.4 Hz, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 50.90, 74.12, 122.04, 127.28, 132.02, 139.46; MS *m/z* (rel. int. %): 238–236 (M^+, 1-1), 202 (1), 200 (1), 158 (1), 156 (3), 108 (7), 107 (100), 105 (5), 104 (2), 103 (4), 102 (1), 91 (6), 89 (1), 79 (60), 78 (8), 77 (42), 76 (2), 75 (2), 51 (31), 50 (13).

*2.14. (R)-(−)-2-Chloro-1-(4-bromophenyl)ethanol (R)-**2f**.* The bioreduction of ketone **1f** (0.467 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 gave (*R*)-**2f** (0.460 g, 97.7% yield) as colorless oil; $[\alpha]_D^{25}$ −34.9 (*c* 1, $CHCl_3$) [lit. −35.87°, *c* 1.1072, $CHCl_3$ for *R* isomer, 99% ee] [14], giving an optical purity of >99% determined by GC using a chiral column; ^1H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

*2.15. (S)-(+)-2-Chloro-1-(4-chlorophenyl)ethanol (S)-**2g**.* The bioreduction of ketone **1g** (0.378 g, 2 mmol) by *Geotrichum candidum* CCT 1205 furnished (*S*)-**2g** (0.363 g, 95.0% yield) as colorless oil; $[\alpha]_D^{25}$ +48.3° (*c* 1.25, $CHCl_3$) [lit. $[\alpha]_D^{20}$ 44.2° (*c* 2.1, $CHCl_3$) for *S* isomer, 96,6% ee] [36], giving an optical purity of >99% determined by GC using a chiral column; IR (film): 3387, 3103, 3090, 3067, 3053, 3020, 2956, 2894, 1598, 1492, 1427, 1410, 1338, 1308, 1252, 1198, 1090, 1075, 1013, 970, 947, 895, 871, 833, 776, 751, 704, 673 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.2 (sl, 1H, OH), 3.58 (dd, 1H, J = 8.4 Hz and 11.3 Hz, CH_2), 3.67 (dd, 1H, J = 3.7 Hz and 11.3 Hz, CH_2), 4.84 (dd, 1H, J = 3.7 Hz and 8.4 Hz, CH), 7.27–7.34 (m, 4H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 50.39, 73.15, 127.16, 128.27, 128.48, 133.82, 138.09; MS *m/z* (rel. int. %): 192-191 (M+, 4), 158 (2), 156 (7), 143 (11), 142 (3), 141 (38), 139 (4), 138 (2), 121 (7), 115 (6), 114 (3), 113 (19), 112 (6), 111 (5), 105 (2), 103 (5), 102 (2), 101 (1), 91 (2), 89 (1), 87 (1), 85 (2), 78 (8), 77 (77), 75 (13), 74 (7), 73 (3), 70 (7), 71 (2), 65 (2), 63 (5), 62 (3), 61 (2), 60 (1), 55 (1), 53 (3), 52 (6), 51 (33), 50 (23), 49 (3), 46 (1), 45 (11), 44 (4), 42 (100), 41 (1), 40 (1).

*2.16. (R)-(−)-2-Cloro-1-(4-chlorophenyl)ethanol (R)-**2g**.* The bioreduction of ketone **1g** (0.378 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 furnished (*R*)-**2g** (0.36 g, 94.2% yield) as colorless oil; $[\alpha]_D^{25}$ −48.3° (*c* 2.1, $CHCl_3$); [lit. $[\alpha]_D^{20}$ 44.2° (*c* 2.1, $CHCl_3$) for *S* isomer, 96,6% ee] [36], giving an optical purity of >99% determined by GC using a chiral column; ^1H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

*2.17. (S)-(+)-2-Chloro-1-(4-methylphenyl)ethanol (S)-**2h**.* The bioreduction of ketone **1h** (0.337 g, 2 mmol) by *Geotrichum candidum* CCT 1205 furnished (*S*)-**2h** (0.329 g, 96.4% yield) as colorless oil; $[\alpha]_D^{25}$ +48.3° (*c* 1.1, $CHCl_3$) [lit. +47.2° (*c* 1.1, $CHCl_3$) for *S* isomer, 92% ee] [32], giving an optical purity of >99% determined by GC using a chiral column; IR (film): 3414, 3094, 3052, 3017, 2970, 2924, 2863, 1611, 1512, 1445, 1411, 1369, 1302, 1280, 1257, 1192, 1181, 1112, 1090, 1071, 1010, 941, 892, 813, 724 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 2,34 (s, 3H, CH_3), 2.50 (sl, 1H, OH), 3.63 (dd, 1 H, J = 8.5 Hz and 11.2 Hz, CH_2), 3.74 (dd, 1H, J = 3.9 Hz and 11.2 Hz, CH_2), 4.85 (dd, 1H, J = 3.9 and 8.5 Hz, CH), 7.20 (d, 2H, J = 8 Hz, Ph), 7.29 (d, 2H, J = 8 Hz, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.17, 50.82, 73.98, 126.05, 129.39, 137.13, 138.22; MS *m/z* (rel. int. %): 171-170 (M^+, 4) 158 (1), 156 (3), 137 (2), 136 (16), 135 (1), 122 (4), 121 (50), 119 (5), 118 (5),117 (8), 115 (4), 107 (2), 105 (1), 103 (1), 102 (1), 94 (4), 93 (49), 92 (11), 91 (45), 89 (4), 78 (5), 79 (2), 77 (30), 75 (1), 74 (1), 67 (3), 66 (2), 65 (20), 64 (2), 63 (10), 62 (4), 60 (15), 59 (2), 57 (4), 55 (1), 53 (4), 52 (5), 51 (18), 50 (9), 46 (1), 45 (9), 44 (3), 43 (100), 41 (10), 40 (4).

*2.18. (R)-(−)-2-Chloro-1-(4-methylphenyl)ethanol (R)-**2h**.* The bioreduction of ketone **1h** (0.337 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 furnished (*R*)-**2h** (0.327 g, 95.7% yield) as colorless oil; $[\alpha]_D^{25}$ −48.3 (*c* 1.1, $CHCl_3$) [lit. +47.2° (*c* 1.1, $CHCl_3$) for *S* isomer, 92% ee] [32], giving an optical purity of >99% determined by GC using a chiral column; ^1H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

*2.19. (S)-(+)-2-Chloro-1-(4-methoxyphenyl)ethanol (S)-**2i**.* The bioreduction of ketone **1i** (0.369 g, 2 mmol) by *Geotrichum candidum* CCT 1205 furnished (*S*)-**2i** (0.370 g, 99.2% yield) as colorless oil; $[\alpha]_D^{25}$ +41.4° (*c* 1, $CHCl_3$) [lit. +40.2°, for *S* isomer, 90,5% ee] [36], giving an optical purity of >99% determined by GC using a chiral column; IR (film): 3400, 3372, 3062, 3031, 2950, 2931, 2907, 2836, 1610, 1520, 1511, 1458, 1443, 1368, 1300, 1250, 1205, 1174, 1115, 1084, 1069, 1030, 1004, 898; 840, 780 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 2.70 (sl, 1H, OH), 3.52 (dd, 1H, J = 8.7 Hz and 11.4 Hz, CH_2), 3.61 (dd, 1 H, J = 3.8 Hz and 11.4 Hz, CH_2), 3.80 (s, 3H, CH_3), 4.78 (dd, 1H, J = 3.8 Hz and 8.7 Hz, CH), 6.90 (d, J = 8.7 Hz, 2H, Ph), 7.20 (d, J = 8.7 Hz, 2H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$) δ 50.70, 55.34, 73.61, 113.78, 127.10, 132.12, 159.62; MS *m/z* (rel. int. %): 186 (M^+, 6), 152 (21), 153 (2), 151 (2), 138 (7), 137 (100), 135 (13), 134 (45), 122 (2), 121 (2), 120 (3), 119 (27), 110 (4), 109 (23), 108 (4), 107 (4), 105 (4), 104 (2), 103 (4), 102 (2), 95 (3), 94 (26), 93 (3), 92 (9), 90 (2), 91(38), 89 (5), 81 (2), 78 (8), 79 (9), 77 (25), 76 (4), 75 (3), 74 (4), 68 (5), 67 (3), 66 (13), 65 (38), 64 (11), 63 (21), 62 (8), 61 (3), 55 (4), 54 (1), 53 (10), 52 (8), 51 (30), 50 (19), 49 (1), 45 (8), 44 (6), 43 (100), 41 (12), 40 (10).

*2.20. (R)-(−)-2-Chloro-1-(4-methoxyphenyl)ethanol (R)-**2i**.* The bioreduction of ketone **1i** (0.369 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 furnished (*R*)-**2i** (0.366 g, 98.0% yield) as colorless oil; $[\alpha]_D^{25}$ −41.5 (*c* 1, $CHCl_3$) [lit. +40.2°, for *S* isomer, 90,5% ee] [36], giving an optical purity of >99% determined by GC using a chiral column; ^1H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

Table 1: Asymmetric reduction of 2-halo-1-(4-substituted phenyl)-ethanones **1a-j** mediated by *Geotrichum candidum* CCT 1205 and *Rhodotorula glutinis* CCT 2182[a].

Ketone	Microorganism	T (°C)	Alcohol	Yield (%)	$[\alpha]_D^{25}$[b]
1a	*Geotrichum candidum*	28	(***S***)-**2a**	96.4	+40.0
1b	"	28	(***S***)-**2b**	95.1	+38.7
1c	"	28	(***S***)-**2c**	96.0	+48.3
1d	"	28	(***S***)-**2d**	98.0	+19.8
1e	"	28	(***S***)-**2e**	97.6	+25.0
1f	"	28	(***S***)-**2f**	99.4	+35.0
1g	"	28	(***S***)-**2g**	95.0	+48.3
1h	"	28	(***S***)-**2h**	96.4	+48.3
1i	"	28	(***S***)-**2i**	99.2	+41.4
1j	"	28	(***S***)-**2j**	97.0	+32.6
1a	*Rhodotorula glutinis*	30	(***R***)-**2a**	99.0	−40.4
1b	"	30	(***R***)-**2b**	97.0	−38.7
1c	"	30	(***S***)-**2c**	95.3	−48.3
1d	"	30	(***R***)-**2d**	97.8	−19.7
1e	"	30	(***R***)-**2e**	98.0	−25.0
1f	"	30	(***R***)-**2f**	97.7	−34.9
1g	"	30	(***R***)-**2g**	94.2	−48.3
1h	"	30	(***R***)-**2h**	95.7	−48.3
1i	"	30	(***R***)-**2i**	98.0	−41.5
1j	"	30	(***R***)-**2j**	98.0	−32.6

[a] 18 h, 2 mmol of ketone/1.5 mL of EtOH was added to 15 g of yeast (wet weight)/400 mL of nutrient broth 1 (malt extract, peptone) for *Geotrichum candidum* or nutrient broth 2 (yeast extract, malt extract, peptone) for *Rhodotorula glutinis*. [b] ee>99%. [c] See Materials and Methods for *c* values and solvent.

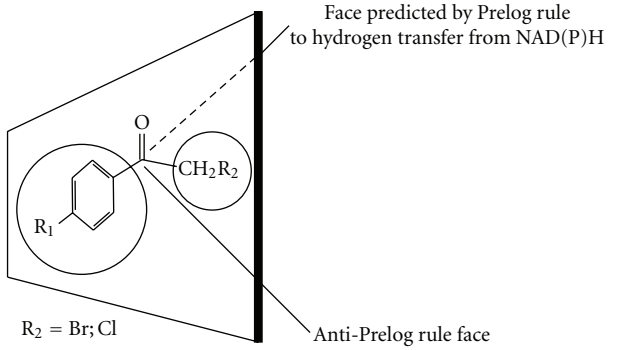


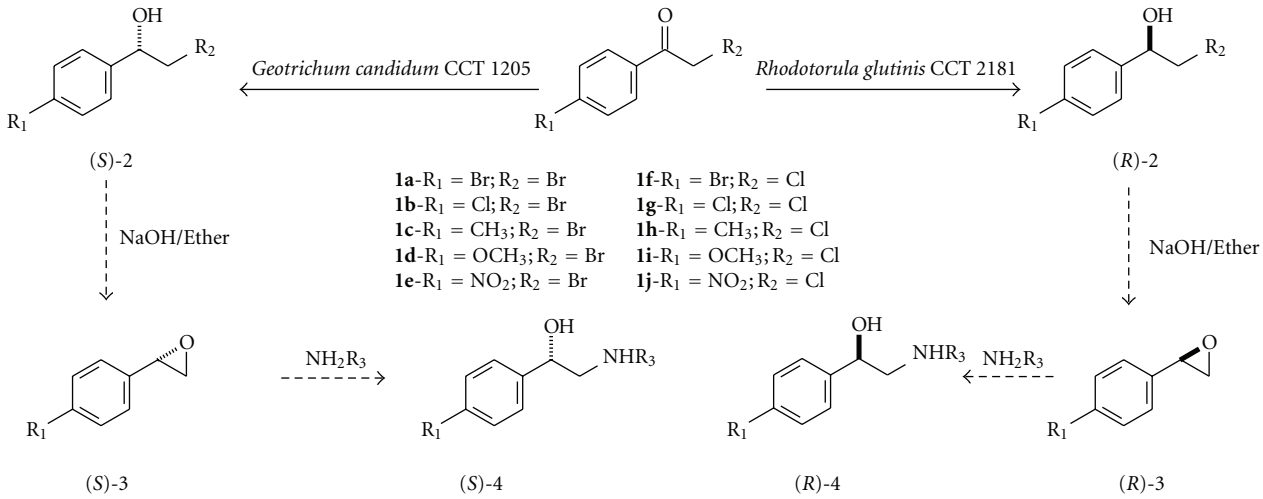
Figure 1: Prelog rule for discrimination of the faces of carbonylic group by the enzymes.

*2.21. (S)-(+)-2-Chloro-1-(4-nitrophenyl)ethanol (S)-**2j**.* The bioreduction of ketone **1j** (0.399, 2 mmol) by *Geotrichum candidum* CCT 1205 furnished (*S*)-**2j** (0.391 g, 97.0% yield) a white solid, mp 87°C (lit. p.f. 87°C) [33]; $[\alpha]_D^{25}$ +32,6° (*c* 1, $CHCl_3$) [lit. +37.2°, *c* 2.0, $CHCl_3$ for *S* isomer, 98,2% ee] [36], giving an optical purity of >99% determined by GC using a chiral column; IR (KBr): 3304, 3051, 3021, 2970, 2923, 2878, 1599, 1506, 1452, 1364, 1323, 1275, 1251, 1204, 1166, 1125, 1075, 1024, 964, 951, 902, 863, 823, 773, 743, 703, 652 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 2.93 (sl, 1H, OH), 3.64 (dd, 1H, J = 8.1 Hz and 11.3 Hz, CH_2), 3.68 (dd, 1H, J = 3.3 Hz and 11.3 Hz, CH_2), 5.03–5.05 (m, 1H, CH), 7.50 (d, 2H, J = 8.7 Hz, Ph), 8.20 (d, 2H, J = 8.7 Hz, Ph); ^{13}C NMR (75 MHz, $CDCl_3$) δ 50.20, 69.38, 123.56, 126.73, 146.59, 146.95; MS *m/z* (rel. int. %): 166 (5), 153 (8), 152 (100), 136 (2), 122 (6), 107 (2), 106 (13), 105 (12), 102 (3), 95 (4), 94 (13), 81 (3), 79 (3), 78 (18), 77 (22), 65 (9), 51 (24), 50 (16), 43 (23), 41 (9).

*2.22. (R)-(−)-2-Chloro-1-(4-nitrophenyl)ethanol (R)-**2j**.* The bioreduction of ketone **1j** (0.399 g, 2 mmol) by *Rhodotorula glutinis* CCT 2182 furnished (*R*)-**2j** (0.395 g, 98.0% yield) a white solid, mp 87°C (lit. p.f. 87°C) [36]; $[\alpha]_D^{25}$ −32,6° (*c* 1, $CHCl_3$) [lit. +37.2°, *c* 2.0, $CHCl_3$ for *S* isomer, 98,2% ee] [33], giving an optical purity of >99% determined by GC using a chiral column; ^1H and ^{13}C NMR and IR spectra and MS analysis were identical to those observed with its (*S*) enantiomer.

3. Results and Discussion

The reduction of ethanones **1a-j** was carried out in 5 mmol/L in a slurry of growing yeast of *Rhodotorula glutinis* CCT 2182 and *Geotrichum candidum* CCT 1205. These ethanones having substituted groups (electron withdrawing groups—EWG: $-NO_2$, –Br, –Cl; electron donating groups—EDG: $-CH_3$, $-OCH_3$) attached to position 4 of benzene ring were studied in order to investigate the influence of these groups in the bioreduction performed by these two microorganisms.



Scheme 2

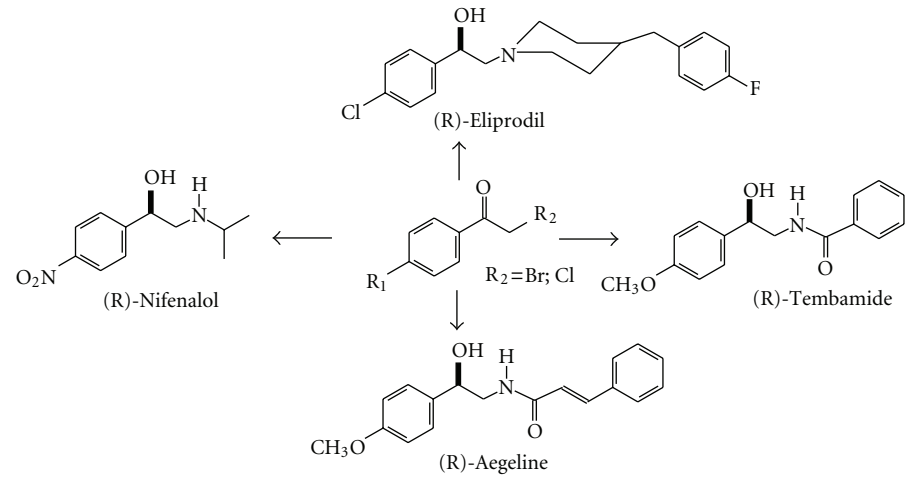


Figure 2: Pharmaceutical useful ethanolamines.

The reaction progress was monitored by GC analysis, and the yields and enatiomeric excesses are shown in Table 1.

The reductions of 2-bromo-1-(4-substituted phenyl)-ethanones **1a-e** and 2-chloro-1-(4-substituted)-ethanones **1f-j** mediated by *Rhodotorula glutinis* CCT 2182 gave the corresponding halohydrins **2a-j** with (*R*) configuration, while the halohydrins **2a-j** with (*S*) configuration were obtained when *Geotrichum candidum* CCT 1205 mediated the reduction of the ethanones **1a-j**.

α-Haloacetophenones have been used as mechanistic probe in the reduction reactions of NADH-dependent horse liver alcohol dehydrogenase [37–40], for identification of reductants in sediments [41] and even in the whole cells [42]. This probe enables the differentiation between reduction processes which proceed through hydride transfer (H^-) or by a multistep electron transfer (e^-, $H^\bullet$ or e^-, H^+, e^- as has been suggested). Acetophenone is the reduction product obtained by electron transfer, while optically active halohydrin is obtained when an enzyme mediates a hydride transfer process. In this work, the reductions of **1a-e** proceed via hydride transfer mediated by an oxireductase, since halohydrins were obtained in high ee and no 4-substituted acetophenone was detected.

Rhodotorula glutinis gives products following the Prelog rule [43], which predicts that, in general, hydrogen transfer from NAD(P)H to the prochiral ethanones **1a-j** occurs to the face of carbonylic group shown in Figure 1, taking into account that the aryl group is larger than the $–CH_2Br$ and $–CH_2Cl$ groups. On the contrary, the *Geotrichum candidum* gives anti-Prelog halohydrins.

The excellent results and complementary enantioselectivities of the produced halohydrins obtained by using *Rhodotorula glutinis* CCT 2182 and *Geotrichum candidum* CCT 1205 in reduction of ethanones **1a-j** are remarkable and highlight the potential of such approach to obtain separately the two isomers of the 1,2-aminoalcohols, by reaction of the easily obtainable epoxy with the appropriated amine (Scheme 2), as an alternative to the approach using the

reduction of α-azido-*para*-substituted acetophenones mediated by those microorganisms [28]. The separate synthesis of two enantiomers is important since the FDA Guidance for Development of New Stereoisomeric Drugs [44] says that "to evaluate the pharmacokinetics of a single enantiomer or mixture of enantiomers, manufacturers should develop quantitative assays for individual enantiomers in *in vivo* samples early in drug development." However, the products of biotransformation of **1b-e** and **1g-j** using *Rhodotorula glutinis* CCT 2182 may be used as important starting material for the preparation of the known pharmaceuticals products with (*R*) configuration: Eliprodil from halohydrins **2b** and **2g**; Tembamide from halohydrins **2c** and **2h**; Aegeline from halohydrins **2d** and **2i**; Nifenalol from halohydrins **2e** and **2j** (Figure 2).

4. Conclusions

The use of *Rhodotorula glutinis* CCT 2182 and *Geotrichum candidum* CCT 1205 in bioreduction reaction of 2-halo-1-(4-substituted phenyl)-ethanones results in an important chiral halohydrins in high ee, excellent yield, and complementary enantioselectivity. These halohydrins may be used as intermediates in the synthesis of optically active substituted styrene oxides and aminoalcohols which have numerous industrial applications.

Acknowledgments

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Research Article

Estolides Synthesis Catalyzed by Immobilized Lipases

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Estolides are vegetable-oil-based lubricants obtained from oleic acid or any source of hydroxy fatty acids. In this work, the estolides synthesis from oleic acid and methyl ricinoleate (biodiesel from castor oil), using immobilized commercial lipases (Novozym 435, Lipozyme RM-IM, and Lipozyme TL-IM) in a solvent-free medium was investigated. Acid value was used to monitor the reaction progress by determining the consumption of acid present in the medium. Novozym 435 showed the best performance. Water removal improved the conversion. Novozym 435 was more active at atmospheric pressure. Novozym 435 was reused four times with conversion reaching 15% after the fourth reaction at 80°C. Estolides produced under the reaction conditions used in this work presented good properties, such as, low temperature properties as pour point (−24°C), viscosity (23.9 cSt at 40°C and 5.2 cSt at 100°C), and viscosity index (153).

1. Introduction

Estolides are a class of polyesters based on vegetable oils that are formed when the carboxylic acid functionality of one fatty acid reacts at the site of unsaturation of another fatty acid [1] or by covalent ester bonds between hydroxyl moiety of one hydroxyl acid and the carboxyl moiety of another hydroxyl acid molecule [2]. These compounds have a variety of potential applications as greases, plastics, inks, cosmetics, viscosity controller for chocolate, emulsifier in margarine, and lubricants [3–5]. As lubricants, estolides have been developed in order to overcome deficiencies associated with some characteristics of vegetable oils, which are known to have poor thermal oxidative stability, low hydrolytic stability, and poor low temperature properties [6]. These vegetable-oil-based lubricants and derivatives have excellent lubricity and biodegradability properties and currently out-perform the commercially available industrial products such as petroleum-based hydraulic fluids, soy-based fluids, and petroleum oils [6, 7].

The conventional chemical route of estolides synthesis using high temperatures (205–210°C) or strong acids as catalysts leads to a lower selectivity, undesired byproducts, colouring, and malodorours of products and cause corrosion of equipments and produce acid effluent [8].

The enzymatic synthesis of ricinoleic acid estolides using lipases (triacylglycerol ester hydrolases E.C.3.1.1.3) have been investigated as an alternative to overcome the common problems that occur in a conventional route. Most of lipases act under mild reaction conditions (low temperature and pressures, and neutral pH), which prevent degradation of

estolides [9] and show high selectivity, including stereoselectivity, giving high purity products. Moreover, immobilized enzymes can be easily separated from the reaction media for reuse [2].

The aim of this work was the synthesis of estolides from oleic acid and biodiesel from castor oil (methyl ricinoleate) catalyzed by immobilized lipases in a medium containing only reagents and enzyme (Figure 1). The effect of several reaction conditions such as temperature, enzyme concentration, molar ratio of reagents, reaction time, water removal by vacuum, and use of molecular sieves and pressure on the reaction was investigated. The stability of Novozym 435 was studied in repetitive batch reuses. The lubricant physical properties were also analyzed. Reagents used in this study were chosen due to availability of oleic acid from different agricultural sources and its abundance owing to genetically engineered high-oleic crops [1] and by the fact that using methyl ricinoleate to produce lubricants will enlarge the applicability of biodiesel from castor oil.

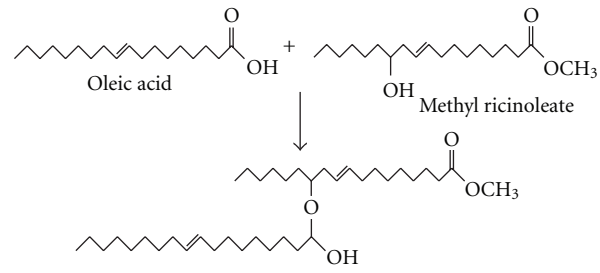

Figure 1: Estolide synthesis from oleic acid and methyl ricinoleate.

2. Experimental

2.1. Materials. The commercial immobilized lipases used were Novozym 435, Lipozyme TL-IM, and Lipozyme RM-IM, all kindly supplied by Novozymes Latin America (Paraná, Brazil). Oleic acid (extra pure) and ethanol (95 wt.%) were purchased from Merck (Darmstadt, Germany). Biodiesel from castor oil was provided by Cenpes/Petrobras (Rio de Janeiro, Brazil). Acetone P. A., butanol (99 wt.%), and sodium hydroxide were supplied by Vetec Química Fina Ltda (Rio de Janeiro, Brazil). Molecular sieves (3 Å) were purchased from SIGMA/Aldrich (St. Louis, USA).

2.2. Reaction System. The enzymatic reaction was carried out in a 15 mL closed batch reactor magnetically stirred and coupled to a condenser. Temperature of the medium was kept constant by circulating hot ethylene glycol through the reactor jacket. A thermostatic bath allowed a close control over the process temperature. The reaction medium contained oleic acid, biodiesel from castor oil, and immobilized lipase.

2.3. Measurement of Lipase Activity. Esterification activities of Lipozyme RM-IM, Lipozyme TL-IM, and Novozym 435 were measured by the consumption of oleic acid at 45°C during the esterification reaction with butanol (0.030 mol oleic acid and 0.030 mol butanol) using an enzyme concentration of 3 wt.%. The reactions were carried out in a 20 mL open batch reactor magnetically stirred. The reactor was kept at the desired temperature by a thermostatic bath. Samples of 100 μL were withdrawn at 0, 5, 10, 15, 20, 30 40, 50, and 60 min, diluted in 30 mL of acetone/ethanol (1 : 2) and titrated with NaOH 0.02 mol·L^{-1}, using an automatic titrator Mettler model DL25. One esterification unit was defined as the amount of lipase that consumed 1 μmol of oleic acid per minute per gram of enzymatic preparation under the experimental conditions described herein. Esterification enzymatic activity was calculated using the formula below:

$$A(\mu\text{mols} \cdot \text{min}^{-1} \cdot \text{g}^{-1}) = \frac{(V_1 - V_2) \times C}{t \times m} \times 1000 \times \frac{V_m}{V_a}. \quad (1)$$

A is esterification activity (μmols·min^{-1}·g^{-1}); V_1 is volume of NaOH consumed in titration of the sample taken at time zero reaction (mL); V_2 is volume of NaOH consumed in titration of the sample taken at time t minutes of reaction (mL); C is NaOH concentration (mol·L^{-1}); t is reaction time (min); m is enzyme mass used in the reaction (g); V_a is volume of sample (mL); V_m is volume of medium (mL).

The enzymatic activities values were maintained during the experimental work.

2.4. Use of Molecular Sieves. Amounts of adsorbent (3 Å molecular sieves) were added into the reaction system to adsorb the byproduct water. Before each reaction, molecular sieves were dried at 300°C during 3 h, and they were added to the reaction medium at the beginning (0 h), after 6 h or 24 h of reaction. The effect of adsorbent amount was studied using 100, 250, 500, and 650 mg of molecular sieves. Reactions were carried out at 80°C, using molar ratio of reagents equal to 1 (0.015 mols of each reagent) and 6 wt.% of Novozym 435 during 48 h.

2.5. Reactions Under Vacuum. The system consisted of a closed batch reactor, equipped with magnetic stirring and coupled to a condenser. A vacuum pump (Edwards Model RV3) was connected to the condenser giving a constant pressure of 0.6 mbar (0.45 mmHg). To evaluate the effect of water removal two strategies were used: the pump was switched on either at the beginning (t = 0 h) or after 6 h of reaction. Reactions were carried out at 80°C, using molar ratio of reagents equal to 1 (0.015 mols of each reagent) and 6 wt.% of Novozym 435 during 48 h.

2.6. Effect of Pressure. The effect of pressure was tested in three levels: atmospheric pressure, 100 and 250 psi using a 50 mL Parr Reactor Model 4843. Nitrogen was used for the system pressurization. Stirring speed was kept constant at 550 rpm. Reactions were carried out at 80°C, using molar

ratio of reagents equal to 1 and 6 wt.% of Novozym 435. The reaction medium was approximately 38 mL (75% of reactor volume).

2.7. Reuse of Novozym 435. The reusability of Novozym 435 was carried out using reagents in a molar ratio of 1 : 1 (0.015 mols of each reagent), 6 wt.% of immobilized lipase and 80°C. After 48 h of reaction, the lipase was separated from the reaction medium by decantation and washed with n-hexane. After that, it was vacuum-filtered. Enzyme was then reused in a new batch.

2.8. Measurement of Reaction Extension. Acid value (AV) was used as an index to show the degree of reaction [10]. The acid value is the number of milligrams of potassium hydroxide necessary to neutralize the free acids in 1 g of sample. AV corresponds to the carboxyl group concentration in the reaction mixture, which decreases due to the condensation of fatty acid. This analysis was chosen to measure the reaction conversion due to the difficulty to identify the reaction products and to quantify methyl ricinoleate consumption. Reaction progress was monitored by taking duplicate samples (100 μL) of reaction medium that was dissolved in 30 mL of acetone/ethanol (1 : 1). Free fatty acids present in reaction medium samples were analyzed by titration with NaOH 0.02 mol·L^{-1} using a Mettler DL 25 autotitrator.

The conversion was calculated using the formula below

$$\text{Conversion}(\%) = \frac{(\text{AV}_{\text{initial}} - \text{AV}_{\text{final}})}{\text{AV}_{\text{initial}}} \times 100. \quad (2)$$

Reproducibility of conversion data was found to be within 2–5%. A blank test was carried out at 100°C in the absence of biocatalyst and no reduction was observed in AV after 24 h of reaction.

2.9. Physical Properties of the Estolides. Physical properties such as viscosity at 40 and 100°C [11], viscosity index [12], pour point [13], acid value [14], and copper strip corrosion [15] were determined for the lubricant obtained in this work.

3. Results and Discussion

3.1. Lipase Activity. The activities obtained for Lipozyme RM-IM, Lipozyme TL-IM and Novozym 435 were 1909, 699, and 3824 μmols of acid·min^{-1}g^{-1} of lipase, respectively.

3.2. Lipase Type Effect. In this work, the synthesis of estolides from oleic acid and methyl ricinoleate was studied using three commercial immobilized lipases: Lipozyme RM-IM, Lipozyme TL-IM, and Novozym 435.

Under the experimental conditions used in this work, the main reaction is the esterification between oleic acid and methyl ricinoleate producing estolide and water. However other side reactions could occur such as the self-condensation of methyl ricinoleate, the transesterification between oleic acid and methyl ricinoleate forming methyl oleate and ricinoleic acid, and the self-condensation of ricinoleic acid.

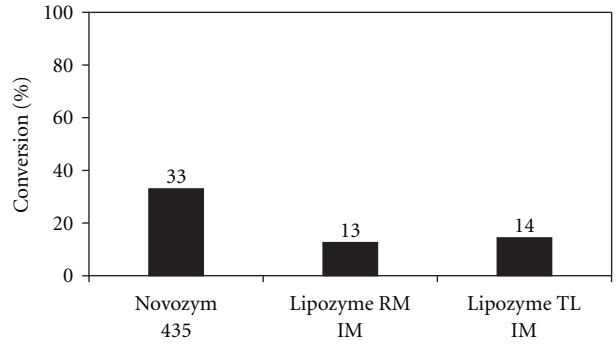

Figure 2: Effect of lipase type on conversion after 48 h at 80°C, using 6 wt.% of immobilized lipase and substrate molar ratio of 1.

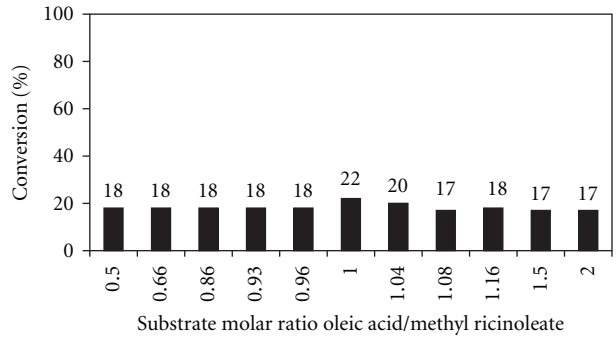

Figure 3: Effect of substrate molar ratio (oleic acid/methyl ricinoleate) on conversion after 24 h at 80°C, using 6 wt.% of immobilized lipase.

The reactions were carried out using oleic acid/methyl ricinoleate molar ratio equal to 1, at 80°C and enzyme content of 6 wt.%. Results are shown in Figure 2. After 48 h, the highest conversion was attained with Novozym 435 (33%).

Conversions obtained with the 1,3-posittionaly specific enzymes (Lipozyme RM-IM and Lipozyme TL-IM) were below 15%, which may be related to the specificity of the lipases studied. It has been reported that reactions involving estolide formation from ricinoleic acid or hydrolysis of estolides have been successfully catalyzed by "random lipases", which present no 1,3-positional selectivity (*Candida rugosa*, *Chromobacterium viscosum*, *Pseudomonas* sp., and *Geotrichum candidum*), but are not well catalyzed by 1,3-specific lipases (*Rhizopus*, *Rhizomucor miehei*, pancreatic lipase, etc.) [4, 5]. According to Peláez et al. [16] 1,3-selective lipases from *Rhizopus delamar* and *R. miehei* did not catalyze formation of estolide. Similar results were obtained by Hayes and Kleiman [9] in reactions with lesquerolic acid (14-hydroxy-11-eicosanoic) and octadecenoic acid.

3.3. Effect of Substrate Molar Ratio. The effect of oleic acid/methyl ricinoleate molar ratio on the conversion was investigated. Reactions were carried out for 24 h, at 80°C and using 6 wt.% of Novozym 435. Figure 3 shows that higher conversion (22%) was attained with stoichiometric molar ratio of reagents. However, molar ratio of reagents did not have significant effect on conversion.

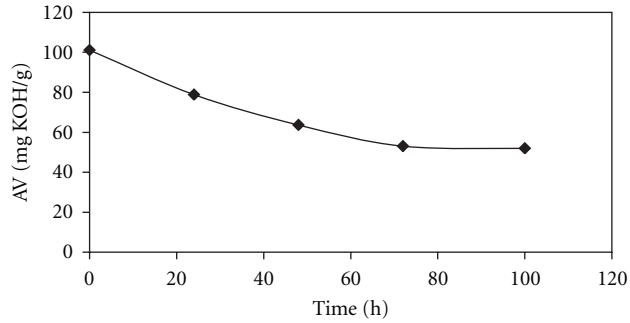

Figure 4: Time course for estolide synthesis from oleic acid and methyl ricinoleate at 80°C, using substrate molar ratio of 1 and 6 wt.% of Novozym 435.

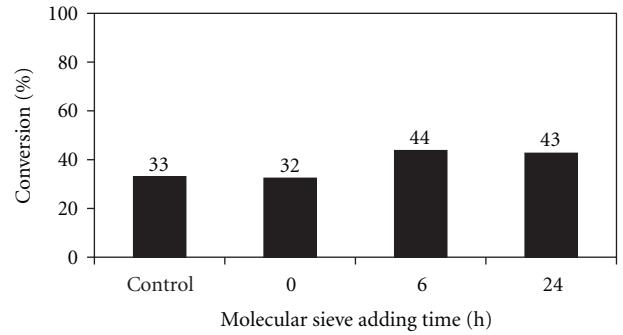

Figure 5: Effect of adsorbent adding time on conversion after 48 h at 80°C, using substrate molar ratio of 1, 6 wt.% of immobilized lipase and 500 mg of molecular sieves. Control: reactions carried out without adsorbent.

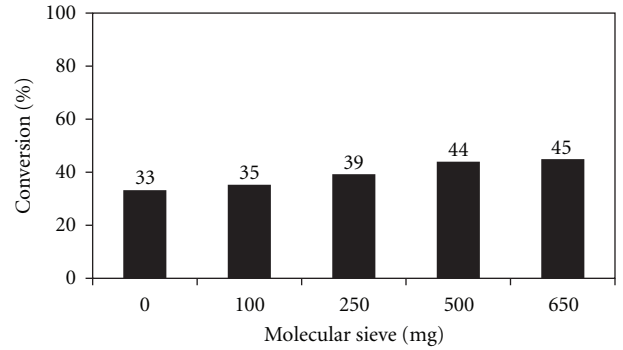

Figure 6: Effect of adsorbent amount (mg) on conversion after 48 h at 80°C, using 6 wt.% of immobilized lipase, substrate molar ratio of 1, and molecular sieves added after 6 h.

The conditions of 6 wt.% of lipase, stoichiometric molar ratio of reagents, and 80°C were used to study the influence of reaction time during 24, 48, 72, and 100 h of reaction. Higher conversions were obtained after 72 and 100 h, as expected (Figure 4). The conversion obtained after 72 and 100 h of reaction (46%) indicates that equilibrium was reached. One of the most common reactions to produce estolides is the self-condensation of ricinoleic acid. Bódalo et al. [8] investigated this reaction using immobilized lipase from *Candida rugosa* and obtained an acid value of 50 after 150 h (72% of conversion), using 16.6 wt.% of lipase at 40°C. These results indicate the applicability of lipases for estolides synthesis through different pathways.

3.4. Molecular Sieves Effect. In esterification reactions catalyzed by lipases water play multiple roles. It is necessary for the catalytic function of lipases because it participates, directly or indirectly, in all noncovalent interactions that maintain the conformation of the catalytic site. On the other hand, the reaction reaches equilibrium and stops when the water content in reaction mixture increases as result of the water formed during the dehydration-condensation reaction [4]. Adsorbents such as alumina, silica gel, and zeolites can be adopted to control water concentration during the reaction process. Molecular sieves present superior drying ability because they cannot coadsorb large hydrocarbon molecules [20].

In order to verify the effect of water removal, reactions were carried out with 500 mg of 3 Å molecular sieves added to the system at the beginning of the reaction, or after 6 or 24 h. The reaction conditions were stoichiometric ratio of reagents and 6 wt.% of Novozym 435 at 80°C. Results are shown in Figure 5. The addition of molecular sieves at the beginning of the reaction ($t = 0$ h) did not improve the final conversion. It was observed that higher conversion values were obtained when molecular sieves were added after 6 or 24 h of reaction. This result can be explained due to excessive stripping of the essential water needed for enzyme activity by the desiccant agent [21].

The effect of adsorbent amount was also studied. The reactions were carried out with 100, 250, 500, and 650 mg of adsorbent added after 6 h of reaction. Results are shown in Figure 6 and it can be seen that conversion increased with increasing molecular sieve amount up to 500 mg.

3.5. Reactions under Vacuum. The use of vacuum as an alternative to removing water from the reaction medium was investigated in reactions using stoichiometric molar ratio of reagents (0.015 mols of each reagent), 6 wt.% of Novozym 435 at 80°C during 48 h. In order to evaluate the effect of water removal two strategies were used: the pump was switched on either at the beginning ($t = 0$ h) or after 6 h of reaction. Results are shown in Figure 7. The use of vacuum (0.45 mmHg) increased the conversion after 48 h in more than 10%. No difference was observed when vacuum was applied in the beginning or after 6 h of reaction.

Bódalo et al. [2] have also observed that the use of a vacuum system (160 mmHg) was efficient to remove water produced on the estolides synthesis from ricinoleic acid catalyzed by *Candida rugosa* lipase. The use of vacuum on estolides synthesis from ricinoleic acid using immobilized lipase from *Candida rugosa* was also investigated by Yoshida et al. [3]. The condition of 110 mmHg was the most advantageous.

The conversion attained after 48 h using 500 mg of molecular sieves added after 6 h of reaction (44%) was similar to that obtained in reactions carried out with vacuum

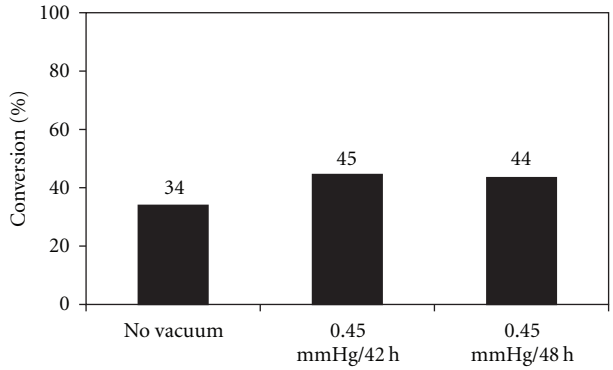

Figure 7: Conversions attained after 48 h under vacuum (0.45 mmHg), using two drying times (42 and 48 h). Reaction conditions: substrate molar ratio of 1, 6 wt.% of immobilized lipase at 80°C.

(0.45 mmHg during 42 h) (45%). So, in light of these results, the addition of molecular sieves for removing water from the medium was a better alternative than using vacuum. Furthermore the operational costs related to vacuum pump are eliminated.

3.6. Effect of Pressure. The hydrostatic pressure has been used to increase the activity and/or stability of several enzymes in different solvents [22]. Lipases stability and activity under high pressures have been investigated in compressed or supercritical fluids and gases including carbon dioxide, propane, butane, and mixtures of butane and propane [22].

The effect of high pressure on estolides synthesis from oleic acid and methyl ricinoleate was studied in a Parr reactor at 80°C operating during 48 h, using reagents in a molar ratio of 1, 6 wt.% of Novozym 435 and stirring speed of 550 rpm. The pressures investigated were 100 and 250 psi (6.8 and 17 atm, resp.). Nitrogen was used to pressurize the system. A pressure increase led to a small reduction in the conversion (less than 10%). These results indicate that the catalytic activity of lipase was not favored by pressure increase in the reaction system.

3.7. Effect of Temperature. Temperature influences the enzymatic reaction rate, enzyme stability, and the velocity of water evaporation from the reaction medium and its viscosity [2]. An increase in temperature can reduce the mixture viscosity, enhance mutual solubility, and improve substrates diffusion process, thus reducing mass transfer limitations and favoring interactions between enzyme particles and substrates. However, high temperatures can lead to higher lipase deactivation [23].

The effect of temperature was studied in a range of 50–100°C. The reactions were carried out using 0.015 mols of each reagent and 6 wt.% of Novozym 435, during 24 h. Results are shown in Figure 8. An increase in temperature until 90°C improved the conversion. Even at 100°C, 30% of conversion was obtained.

3.8. Effect of Catalyst Amount. The effect of enzyme concentration on the fatty acid conversion was studied in a range of

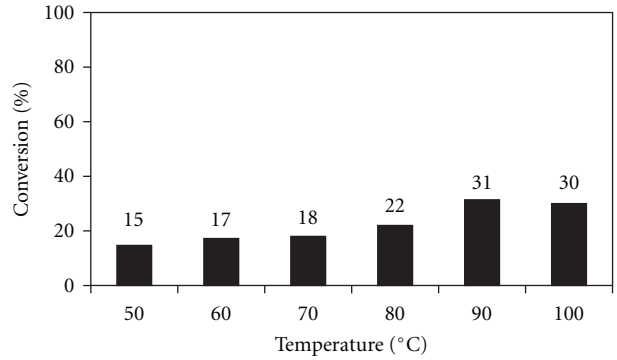

Figure 8: Effect of temperature on conversion after 24 h, using 6 wt.% of immobilized lipase and substrate molar ratio of 1.

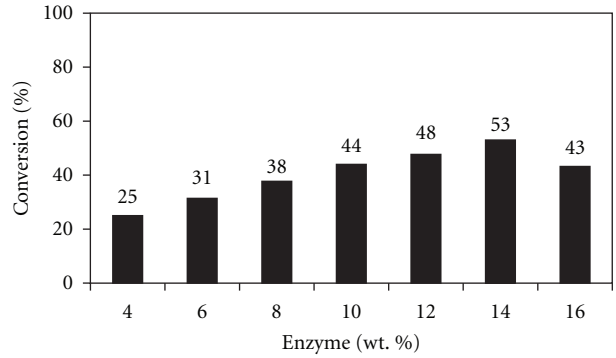

Figure 9: Effect of lipase concentration on conversion after 24 h at 90°C, using substrate molar ratio of 1.

4 and 16 wt.% of Novozym 435 at 90°C using stoichiometric reagents. Results are shown in Figure 9. A linear correlation between catalyst amount and conversion was found until 14 wt.% of lipase (conversion of 53%). Loading 16 wt.% of lipase caused significant conversion decrease (43%) that can be explained taking into account the phenomenon of enzyme agglomeration.

Similar results were obtained by Bódalo et al. [2] that studied the influence of the enzyme concentration on estolides synthesis from ricinoleic acid catalyzed by immobilized *Candida rugosa* lipase (concentrations of 8.3; 16.6 and 33 wt.%). The authors observed that when the concentration of immobilized enzyme was increased, the reaction progressed faster and a lower acid value was reached. However, changes in reaction rate and final acid value were more noticeable when the enzyme concentration varied from 8.3 to 16.6 wt.% than when the enzyme concentration was increased to 33 wt.%.

The behavior of leveling-off of esterification at higher enzyme concentration has also been reported in other enzymatic reactions [23]. Clumping leads to aggregate formation and inhomogeneous enzyme distribution. Then, just the fraction of the catalyst that remains on the outer surface of the agglomerates is truly available for catalysis. Substrates have reduced access to catalytic molecules inside agglomerates, being the efficiency of the milligram of catalyst added to reaction mixture seriously reduced [24].

Table 1: Comparison between the properties of the product obtained after reaction with oleic acid and methyl ricinoleate using Novozym 435 (6 wt.%) at 80°C and those of estolides reported in the literature.

Lubricant	Pour point (°C)	Viscosity at 40°C (cSt)	Viscosity at 100°C (cSt)	Viscosity index
Product of this study (oleic acid + methyl ricinoleate)	−24	23.9	5.2	153
Cermak and Isbell [17][a]	−27	123.4	16.7	146
Cermak et al. [18][b]	−54	34.5	7.6	196
Hayes and Kleiman [9][c]		126.3	23.11	191
Cermak and Isbell [19][d]	−24	389.1	37.7	143

[a]Cuphea-oleic estolides.
[b]Castor oil estolides.
[c]Oleic acid estolides.
[d]Oleic acid estolides + octanoic acid.

Table 2: Comparison between the properties of the product obtained after reaction with oleic acid and methyl ricinoleate using Novozym 435 (6 wt.%) at 80°C and those of commercial lubricants.

Lubricant	Viscosity at 40°C (cSt)	Viscosity at 100°C (cSt)	Viscosity index
Product of this study (oleic acid + methyl ricinoleate)	23.9	5.2	153
Light neutral solvent	30–40	5	125
Medium neutral solvent	40–50	7	95
Heavy neutral solvent	70–300	10–20	95
Mineral oil Spindle 09	10.7	2.7	95
Lubrax Unitractor[a]	54	9.3	156

[a]Biodegradable synthetic ester marketed by BR for tractors.

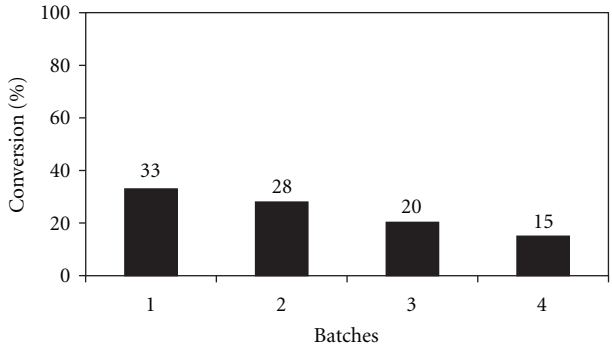

Figure 10: Effect of repeated batch reactions on conversion after 48 h for 6 wt.% of Novozym 435 at 80°C.

3.9. Reuse of Novozym 435. Immobilization provides an attractive possibility for consecutive use of the same biocatalyst [9]. Catalyst reusability was studied with reactions at 80°C, using 6 wt.% of Novozym 435 and reagents in a molar ratio of 1 : 1 during 48 h (Figure 10). After each batch reaction, the enzyme was recovered, washed with n-hexane, and dried under vacuum before reuse.

As shown in Figure 10, in the first batch experiment, the conversion attained was 33%. The conversion has decreased after successive use of the catalyst. After the fourth batch reuse, the conversion was half that obtained in the first experiment. The decrease on lipase activity when it was reused was also observed by other authors [3, 9].

3.10. Physical Properties of the Product. The physical properties of some estolides reported in the literature [9, 17–19] and of the product obtained after reaction between oleic acid and methyl ricinoleate using Novozym 435 (6 wt.%) at 80°C are presented in Table 1. The kinematic viscosities obtained at 40 and 100°C (23.9 and 5.2 cSt, resp.) were lower than the values reported in the literature [9, 17–19]. The viscosity index was higher than the desirable value for lubricants (150). Pour point (−24°C) showed a value similar to that obtained by Cermak and Isbell (−27°C) [17] indicating the potential application of the biolubricant at lower temperatures. According to the corrosion test on copper strip, the product presents no corrosiveness. Table 2 shows a comparison between the properties of some commercial lubricants and the product obtained in this study. According to the viscosity data, the estolide from oleic acid and biodiesel from castor oil is similar to light neutral solvent. However, the product obtained in this study has a high acid value (56.3 mg $KOH \cdot g^{-1}$) that was due to the presence of oleic acid.

4. Conclusions

Estolides synthesis from oleic acid and biodiesel from castor oil catalyzed by three immobilized lipases was studied and can be performed in a solvent-free system. Novozym 435 was the most efficiently biocatalyst tested. Novozym 435 was reused four times, however with partial loss of activity. Despite the high acid value of the product, the reaction route investigated to obtain a lubricant using a clean technology (enzymatic catalysis) allowed us to obtain a noncorrosive product with good low temperature properties, which can be proved by comparing its properties to data reported in the literature regarding the same subject. Therefore, it was possible to establish some conditions for the biolubricant synthesis from reagents present in abundance in Brazil.

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Research Article

Characterization of the Recombinant Thermostable Lipase (Pf2001) from *Pyrococcus furiosus*: Effects of Thioredoxin Fusion Tag and Triton X-100

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In this work, the lipase from *Pyrococcus furiosus* encoded by ORF PF2001 was expressed with a fusion protein (thioredoxin) in *Escherichia coli*. The purified enzymes with the thioredoxin tag (TRX-PF2001Δ60) and without the thioredoxin tag (PF2001Δ60) were characterized, and various influences of Triton X-100 were determined. The optimal temperature for both enzymes was 80°C. Although the thioredoxin presence did not influence the optimum temperature, the TRX-PF2001Δ60 presented specific activity twice lower than the enzyme PF2001Δ60. The enzyme PF2001Δ60 was assayed using MUF-acetate, MUF-heptanoate, and MUF-palmitate. MUF-heptanoate was the preferred substrate of this enzyme. The chelators EDTA and EGTA increased the enzyme activity by 97 and 70%, respectively. The surfactant Triton X-100 reduced the enzyme activity by 50% and lowered the optimum temperature to 60°C. However, the thermostability of the enzyme PF2001Δ60 was enhanced with Triton X-100.

1. Introduction

One of the main limitations for the use of enzymes in industrial processes is their low stability under operational conditions (e.g., high temperatures, organic solvents, and extremes of pH). Enzymes from thermophilic and hyperthermophilic microorganisms are inherently more stable towards a variety of enzyme denaturants and thus represent promising alternatives for the development of industrial biocatalytic processes [1]. In addition to the potential biotechnological applications of these enzymes, the structural requirements that underlie their superior thermodynamic stability are of great interest to researchers [2].

Carboxylesterases (E.C. 3.1.1.1) and lipases (E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of ester bonds involving a carboxylic acid of variable chain length, that is, from C2 to C18 or more. In organic media, these two classes of enzymes also possess the ability to catalyze several other types of biotransformations such as esterification, transesterification, alcoholysis, aminolysis, and acidolysis [3]. These unique properties have increased the biotechnological interest of enzymes for a number of important industrial biotransformations, either as hydrolytic or as synthetic catalysts [4]. For instance, they are used by organic chemists for chiral molecule preparation by optical resolution from racemic mixtures; sugar modification and flavor ester synthesis for the food industry; biodiesel production and waste treatment [3–9]. Despite their significant biotechnological potential, the use of more resistant enzymes (extremozymes) could open up more possibilities for lipases and esterases in industrial processes.

A number of lipases and esterases from extremophilic organisms have been cloned in *E. coli*, but in general their expression levels and purification yields are low. This effect could be caused by the hydrophobic nature of lipases that causes folding problems. In a previous work, Almeida et al. [10] cloned the gene PF2001 from *P. furiosus* fused to a thioredoxin tag (TRX-PF2001Δ60) in *E. coli*. The enzyme was characterized as an esterase according to its substrate preference to MUF-Hep ($C < 10$). This enzyme was immobilized on microporous polypropylene at low ionic strength, showing the hyperactivation phenomenon [11]. Recently, the same enzyme was immobilized on more hydrophobic supports showing, once more, the hyperactivation phenomenon. The immobilization on hydrophobic supports under low ionic strength and hyperactivation is a characteristic of lipases suggesting that PF2001 enzyme is a lipase and not an esterase [12].

In this work, we investigated the effects of the TRX tag on the lipase activity of the purified enzyme. To this end, the purification of the recombinant *P. furiosus* lipase (TRX-PF2001Δ60) was carried out, and the thioredoxin tag was removed. The purified enzyme with and without the thioredoxin tag (PF2001Δ60) was then characterized. Furthermore, the influence of Triton X-100 (a surfactant commonly used in esterase/lipase assays) on the optimum temperature and thermostability was investigated.

2. Materials and Methods

2.1. Enzyme and Chemicals. Calf intestine enterokinase was purchased from Roche (Nutley, NJ, USA); Isopropyl-β-D-thiogalactopyranoside (IPTG), 4-methylumbelliferyl-acetate (MUF-Ace), 4-methylumbelliferyl-heptanoate (MUF-Hep), and 4-methylumbelliferyl-palmitate (MUF-Pal) were purchased from Sigma (Sigma, Chemicals, USA.). All other chemicals were of the highest reagent grade commercially available.

2.2. Expression. The *E. coli* strain BL21 (DE3) pLysS containing the plasmid with the ORF PF2001 and the thioredoxin tag, obtained by Almeida et al. [10], was grown in LB medium (0.5% yeast extract, 1% tryptone, and 0.5% NaCl) containing ampicillin (100 μg/mL) and chloramphenicol (12.5 μg/mL). After incubation with shaking at 35°C until $A_{600\,nm}$ reached 0.3, the induction was carried out by adding IPTG at a final concentration of 0.5 mM and incubation for 3 h at 35°C. Cells were collected by centrifugation, resuspended in sodium phosphate buffer (50 mM, pH 7.0) and disrupted by sonication. The lysed cells were centrifuged and the clarified supernatant was used for affinity purification.

2.3. Purification. A Hitrap Chelating resin (bed volume 1 mL) (Amersham Biosciences/GE Healthcare) was washed with 10 mL of sterile water and charged with Ni^{2+} using 5 mL of a 100 mM $NiSO_4$ solution. After a second wash with 10 mL of sterile water, the charged resin was equilibrated with 10 mL of sodium phosphate buffer (50 mM, pH 7.0). Eight milliliters of the clarified supernatant of the cell lysate was applied directly to the column. A washing step with 100 mM imidazole in equilibration buffer was done in order to elute nonspecific ligands. Elution of the TRX-PF2001Δ60 was carried out with 150 mM imidazole in the equilibration buffer. Fractions of 1 mL were collected at a flow rate of 0.2 mL/min and monitored by their absorbance at 280 nm.

2.4. Enterokinase Hydrolysis. Twenty-five micrograms of purified enzyme were dialyzed against Tris-Cl buffer (50 mM, pH 7.5). The dialyzed enzyme was treated with 0.6 μg of enterokinase at 4°C for 18 h to remove the TRX tag. The digestion mixture was analyzed by SDS-PAGE and zymography.

2.5. Electrophoresis and Zymogram Analysis. Nonreducing gel electrophoresis (SDS-PAGE) was carried out with a 10% separating gel on a vertical slab minigel apparatus (Bio-Rad) at 120 V for 1 h [13].

After the run, the gels were soaked for 30 min in 2.5% Triton X-100 at room temperature, briefly washed in 50 mM sodium phosphate buffer, pH 7.0, and covered by a solution of 100 μM MUF-Hep in ethylene glycol monomethylether. Soon after, the bands became visible under UV illumination [14]. Following zymogram analysis, the gels were silver-stained, and the protein bands were visualized.

2.6. Enzymatic Activity Assay. Enzyme assays were carried out using 4-methylumbelliferone (MUF) derivatives as substrates (MUF-Ace, MUF-Hep and MUF-Pal) in a Varian Cary Eclipse spectrofluorimeter, as described elsewhere [10, 14]. The reaction mixture contained 0.6 mL of 50 mM sodium phosphate buffer pH 7.0 containing 0.1% gum arabic and 2.4 μL of MUF-derivative substrate stock solution (25 mM in ethylene glycol monomethylether). The enzymes TRX-PF2001Δ60 and PF2001Δ60 (0.5 μg) were added to start the reaction. Enzymatic activity was determined at 70°C by measuring the increase of fluorescence emission (λ_{ex} = 323 nm and λ_{em} = 448 nm) due to the release of MUF. All rates were measured during the linear part of the progress curve. A calibration curve was constructed with 4-methylumbelliferone (MUF). One unit of activity was defined as the amount of enzyme required to release 1 μmol of MUF per minute under the conditions described above. Protein concentration was determined by the Bradford method [15]. All tests were carried out 3 times in triplicate.

2.7. Temperature and pH Effects. For optimum temperature determination, the reaction was assayed using MUF-Hep as substrate at 50, 60, 70, and 80°C with 50 mM phosphate buffer pH 7.0.

For optimum pH determination, the reactions were performed at 70°C with 100 mM BIS-TRIS-propane buffer at pH 6, 7, 8, and 9.

2.8. Thermostability Analysis. The enzymes TRX-PF2001Δ60 and PF2001Δ60, in sodium phosphate buffer (50 mM pH 7.0) containing 0.1% gum arabic and 0.4% Triton X-100, were preincubated for different times at 55, 75, and 95°C.

Tubes were removed periodically and assayed for lipase activity using MUF-Hep at 70°C. The Triton X-100 influence on thermostability was determined for PF2001Δ60 at 70°C with and without 0.4% Triton X-100.

2.9. Substrate Preference and Influence of Ions and Inhibitors. The substrate preferences of TRX-PF2001Δ60 and PF2001Δ60 were determined using 100 μM of the MUF-Ace, MUF-Hep, and MUF-Pal substrates. The effects of metal ions on lipase PF2001Δ60 activity were assessed by measuring the residual activity after preincubation in phosphate buffer 50 mM with 10 mM of metal ion at 70°C and pH 7.0 for 10 min. The metal ions used were KCl, $MgCl_2$, $FeCl_2$, and $CaCl_2$. To determine the effects of enzyme inhibitors, the PF2001Δ60 enzyme was pre-incubated as above, and then the substrate was added in order to measure lipase activity. The inhibitors and concentrations used were sodium dodecyl sulfate (SDS) 1 mM, dithiothreitol (DTT) 1 mM, ethylene diamine tetraacetic acid (EDTA) 1 and 10 mM, and ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) 1 and 10 mM.

2.10. Data Analyses. Lipase activity was analyzed using MINITAB v.14 package. The values expressed were based on the average of triplicate experiments. Experimental errors were between 5 and 10%, and averages were compared using t-test ($P < 0.05$).

3. Results and Discussion

In a previous work, we reported the identification and cloning of a novel lipase from *Pyrococcus furiosus* encoded by the ORF PF2001 [10]. The enzyme was cloned and expressed without the first 60 nucleotides (a hypothetical signal peptide). A thioredoxin tag was fused to the amino terminal domain of the enzyme. The enzyme in a crude extract from *E. coli* presented the best activity at 60°C (pH 7.0). This temperature is 40°C lower than the optimal *P. furiosus* growth temperature. In order to understand the reasons for this large difference, and to validate the fusion of the TRX tag as a method for expressing the extremophilic lipases, we performed in this work the purification of the enzyme and its characterization before and after thioredoxin tag removal.

3.1. Purification and Enterokinase Hydrolysis. The TRX-PF2001Δ60 purification results are summarized in Table 1. The enzyme was completely purified with a yield of 89.8% (Figure 1, lane 3). A purification of 6.6-fold was achieved. The specific activity of the purified enzyme was 8.9 U/mg using MUF-Hep as the substrate.

The purified enzyme was cleaved with enterokinase, and the hydrolyzed product showed two protein bands (about 48.8 and 26.5 kDa, representative of TRX-PF2001Δ60 and PF2001Δ60, resp.) in the SDS-PAGE analysis (line 4, Figure 1(a)). Both proteins rendered a positive signal in the zymogram analysis. Therefore, the PF2001Δ60 preparation also contained some of TRX-PF2001Δ60. However, densitometry analyses show that less than 10% of the enzyme remains in the TRX-PF2001Δ60 form (Figure 1(a)). Furthermore, the zymogram (Figure 1(b)) is not a quantitative analysis. So the influence of this form of protein is minimal, within experimental error.

The purified enzyme was stable at −20°C for several months (data not shown).

3.2. Effects of Thioredoxin Tag

3.2.1. Optimal Temperature. The activities of TRX-PF2001Δ60 and PF2001Δ60 increased linearly from 50 to 70°C. At 80°C, the measured activities were higher than at 70°C, but the slope of the curve over this range was less steep than between 50 and 70°C. Among the temperatures tested, the temperature at which both enzymes show maximum activity was 80°C. This temperature was considered as the optimal temperature for both enzymes (Figure 2(a)).

At 50°C, TRX-PF2001Δ60 and PF2001Δ60 exhibited the same level of activity (3 U/mg), but the difference between them increases with temperature. At 80°C, the enzyme without the thioredoxin tag is twice as active as the enzyme with the thioredoxin tag.

Thioredoxin is a peptide of 11.7 kDa, responsible for 24% of the total molecular weight of the recombinant fusion protein. Although this tag is considered a thermostable protein [16], Pedoni et al. [17] demonstrated that 90% of the enzyme is denatured at 90°C. These facts may explain the reason that the lipase with and without thioredoxin tag has the same optimal temperature, but with TRX-PF2001Δ60 presenting lower activity at higher temperatures. We believe that the TRX-tag does not modify the lipase structure, but it diminishes the quantity of active enzyme.

3.2.2. Optimal pH. The enzymes TRX-PF2001Δ60 and PF2001Δ60 showed optimum activity in pH 7.0. The enzyme with and without the thioredoxin tag demonstrated 73 and 90% of its maximum activity, respectively, at pH 8.0 (Figure 2(b)). The optimal pH for lipases and esterases according to Petersen is determined by the electric charge distribution on the active site surface [18]. These results suggest that the thioredoxin tag is sufficiently distant from the active site to cause any effect on the charge distribution.

3.2.3. Thermostability. The resistance to heating in the presence of Triton X-100 was investigated at the temperatures of 55, 75, and 95°C and showed that the enzyme is endowed with high thermostability. Around 100% activity was recovered after 6 h pre-incubation at 55 and 75°C. However, less than 50% activity was retained after 10 min at 95°C. No differences were observed between PF2001Δ60 and TRX-PF2001Δ60 (Figure 3).

3.3. Substrate Preference and Effects of Ions and Inhibitors. Among the different substrates tested, the enzyme was more active towards MUF-Hep. The activities towards MUF-Ace and MUF-Pal were, respectively, equivalent to 20.8 and 6.4% of the activity towards MUF-Hep.

Table 1: Summary of the purification of the recombinant TRX-PF2001Δ60 from *P. furiosus*.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Soluble Ext.	16.9	22.5	1.34	100	—
Ni-NTA	2.28	20.4	8.9	89.8	6.63

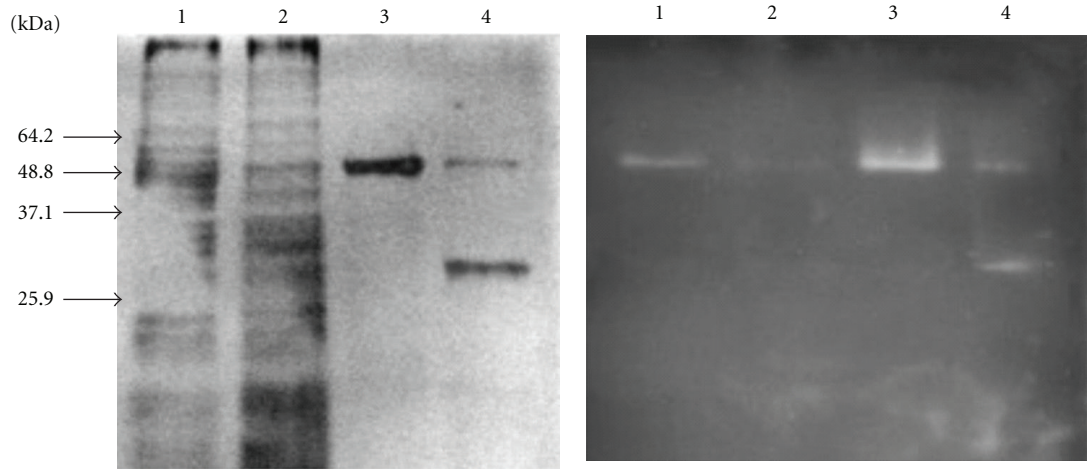

Figure 1: SDS-PAGE (a) and zymography (b), on a 15% polyacrylamide gel, of expressed and purified protein encoded by the PF2001Δ60 gene. (1) soluble extract; (2) flow-through; (3) recombinant lipase eluted with 150 mM imidazole; (4) lipase treated with enterokinase to remove the thioredoxin tag.

Table 2: Effects of metal ions, chemicals, and detergents on PF2001Δ60 lipase activity. The enzyme (0.5 μg) was incubated at 70°C in 50 mM sodium phosphate buffer (pH 7.0) in the presence of the tested agent using MUF-Hep as substrate. After 10 min, the mixture was assayed for residual activity using the standard lipase assay. The activity of the control was considered 100%.

Chemical	Concentration (mM)	Relative activity (%)
KCl	10.0	107.5
$MgCl_2$	10.0	77.9
$CaCl_2$	10.0	54.8
SDS	1.0	37.0
DTT	1.0	94.6
Triton X-100	6.4	47.0
EDTA	1.0	97.8
	10.0	195.7
EGTA	1.0	103.4
	10.0	169.0
PMSF	1.0	0.0

In order to define the ion and inhibitor effects on the PF2001Δ60, enzyme the activity assays were performed in the presence of the above-mentioned substrates.

The influence of mono- and divalent metal ions was studied at a concentration of 10 mM. K^+ did not affect the activity of the enzyme significantly, but significant activity loss was observed with Mg^{2+} and Ca^{2+} (20 and 45%, resp.). This suggests that PF2001Δ60 enzyme is not a metalloenzyme. This was reinforced by a significantly higher activity of the enzyme in the presence of 10 mM of metal chelators: EDTA (activity increased by 96%) and EGTA (69%) (Table 2).

PF2001Δ60 retained full activity in the presence of the disulfide bond reducing agent DTT. The enzymatic stability against DTT suggests that the Cys166 and Cys167 are not disulfide bonded, or that this covalent bond does not affect the protein structure.

In the presence of the detergents: SDS (1mM) and Triton X-100 (0.4%–0.6 mM), the activity decreased by 63% and 53%, respectively. Similar inhibitory effects on microbial lipases and esterases activity have been observed by other groups [19–22].

Because Triton X-100 has traditionally been used in a concentration of 0.4% as an emulsifier to measure lipase and esterase activities, its effects were further investigated.

3.4. Effects of Triton X-100 on Optimal Temperature and Thermostability. Figure 4(a) shows that the presence of Triton X-100 in the reaction mixture drastically modifies the temperature dependence profile of the enzyme. Using 0.4% Triton X-100, the optimal temperature of the enzyme was shifted from 80°C to 60°C. This data explains the results described in our previous report [10] that the optimal reaction temperature for this enzyme was 60°C. In those experiments, the enzyme activity was measured in a reaction mixture containing 0.4% of Triton X-100 because this nonionic surfactant is frequently used to help dissolve lipids in water.

The Triton X-100 cloud point temperature is probably one of the main reasons for shift in optimal temperature of the enzyme. At 60°C, most non-ionic surfactants in aqueous

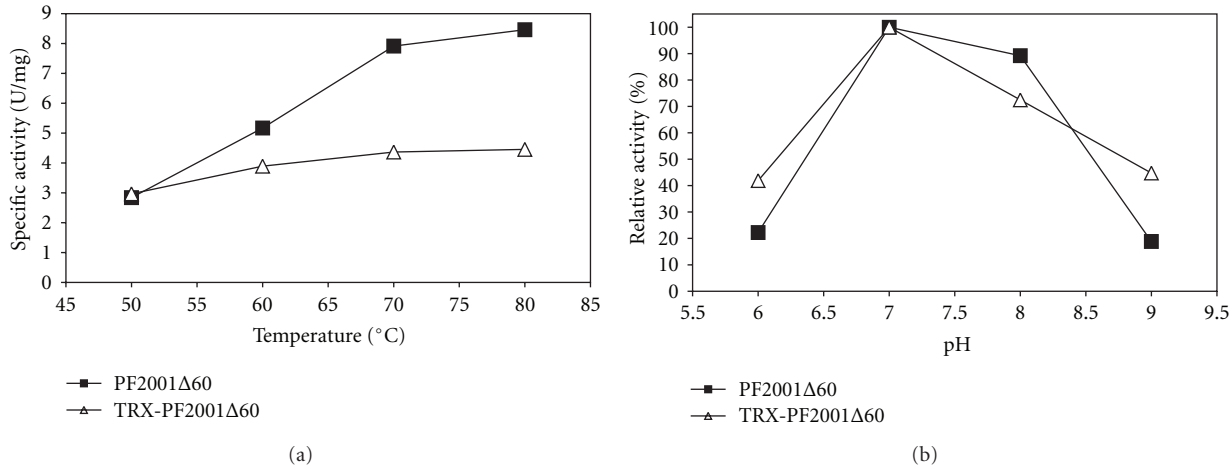

Figure 2: (a) Effects of temperature on lipase activity. The purified lipases, TRX-PF2001Δ60 and PF2001Δ60, were assayed at temperatures ranging from 50 to 80°C, in 50 mM phosphate buffer, pH 7.0. (b) Effects of pH on lipase activity. The purified lipases, TRX-PF2001Δ60 and PF2001Δ60, were assayed at pH ranging from 5.0 to 9.0, in 100 mM BIS-TRIS-propane buffer at 70°C. All assays were carried out without Triton X-100.

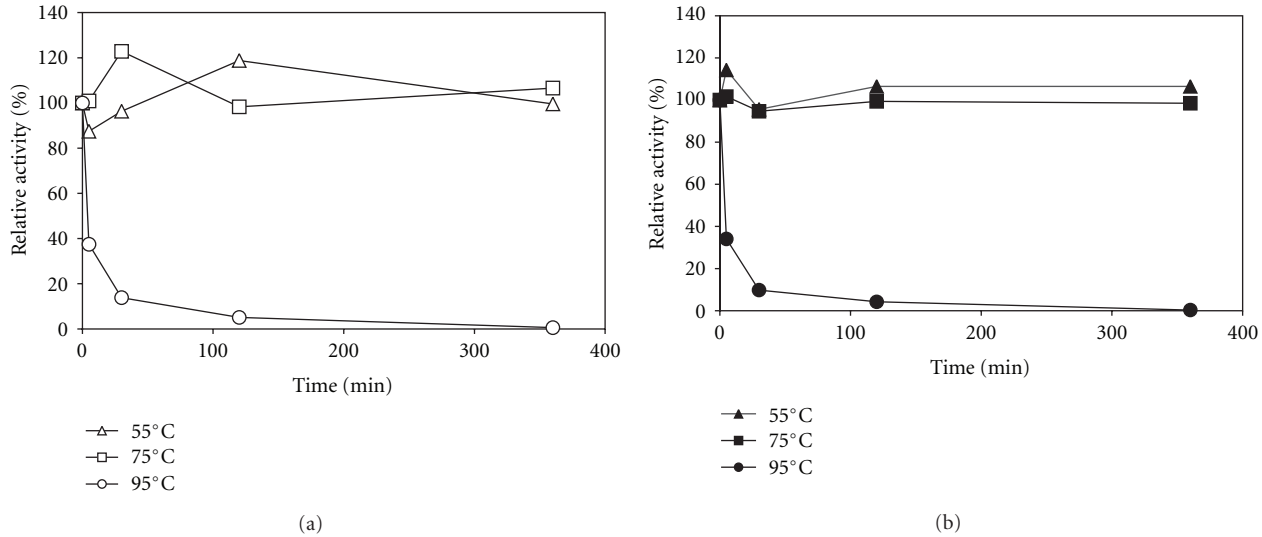

Figure 3: Thermal stability of the purified enzyme, with (a) and without (b) the thioredoxin tag. The enzyme samples in phosphate buffer (50 mM pH 7.0) containing 0.1% gum arabic and 0.4% Triton X-100 were incubated at the indicated temperatures for 5 min, 30 min, 2, and 6 h. The residual enzyme activity was assayed at 70°C, pH 7.0, using the substrate MUF-Hep.

solutions form micelles and become turbid when heated. Above this temperature, the micellar solution separates into a surfactant-rich phase, in which the surfactant concentration is close to the critical micellar concentration.

Although the presence of Triton X-100 affects the activity of the enzyme at higher temperatures, the thermostability was higher when the enzyme was incubated with the detergent. At 70°C, 89% of enzyme activity was recovered after 85 min of incubation in the presence of Triton, but only 40% was recovered when Triton X-100 was not used in the incubation mixture (Figure 4(b)). Studies using this enzyme immobilized on Butyl and Octadecyl-Sepabeads also showed an increase in enzyme thermostability in the presence of Triton X-100 [12]. We believe that interactions between the Triton X-100 molecules and the enzyme provided a more rigid external backbone for the lipase molecules. Also, the effect of higher temperatures in breaking the interactions that were responsible for the proper globular, catalytic active structure, became less prominent, thus increasing the thermal stability of the immobilized lipase.

In spite of its high optimal temperature and thermostability, it is curious to observe the rapid denaturation of PF2001Δ60 at 95°C with or without Triton X-100, a temperature close to the optimal growth temperature of *P. furiosus*. Similar effects were observed when other enzymes were heterologously expressed in *E. coli*; where the enzymes appeared to be significantly less thermostable and barostable than in their natural hosts [23]. These results suggest that

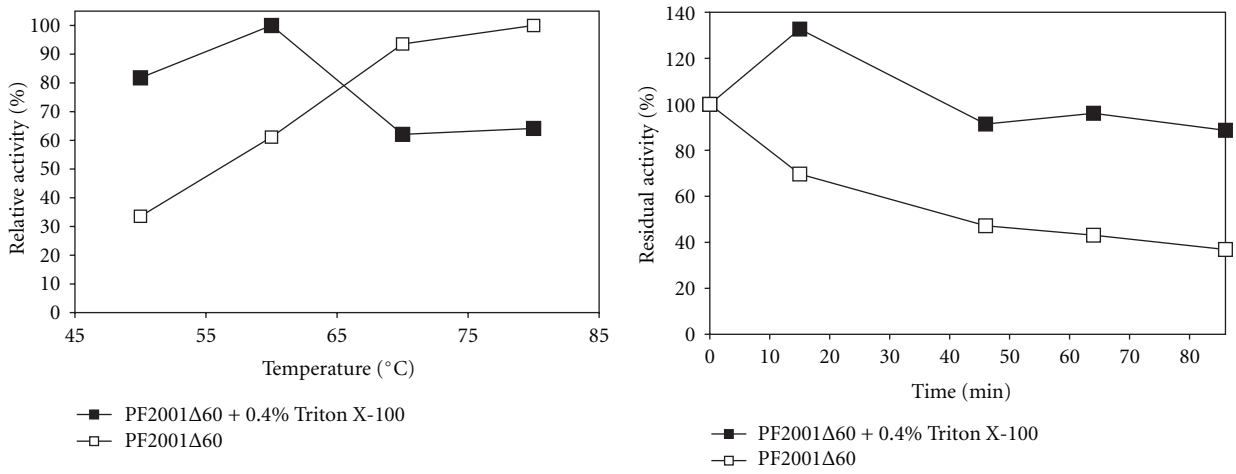

Figure 4: (a) Effect of Triton X-100 on optimal temperature. The lipase PF2001Δ60 was assayed at temperatures ranging from 50 to 80°C, in 50 mM phosphate buffer, pH 7.0, in the presence and absence of Triton X-100. (b) Effects of Triton X-100 on thermal stability of the PF2001Δ60. The enzyme samples in phosphate buffer (50 mM pH 7.0) containing 0.1% gum arabic were incubated at 70°C for 85 min and in the same conditions with 0.4% Triton X-100. The residual enzyme activity was assayed at 70°C, pH 7.0, using the substrate MUF-Hep.

PF2001Δ60 might be stabilized in part by association with other cellular components.

4. Conclusions

The purified recombinant lipase exhibits its highest activity at 80°C—one of the highest temperatures described for a lipase with medium chain length substrate preference. Furthermore, the TRX tag did not influence the optimal pH and optimal temperature of the enzyme; however, the temperature profile was influenced by the TRX tag, reducing its specific activity at 80°C by 50%.

In addition, we show here that Triton X-100, a commonly used detergent in lipase essays, influenced the enzymatic performance; it shifted the optimal temperature to 60°C, diminished the enzyme activity by 50, and stabilized the enzyme towards the temperature.

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Research Article

Amylolytic Microorganism from São Paulo Zoo Composting: Isolation, Identification, and Amylase Production

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Composting is a way of transforming the organic waste into fertilizer, minimizing the use of inorganic compounds that may contaminate the environment. This transformation is the result of the microorganism action, converting complex carbon sources into energy. Enzymes that are exported by the microorganisms to the surrounding environment mediate this process. The aiming of the present work is to prospect the compost produced by the organic composting unit (OCU) of the Fundação Parque Zoológico de São Paulo (FPZSP) to find novel starch hydrolyzing organisms (SHO) that secrete large amounts of amylases under harsh conditions, such as high temperature. We found five bacterial isolates that have amylolytic activity induced by soluble starch and 39°C temperature of growth. These bacterial strains were identified by MALDI-TOF (**M**atrix-**a**ssisted **l**aser **d**esorption/**i**onization-**T**ime **o**f **F**light) analysis, a rapid and efficient methodology for microbe identification in large scale. Our results present amylolytic strains that belong to diverse taxonomic groups (*Solibacillus silvestris*, *Arthrobacter arilaitensis*, *Isoptericola variabilis*, and *Acinetobacter calcoaceticus*); some of them have never been associated with this kind of hydrolytic activity before. The information regarding enzyme induction will be important to optimize the production by the bacterial isolates, which may be a great value for biotechnological applications.

1. Introduction

Amylases produced by plants, animals, and microorganisms have a wide range of industrial applications, such as pharmaceutical, animal feed, paper, textile, food industry, and renewable energy [1–3]. Most amylases used in industry are from microbial source due to several factors, for example, the great microbial genetic diversity present in the environment, high enzymatic activity in a wide range of conditions (extreme pH, temperature, osmolarity, pressure, etc.), and simple and cost effective production [4]. Industrial processes require robust enzymes, especially because large amounts of heat are generated during the process, accumulation of inhibitors, high salt concentrations, and so forth; therefore, hydrolytic microbes, which have their hydrolases induced by temperature, may produce thermotolerant enzymes and, consequently, are highly desirable [5, 6].

Composting is the transformation of residual organic matter into fertilizer accomplished by microorganism, which secrete, among other things, hydrolytic enzymes to assimilate diverse energy sources available in the substrate in order to survive. Compost piles can reach up to 80°C due to microbial metabolism, favoring the development of thermophylic organisms. In this process, the interaction between biotic and nonbiotic factors leads to constant transformation of the complex microbial community over time, which is the mechanism underlining organic matter transformation [7–10].

In Brazil, the São Paulo zoo has an organic compost production unit (OCU), where all the waste from about 3000 native and exotic animals are mixed with plant debris from the Atlantic Forest in a ratio of 30 parts of carbon to 1 part of nitrogen in aerobic condition to produce 600 tons compost per year. This is a self-sustainable process since the final compost generated is used in the zoo farm to fertilize the land, which produces most of the food consumed by the animals in the park. In this work, we used this compost as the starting material to prospect amylolytic enzymes produced by novel thermophylic starch-hydrolyzing organisms (SHOs). To our knowledge, compost comprising these properties has never been screened for amylolytic microorganisms. Here, we describe the isolation and screening of a compost microbe collection (387 entries) for amylolytic activity. Five bacterial isolates from diverse taxonomic groups, identified by MALDI-TOF spectrometry and 16S gene sequencing, were capable of using starch as the major carbon source and secrete amylolytic enzymes to the extracellular environment in response to a combination of soluble starch and 39°C growth temperature. Since many hydrolyzing enzymes are subjected to catabolism repression [11], characterization of induction profile is important for amylase production, fermentation strategies, enzyme purification, and SHO utilization in biotechnology.

2. Material and Methods

2.1. Compost Sampling. Samples were collected according to the method described by Bitencourt et al. [12]. In brief, the temperature of several piles (8 m^3 each) was measured in five different points. Those that displayed temperatures higher than 45°C were probed with a soil auger at 1 meter depth. Organic wastes present in sampled compost piles were food waste, droppings and excreta, beds of native and exotic wild animals, animals carcasses, wood chips from gardening and Atlantic Forest plant waste. Phases of decomposition were considered active degradation (before aeration) and mature compost (after aeration). Pile aeration was achieved by mechanical revolving of the material after 50 to 60 days after composting started.

2.2. Microbial Isolation and Morphological Characterization. All methods of isolation used in this work are suitable to aerobic organisms. Once the samples were removed from the piles at the desired temperature, one gram of compost was weighted and diluted in 5 mL of 0.9% sterile saline, vortex, and allowed to decant for 2 hours. Serial dilutions were prepared and spread on nutrient agar plates (Himedia, India) and incubated at 30°C. After 24 and 48 hours, plates containing isolated colonies were selected, and the isolated colonies were transferred individually to obtain single purified colonies. In order to classify the isolated microorganisms in the main groups of bacteria, yeast and fungi, microscopic analysis of purified colonies was carried out in fresh preparations analyzed in optic microscope (Axiscope, Zeiss). Gram staining was conducted with commercial kit (LaborClin, Brazil) and confirmed by growth on MacConkey agar (Himedia, India) plates. Bacteria capable of growth in this selective medium were considered Gram negative.

2.3. Enzymatic Activity on Agar Plates. The purified colonies were point inoculated on starch induction medium containing 0.6% peptone, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 1% soluble starch, 2% agar, pH 7.0 for bacteria and 1.4 g/L de KH_2PO_4, 10 g/L de NH_4NO_3, 0.5 g/L de KCl, 0.1 g/L de $MgSO_4.7H_2O$, 0.01 g/L de $FeSO_4.7H_2O$, 2% soluble starch, and 2% agar, pH 5.5 for yeast and filamentous fungi [13], incubated at 30°C, 39°C, and 45°C for 48 to 72 hours. The starch present in the bacteria induction medium was considered the major carbon source. However, it was not the sole carbon source, since peptone, which is considered a complex additive, was added to the medium. After the incubation period, the plates were exposed to iodine crystals during 5 minutes to reveal the starch degradation zone [14]. Microorganisms were considered amylolytic when they were able to grow in the presence of starch as the major carbon source and formed a degradation zone around the colony. All experiments were done in triplicate at 3 temperatures (30, 39, and 45°C), and a starch hydrolytic index (SHI) was assigned to each isolate. SHI was calculated as the sum of colony and degradation zone diameter (cm) divided by colony diameter.

2.4. Growth Evaluation and Amylase Production. each isolate under investigation was cultured for 16 hours in 50 mL of LB (Luria Bertani) complex medium (10 g/L NaCl, 10 g/L Tryptone and 5 g/L Yeast Extract, pH 7.0) at 150 rpm at 30°C. Cells were harvested by centrifugation, washed twice in sterile distilled water, and inoculated (OD_{600}0,3) in 50 mL of starch medium (0.6% peptone, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 1% soluble starch) or LB medium. The cultures were incubated on a rotary shaker (150 rpm) at 30, 39 and 45°C. Samples of the supernatant were collected at specific time points (0, 2, 4, 6, 16, 18, 24, and 26 hours) to evaluate the growth pattern at OD600. The aliquots drawn for the enzymatic assays were centrifuged to remove the cells followed by filter sterilization (0,22 μm).

2.5. Determination of Enzymatic Activity. The enzymatic activity was determined according to Smith & Roe [15] adapted by Medda & Chandra [16]. Briefly, a total of 0.5 mL of cell-free supernatant of the cultures were assayed for amylolytic activity in 1 mL of 1% soluble starch diluted in 50 mM acetate buffer pH 5.6 during 15 minutes at 96°C for all samples taken from all time points. The cell pellet extract (0.5 mL) was also assayed for the presence of intracellular amylolytic enzymes. In brief, pellet was washed two times in water, resuspended in the reaction buffer, and submitted to 3 cycles of 1 minute ultrasonic liquid processing (LKB Instruments, Australia) under ice bath during the entire procedure. A sample of the cell extract was observed under the microscope to ensure cell lyses. Reducing sugar for supernatant and cellular extract was assayed by dinitrosalicylic acid (Sigma, USA) method according to standard protocol by Miller [17]. Protein concentration of each time point was determined by Bradford method [18], using the Bio-Rad protein assay kit (BioRad, USA) according to the instructions provided

by the manufacturer. All growth curves and reducing sugar assays were performed in triplicates ($n = 9$). One unit was considered the amount of enzyme required to generate 1 μmol of glucose per minute at 96°C. Specific activity was defined as U/mg of protein. Maximum total units refer to all units produced in 50 mL culture at the time point in which the highest specific activity was achieved.

2.6. Bacterial Identification by Mass Spectrometry. Taxonomic identification was done by MALDI-TOF analysis [19], which provides protein profiles from each isolate. The methodology employed was the ethanol/formic acid extraction, which is suggested by Bruker Daltonics. In brief, cells from single colonies were recovered by scraping the plate and transferring the material into a tube with 300 μL of sterile water and mixing carefully. Absolute ethanol (900 μL) was added, mixed, and centrifuged at 10.000 g for 2 minute at room temperature (25°C). The supernatant was discarded and the pellet air dried. The dry pellets were mixed thoroughly with 50 μL 70% formic acid (Merck, USA) and 50 μL acetonitrile (Merck, USA). The suspension was centrifuged at 10.000 g for 2 minutes, and 1 μL of the supernatant was place onto an Micro Scout Plate (MSP) 96 polished steel target (Bruker Daltonics GmbH, Germany) and dried at room temperature (25°C). Each sample was overlaid with 1 μL of the matrix solution which consisted of a saturated solution of a-cyano-4-hydroxy-cinnamic acid (Sigma, USA) in 50% acetonitrile—2.5% trifluoroacetic (Sigma, USA), and the matrix/sample was cocrystallized by air drying at room temperature (25°C). Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonics) using FlexControl software (version 3.0, Bruker Daltonics). Spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.6 kV; lens voltage, 7.5 kV; mass range, 2000 to 20 000 Da). For each spectrum, 240 shots in 50-shot steps from different positions of the target spot (automatic mode) were collected and analyzed. Spectra were internally calibrated by using *Escherichia coli* ribosomal proteins. The raw spectra were imported into the BioTyper software (version 2.0, Bruker Daltonics), processed by standard pattern matching with default settings, and the results reported in a ranking table. Outcomes of the pattern-matching process were expressed as proposed by MALDI-TOF biotyper (MT) manufacturer with ID scores ranging from 0 to 3. Scores < 1.70 were considered not to have a generated reliable ID; a score of 1.7 < ID < 1.9 was considered ID to genus, and a score > 1.9 was used for reliable species ID.

2.7. Bacterial Identification by 16S DNA Sequencing. gDNA was extracted from 10 mL overnight cultures with Axygen bacterial genomic DNA extraction kit (Axygen Scientific, USA). Small Subunit RNA (16S) was amplified by PCR (polymerase chain reaction), using 1 U Taq Polymerase (New England Biolabs, USA), 1x Buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2 mM $MgCl_2$, 200 μM dNTP's (Fermentas, Finland), 0.2 μM of each primer (PRCP005 5′ GTGCCAGCMGCCGCGG 3′ and PRCP006 5′ ACGGGCGGTGTGTRC 3′, IDT, USA). Amplification condition: 1 cycle 94°C for 5 minutes, 35 cycles: denaturing at 94°C for 30 seconds, primers annealing at 54°C for 30 seconds, and extension at 72°C for 1 minute, a final extension of 72°C for 7 minutes. Single-band amplicons were purified with PCR Clean up Kit (Axigen Scientific, USA) and double stranded sequenced with BigDye Terminator (Applied Biosystems, USA) according to the manufacturer's instructions. The sequences were aligned using SeqMan module of software Lasergene (DNAStar, USA) and blasted against Genebank and Ribosomal Database Project (RDP). Identification at species level was considered for sequence similarities above 97%.

3. Results and Discussion

3.1. Microorganism Isolation from São Paulo Zoo Park Organic Compost Unit. Three samples of different compost piles were removed from São Paulo zoo OCU over an approximately 5-month period. The properties (composition, temperature, pH and decomposition phase) of the compost pile are summarized in Table 1.

A total of 387 microorganisms were isolated at 30°C from these samples as described in materials and methods. Among these, under microscopic examination, 67% were classified as bacteria, 20% as filamentous fungi, 11% as yeast, and 2% remains undetermined, due to ambiguous morphology, which impaired positive classification. Table 1 shows that, as the temperature in the compost pile rises, there is a decrease in microbial population, which is reflected by the lower number of isolates in compost pile 2 ($n = 38$) compared to piles 1 and 3 ($n = 183$ and $n = 166$, resp.).

3.2. Amylolytic Microorganism Screening. Fifty-five microorganisms were classified as SHO (14%). Considering each compost pile individually, the SHO percentage is very similar (Table 1). Among these SHO isolates, 10 amylolytic bacteria were chosen for further studies. The selection criteria was (i) the ability to grow in 1% starch medium as the major carbon source at 30, 39, and 45°C and (ii) the production of a SHI of, at least, 1.5 in total average (Table 2). Also, gram staining and colony macromorphology were taken into account, in order to guarantee the analysis of a greater diversity of microorganisms (data not shown).

None of the yeast and filamentous fungus isolated was classified as SHO. It is possible that the high temperature observed at the collection points taken for this work (Table 1) exterminated most of these organisms, or the isolation conditions did not favor them. Therefore, samples collected from piles with a lower temperature and a different isolation strategy may be necessary to augment the number of filamentous fungus and yeast capable of starch degradation. An enrichment liquid media containing soluble starch as the sole carbon source, low pH and the antibiotics addition to the media maybe a way to preferentially isolate amylolytic yeasts and fungi. The compost sample could be directly added to this media and cultured overtime. After several passages in liquid media, the enriched culture could be inoculated in solid starch media to obtain single colonies.

Table 1: Compost pile properties.

Properties	Pile 1	Pile 2	Pile 3
Date	19.02.2009	12.05.2009	18.07.2009
Average temperature	48.7	72.6	55.2
pH	—	6.1	6
Decomposition phase	*Active degradation	Active degradation	**Mature
Number of isolates	183	38	166
SHO	15%	13.1%	12.6%

*Active degradation: before aeration.
**Mature: after aeration.

Table 2: Starch hydrolytic index (SHI) on 1% starch solid medium at 3 different temperatures for 10 SHO chosen for further studies.

Isolate	SHI 30°C	SHI 39°C	SHI 45°C	Average
URX291	1.6 ± 0.2	1.9 ± 0.5	2 ± 0.8	1.8 ± 0.2
URX303	1.7 ± 0.3	1.8 ± 0.8	1.9 ± 1.0	1.8 ± 0.1
URX336	1.5 ± 0.3	1.7 ± 0.1	1.5 ± 0.5	1.6 ± 0.1
URX350	1.8 ± 0.4	1.9 ± 0.5	1.8 ± 0.5	1.8 ± 0.1
URX356	1.6 ± 0.2	1.9 ± 0.3	2.0 ± 0.8	1.8 ± 0.2
URX377	2.1 ± 0.1	2.0 ± 0.3	1.8 ± 0.2	2.0 ± 0.2
URX379	1.9 ± 0.3	1.7 ± 0.2	2.0 ± 0.7	1.9 ± 0.2
UED487	1.6 ± 0.3	1.9 ± 0.5	2.0 ± 0.6	1.8 ± 0.2
UED 641	2.3 ± 1.1	2.0 ± 0.5	1.6 ± 0.2	2.0 ± 0.3
UED 644	2.6 ± 1.2	1.7 ± 0.4	1.9 ± 0.5	2.1 ± 0.5

Each data point is the result of 3 replicates for each temperature.

3.3. Quantification of the Amylolytic Activity in Liquid Culture. Growth curves in starch liquid medium were performed at 30, 39, and 45°C for ten selected isolates; however Figure 1 shows the growth pattern for five isolates chosen based on their enzymatic activity in response to temperature. Figures 1(a) and 1(b) show that the performance of each isolate follows a similar trend of growth under these conditions (30°C versus 39°C). At 45°C, all isolates presented a reduce growth and isolates URX303 and URX350 grew to a lower extent compared to other three isolates, especially at the end of the growth period (Figure 1(c)).

The amylolytic activity in the supernatant of the cultures grown at three temperatures (30, 39, and 45°C) was measured over a 26-hour period (8 time points) in order to establish the temperature induction pattern for the extracellular starch degrading enzymes. Activity was determined by measuring the reducing sugar released during the incubation of cell-free supernatant with soluble starch, according to the conditions described in Material and Methods. Also, the amount of reducing sugar released by enzymes present in the cell pellet extract was assayed, but no activity was detected (data not shown), suggesting that most of the amylases produced are exported to the extracellular fraction and any active enzyme that remained inside the cells was probably at concentrations bellow the sensitivity of this assay. In accordance to our results, Ryan et al. [20] reported the analysis of *Bifidobacteria* strains from the Japan Collection of Microorganisms (JCMs) that produces extracellular starch hydrolyzing enzymes. In this report, cell-free supernatants and cellular extracts were qualitative assayed for amylolytic activity on plates containing starch, amylopectin, or pullulan. They found that the amylolytic activity was present only in the extracellular fraction. In our assays cellular extracts showed no activity.

Out of the 10 isolates analyzed for extracellular amylolytic activity, 5 isolates (URX291, URX303, URX350, UED641, and UED644) clearly showed induction in the presence of starch as the major carbon source at 39°C growth temperature (Figures 2(a) to 2(e) and Table 3). Moreover, none of these 5 chosen isolates showed significant extracellular enzymatic induction at 30°C and 45°C, indicating that the optimum temperature for amylase secretion in this group of isolates is 39°C (Figures 2(a)–2(e)). Therefore, all subsequent enzymatic analysis was performed with supernatant harvested at 39°C and 30°C. The latter temperature of cultivation was employed as a control.

In order to verify if expression of the amylolytic enzymes is dependent upon substrate induction, besides temperature, the specific activity (U/mg of protein) was calculated for all 5 isolates in starch induction medium and LB at all 8 time points collected during growth at 30 and 39°C. Table 3 shows the time points in which the highest specific activity can be found for the 5 isolates in each cultivation condition (maximum specific activity). All isolates had higher specific activity in starch at 39°C. Isolates URX303 and UED644 had the highest specific activity (0.278 at 26 hours and 0.230 at 2 hours, resp.), showing that URX303 has the longest time of induction response whereas UED644 had the fastest among these isolates.

Table 3: Maximum specific activity (U/mg of protein) and the total units produced at the time point (hours) where the highest specific activity was found for each of the 5 isolates that showed enzymatic induction. 30°C and 39°C are the temperatures of growth where supernatant samples were collected to conduct the enzymatic assays. LB indicates the complex medium used, and starch refers to the induction medium containing starch as the major carbon source, in addition to a trace of peptone. All data points are average of 3 replicates of growth curves and 3 replicates of enzyme assay per time point (equals to 9 replicates).

Strain	30°C						39°C					
	LB			Starch			LB			Starch		
	U/mg	Total units	Time point	U/mg	Total units	Time point	U/mg	Total units	Time point	U/mg	Total units	Time point
URX291	0.079 ± 0.007	4	6	0.022 ± 0.001	4.3	16	0.043 ± 0.002	5.9	18	0.114 ± 0.016	8.2	6
URX303	0.098 ± 0.002	6.8	18	0.015 ± 0.002	2.7	16	0.036 ± 0.011	4.5	16	0.278 ± 0.024	25.2	26
URX350	0.097 ± 0.007	6.5	16	0.022 ± 0.004	2.2	16	0.037 ± 0.001	4.7	16	0.134 ± 0.014	19.4	18
UED641	0.094 ± 0.005	6.1	16	0.029 ± 0.002	2.9	4	0.035 ± 0.001	4.4	16	0.120 ± 0.006	8.7	2
UED644	0.160 ± 0.027	9.2	24	0.037 ± 0.003	3.2	2	0.035 ± 0.001	4.3	16	0.230 ± 0.024	19.4	2

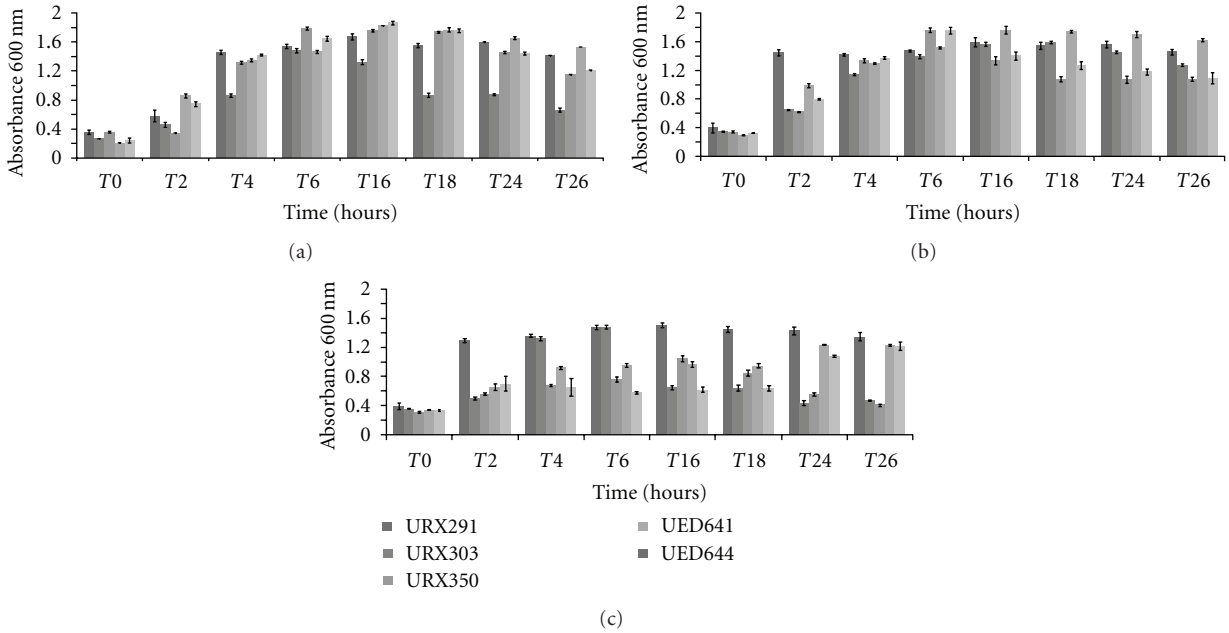

Figure 1: Growth pattern of five isolates over 26-hour period. (a) 30°C, (b) 39°C, and (c) 45°C. All growth curves were done in triplicates for all three temperatures, the bars represent standard deviation.

Orlando et al. [21] reported a *Bacillus subtilis* strain that presented a specific activity of 0.83 U/mg of protein in crude extract. However, the authors used much larger culture volume (50 liter tank) and longer fermentation (42 hours). Mabrouk et al. [22] also reported the cloning and expression of a *Bacillus sp.* amylase in heterologous system and was able to achieve 0,93 U/mg of protein in crude extract. Carvalho et al. [23] also studied an unidentified bacteria isolated from petroleum contaminated soil that presented the maximum amyloglucosidase activity at 0.18 U/mL, using starch as the sole carbon source. A screen for starch degrading *Bifidobacterial* strains by Ryan et al. [20] reported that 11 of these microorganisms belonging to various species are able to produce a range of specific activities (from 0.84 to 1.35 U/mg of protein).

Kubrak et al. [24] reported a *Bacillus sp.* BKL20 strain isolated from natural can reach up to 7 U/mg of enzyme specific activity. However, the cultivation parameters (starch, peptone and yeast extract concentration and temperature) have been optimized for this strain reflection an increment of activity.

Table 3 shows that the amylase induction is dependent on the combination of starch-containing media and 39°C temperature of culture for isolates URX303, URX350 and

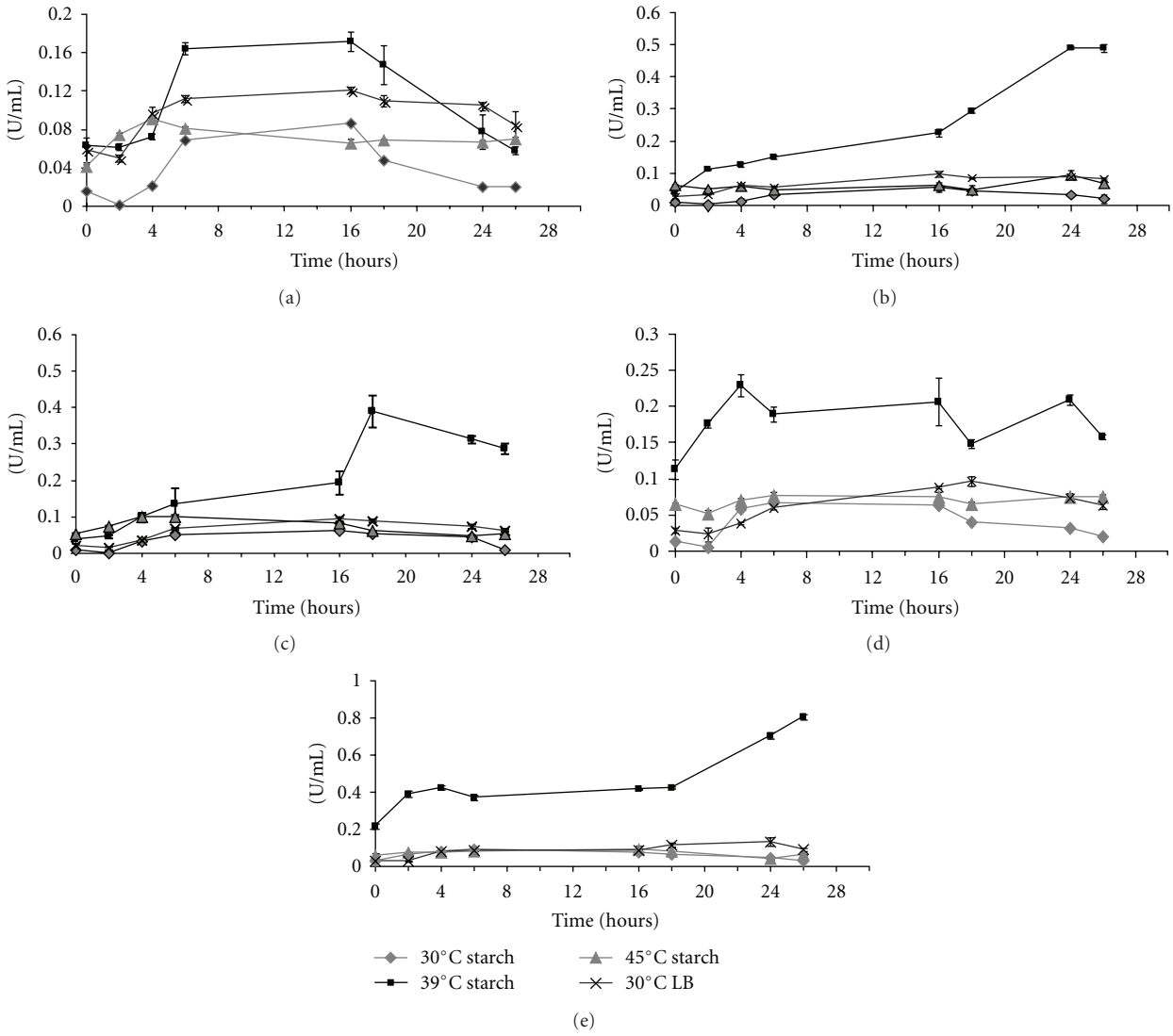

Figure 2: Kinetics of amylase activity in the culture supernatants of five isolates (a) URX291, (b) URX303, (c) URX350, (d) UED 641, and (e) UED644 in starch medium for 26 hours at 30 (diamonds), 39 (squares), 45°C (triangles), and in LB rich medium at 30°C (crosses). $n = 9$. Vertical lines represent error bars.

UED644 (9.3, 8.8, and 6.0 fold induction over 30°C, resp.). On the other hand, the induction for these isolates is less pronounced when they are switched from LB to starch at 39°C (5.6, 4.1, and 4.5 fold induction, resp.). For isolates URX291 and UED641, the fold-induction for amylase production on starch-containing medium at 30°C versus 39°C was 1.9 and 3, respectively.

The enzyme activities reported in this work are relevant once compared to the activities available in the literature and cited above, especially considering that no optimization of the growth conditions has been performed for each isolate. Furthermore, these results are of great interest in other aspects, such as for the optimization of fermentation procedure, protein purification, and microbial characterization. The knowledge of the induction kinetics regarding substrate and temperature will allow the harvesting of the largest amounts of protein, which is an important factor regarding production and often a limiting step in protein chemistry. Moreover, this information will be valuable for enzyme production by SHO, considering that they can be used in biotechnological processes.

3.4. SHO Identification. We believe that the São Paulo Zoo OCU has the potential to host a large number of novels, not yet described microorganisms, which could be useful for hydrolytic enzyme search. In order to evaluate this possibility, we used two different tools to identify the selected amylolytic microbes and attribute at least genus level identification to each one of them. As a first screen, we generated mass spectrometry profiles and compared them to the Bruker Daltonics database using Biotyper software. Table 4 shows the results found by this technology. Five out of ten microorganisms could not be identified because no similar profiles were found in the database. Since the database is composed roughly of 4000 entries from clinical origin, and we are prospecting environmental microbes, this outcome is

Table 4: Microorganism identification based on 16S gene sequence and protein profile by MALDI-TOF.

Isolate	Biotyper	16S (SSU RNA)	Accession #
URX291	*Bacillus sp.*	*Bacillus megaterium* DSM319 (99%)	CP001982.1
URX303	(−)	*Bacillus safensis* DSM 19292 (99%)	JF798363.1
URX336	(−)	*Bacillus subtilis* CCGB:1266 (99%)	GU434357.1
URX350	(−)	*Enterobacter asburiae* DSM 17506 (100%)	HQ242717.1
URX356	(−)	*Solibacillus silvestris* BCHCNZ317 (98%)	GU188937.1
URX377	*Arthrobacter sp.*	*Arthrobacter arilaitensis* RE117 (97%)	FQ311875.1
URX379	*Arthrobacter sp.*	*Arthrobacter arilaitensis* RE117 (96%)	FQ311875.2
UED487	(−)	*Isoptericola variabilis* NBRC 104115 (99%)	AB489221.1
UED641	*Acinetobacter sp.*	*Acinetobacter calcoaceticus* DSM3000 (99%)	X81668.1
UED644	*Bacillus cereus*	*Bacillus cereus* BCHMAC21 (98%)	GU188894.1

(−) no matches found in the Bruker Daltonics database.

not surprising. In order to validate MALDI-TOF results and attempt positive identification of the unidentified isolates, we conducted molecular identification by 16S gene sequencing (Table 4).

All isolates showed high sequence similarities to 16S entries in the GeneBank and at Ribosomal Database Project (RDP). URX 291, URX336, URX303, and UED644 have high similarity sequences at species level to *Bacillus megaterium* (99%), *B. subtilis* (99%), *Bacillus safensis* (100%), and *B. cereus* (98%), respectively. Except for URX303 (*B. safensis*), all isolates belonging to this genus have already been reported as SHO, and all of them were reported as extracellular producers and had at least alpha-amylase gene cloned and expressed in heterologous system, [21, 25–27].

However, six isolates (URX350, URX356, URX377, URX379, UED487, and UED641) showed similarity sequence to species that, either, have been described recently, therefore have limited information regarding biological properties (URX356 and UED487), or are well known, but have never been associated with amylolytic activity (URX350, URX377, UED641, and URX379).

URX377 and URX379 were both considered as *Arthrobacter arilaitensis*. To our knowledge this microorganism has never been associated with this enzymatic activity. However, Gratia et al. [28] demonstrated that *Arthrobacter psychrolactophilu* is an extremophile microbe capable of producing extracellular amylases under low growth temperature, and it is able to clarify wastewater containing starch and protein at 10°C. Other isolates belonging to the *Arthrobacter genus* have been reported as useful to bioremediation since they can consume p nitrophenol [29].

Isolate UXR356 was identified by 16S sequencing as *Solibacillus sylvestris*, and no correlation between this species and hydrolytic activity could be found in the literature suggesting that this is novel information. Similarly, UED487 was identified as *Isoptericola variabilis*, which has not been the target of many reports. However, another species in the genus (*Isoptericola jiangsuensis*) has been associated with chitin-hydrolyzing activity [30].

UED641 was identified as *Acinetobacter calcoaceticus*, which has been reported as an exoenzyme producer [31]. However, since this species is considered an opportunistic pathogen and explored under this perspective, it has not been investigated for biotechnological applications.

4. Conclusions

In this paper, we describe 10 amylolytic bacterial strains belonging to very diverse taxonomic groups, identified in a collection of 387 microbes from São Paulo Zoo composting unit, an exotic substrate that has never been prospected before for hydrolytic microorganisms. The increment on amylases activity, observed on the supernatant of five bacterial strains, showed strong induction in response to the combination of starch and temperature (39°C) during a 26-hour period, whereas the other five bacterial isolates did not present such an induction. Initially, we asked the question whether the São Paulo Zoo compost would be a suitable substrate for the discovery of novel hydrolytic microorganisms, producing extracellular robust enzymes for biotechnological application. The data presented demonstrates that not only this is true, but also SHO isolated here shows a distinct pattern of enzyme expression dependent upon substrate induction and high temperature, which are interesting aspects for enzymes aiming biotechnological application.

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Research Article

Production and Partial Characterization of Cellulases from *Trichoderma* sp. IS-05 Isolated from Sandy Coastal Plains of Northeast Brazil

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This study evaluated the production of cellulolytic enzymes by *Trichoderma* sp. IS-05 strain, isolated from sand dunes, according to its ability to grow on cellulose as carbon source. Wheat bran was tested as the carbon source and peptone tested as the nitrogen source. Different concentrations of carbon and nitrogen were tested using a factorial design to identify optimal cellulase activity production. The results showed that media containing wheat bran 4.0% (w/v) and peptone 0.25% (w/v) lead to the highest production, 564.0 $U\,L^{-1}$ of cellulase, obtained after 2 days of fermentation. The pH and temperature profile showed optimal activity at pH 3.0 and 60°C. As for thermostability, the cellulase was most tolerant at 60°C, retaining more than 59.6% of maximal activity even after 4 hours of incubation. The combination of acid pH, high temperature tolerance, and production of cellulase from agro-industrial residues by *Trichoderma* sp. IS-05 offers possibilities condition for the biomass hydrolysis process to produce bioethanol.

1. Introduction

Cellulosic material is the most abundant renewable carbon source in the world. It is a linear polymer of 8000–12000 glucose units linked together by β-1,4-glycosidic bonds, is a major component of plant biomass, and is naturally degraded by cellulolytic fungi and bacteria. Cellulose hydrolyses to glucose, which can then be used for production of ethanol [1], organic acids [2], and other chemicals [3]. This hydrolysis is carried out via the synergic action of three cellulolytic enzymes: endo-β-D-glucanase (EC 3.2.1.4), exo-β-D-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [4, 5].

Lignocellulosic residues from agriculture and forestry have potential as cheap and renewable feedstocks for the large-scale production of fuels and chemicals. The biodegradation of cellulose to soluble sugar is a process which is only possible after the action of complex cellulolytic produced by cellulolytic microorganisms. In recent years, increased scientific attention has been given to this process due to its environmental and economic significance [6]. Wheat bran is one of the most common agroindustrial residues used as raw material for various processes and products. Industrial wheat bran usually accounts for 14–19% of the grain and comprises the outer coverings, the aleuronic layer, and the remnants of the starchy endosperm. It consists mainly of starch, (glucurono)arabinoxylans, cellulose, β-glucan, protein, and lignin [5].

Significant research efforts have been invested in evaluating and understanding the enzymatic hydrolysis of lignocellulosic substrates by cellulases produced by species of the fungus *Trichoderma* [7–10]. Commercial products of various *Trichoderma* isolates have long been available for cereal foods, brewing, and fruit and vegetable processing and have also been widely evaluated and applied for bioethanol production processes [11].

Studies dealing with cellulase production by fungi using low-cost residues are abundant in the literature. *Trichoderma* species, one of a wide range of cellulase-producing organisms, is ubiquitous in Brazil, being commonly found in lignocellulosic residues and soils. The present work reports on cellulolytic enzyme production by a strain, identified in our laboratory as IS-05 that was isolated from sand dunes on the coastal plains, in Guaibim, BA, Brazil. An experimental design was carried out to study cellulase production using wheat bran (WB) as the carbon source and peptone as the nitrogen source. Crude enzyme preparations (culture supernatants) were used to perform preliminary studies for the temperature and pH activity profile and effect of metal ions on enzyme activity.

2. Materials and Methods

2.1. Isolation, Selection, and Inoculums Preparation of the Fungal Strain. *Trichoderma* sp. IS-05 was collected from the sand dunes at Guaibim beach, BA, Brazil and then isolated and identified by morphological aspects [12]. The crude sample was diluted in 0.85% (*w/v*) saline solution (1 : 10), stirred at 150 rev.min^{-1} for 45 min and serial diluted. Dilutions were plated on microcrystalline cellulose-salt mineral medium agar consisting of (g L^{-1}) 2.6 $(NH_4)_2SO_4$; 2.0 NaCl; 3.0 KH_2PO_4; 6.0 K_2HPO_4; 0.2 $MgSO_4 \cdot 7H_2O$; 0.02 $CaCl_2 \cdot 2H_2O$; 10.0 microcrystalline cellulose (Sigma); 15.0 agar; 1.0 mL trace solution (0.64 g $CuSO_4 \cdot 5H_2O$; 0.15 g $ZnSO_4 \cdot 7H_2O$; 0.11 g $FeSO_4 \cdot 7H_2O$; 0.79 g $MnCl_2 \cdot 4H_2O$; 100 mL distilled water). The inoculated plates were incubated for 10 days at 30°C, and the grown fungi were cultivated in pure culture.

The cellulase-producing capacity of the strain was carried out using filter paper cellulose, in Mandel's media [13], pH 6.0 (g L^{-1}): 2.0 K_2HPO_4, 1.4 $(NH_4)_2SO_4$, 0.3 $MgSO_4 \cdot 7H_2O$, 0.3 $CaCl_2$, 0.005 $FeSO_4 \cdot 7H_2O$, 0.00156 $MnSO_4 \cdot H_2O$, 0.0014 $ZnSO_4 \cdot 7H_2O$, 0.002 $CoCl_2$, 3.0 yeast extract, supplemented with 0.6 g of Whatman Filter Paper Grade N°1 (1.0 × 1.0 cm). The flasks were incubated at 28°C, stirred at 180 rev.min^{-1} for 7 days and daily aliquots of 2.0 mL were collected, filtered, and the cellulase activity was analyzed as described below.

For inoculums production, fungal spores of culture grown on Potato Dextrose Agar (PDA) at 28°C for 15 days, were harvested in sterile saline solution (0.85% *w/v*), as described by Hopwood et al. [14]. Spores were maintained in 20% (*v/v*) glycerol at −20°C.

2.2. Cellulase Production Using Experimental Design. Cellulase activity was measured after cultivation in 250 mL Erlenmeyer flasks containing 50 mL of mineral salts solution [5], pH 6.5 (g L^{-1}, KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 2.6; K_2HPO_4, 6.0; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 2.0 and $CaCl_2$, 0.002) supplemented with a trace element solution (g L^{-1}, $FeSO_4 \cdot 7H_2O$, 1.1; $MnCl_2 \cdot 4H_2O$, 7.9; $ZnSO_4 \cdot 7H_2O$, 1.5 and $CuSO_4 \cdot 5H_2O$, 6.4). Wheat bran (WB) was added as the main carbon source, and peptone was used as the main nitrogen source. The initial pH of the medium was adjusted to 5.0. Culture medium was inoculated with 50 μL of spore suspension (6.22×10^9 spores mL^{-1}), incubated at 28°C, and shaken for 6 days. At periodical intervals (24 hours), flasks were collected, its whole content centrifuged (2500 g for 10 min), filtered, and the supernatants were tested in cellulase activity assays. The supernatants were preserved at −20°C. Results were presented as an average of duplicates.

Optimization of the concentration of WB (C source) and peptone (N source) at 200 rev.min^{-1} was carried out by employing a response surface methodology. As the dependent variable we used the cellulase activity (UL^{-1}) and the independent variables we used the C source (WB) and N source (peptone) concentrations. A 2^2 full factorial central composite rotational design (CCRD) was used to generate 11 run combinations as shown in Table 1 [15]. This design is represented by a second-order polynomial regression model, (1), to generate contour plots:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2, \quad (1)$$

where Y is the predicted response (cellulase activity); X_1, X_2 and X_3 the coded forms of the input variables (WB and peptone, resp.); b_0 a constant; b_1 and b_2 the linear coefficients; b_{12} a cross-product coefficient; b_{11} and b_{22} the quadratic coefficients. The test factors were coded according to the following regression equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i}, \quad (2)$$

where x_i is the coded value and X_i the actual value of the independent variable, X_0 is the actual value at the center point and ΔX_i is the step change value.

ANOVA (analysis of variance) was used to estimate the statistical parameters. The significance of the regression coefficients was determined by the Student's t-test; the second-order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination, R^2. STATISTICA (version 7.0) software from StatSoft Inc. was used for the regression and graphical analysis.

The same medium used in the preliminary tests, supplemented with different combinations of WB as the carbon source and peptone as the nitrogen source, was used for the experimental design (Table 1). Conditions for the inoculation, incubation, and filtration of the supernatant were used as preliminary studies on cellulose production (data not show).

Based on the CCRD experiment, a validation was carried out using, in triplicate, the conditions suggested by the model. The concentration of peptone was fixed at 0.25% (*w/v*) and the WB concentrations used were 4.2%, 4.4%, 4.6%, and 4.8% (*w/v*), generating 4 validation assays. The system was incubated at 28°C/200 rev min^{-1} for 2 days. The supernatant was collected as described above.

2.3. Enzyme Assays. Cellulase (filter paper activity) was assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman N°1 filter paper (1.0 cm × 6.0 cm ≅ 50 mg) as substrate in 50 mM sodium citrate buffer (pH 4.8) at 50°C after 30 min. One unit (U) of cellulase activity corresponded to 1 μmol of glucose

Table 1: Values of independent variables (WB concentration X_1 and peptone concentration X_2, resp.) used in CCRD, showing the values observed and predicted by the mathematical model for cellulase production by *Trichoderma* sp. IS-05.

Run	Coded setting levels		Actual levels (% *w/v*)		Cellulase activity ($U\,L^{-1}$)	
	X_1	X_2	X_1	X_2	O	P
1	−1	−1	2.0	0.05	8.2	102.2
2	+1	−1	4.0	0.05	447.3	463.0
3	−1	+1	2.0	0.25	83.6	102.2
4	+1	+1	4.0	0.25	563.6	463.1
5	−1.41	0	1.59	0.15	21.3	−55.7
6	+1.41	0	4.41	0.15	389.9	453.2
7	0	−1.41	3.0	0.009	465.3	366.6
8	0	+1.41	3.0	0.291	281.8	366.7
9	0	0	3.0	0.15	275.3	285.1
10	0	0	3.0	0.15	288.4	285.1
11	0	0	3.0	0.15	291.6	285.1

Results are the mean of two experiments, O = observed; P = predict.

Table 2: Statistical analysis of variance (ANOVA) for the model of cellulase production at different levels of concentration of WB and peptone.

Source of variations	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P* value*
Regression	287810.6	3	95936.9	14.4	0.007
Residue	46549.1	7	6649.9		
Total SS	334359.7	10			

* Statistically significant at 90% of confidence level. $R^2 = 0.86$.

equivalent released per minute under the assay conditions [16]. Reducing sugars were assayed by the dinitrosalicylic acid (DNS) method [17].

All assays were conducted in duplicates, and results expressed as average values. Variations in the multiple assays were <5%.

2.4. Partial Crude Enzyme Characterization. Temperature profile for cellulase activity was determined by assaying activity at different reaction temperatures (20 to 80°C) in 50 mM sodium citrate buffer (pH 4.8). In the same way, cellulase activity was assayed in different reaction buffers 50 mM (glycine-HCl for pH 2.0–3.0; sodium citrate for pH 3.0–6.0; citrate phosphate for pH 6.0-7.0; phosphate for pH 7.0-8.0; Tris HCl for pH 8.0–10.0) at 60°C to determine the effect of pH on activity. For comparison, some tests (pH and temperature effect) were carried out using the commercial cellulase CAREZYME by Novozyme.

To determine the thermal stability, the crude supernatant was incubated at 60°C and the residual cellulase activity was measured at various time periods (0.5, 1, 2, 4, and 6 h).

The influence of various metal ions on cellulase activity was evaluated with enzymatic assay at pH 3.0 and 60°C after addition of each ion (potassium, barium, iron, calcium, sodium, cobalt and mercury in the chloride form and zinc, manganese, copper, magnesium in the sulfate form) at 10 mM final concentration [5]. The influence of ethylenediamine tetraacetic acid (EDTA) was also tested at the same concentration.

3. Results and Discussion

The fungal strain IS-05, identified as *Trichoderma* sp., was capable of degrading microcrystalline cellulose in a solid plate medium and was therefore selected for further studies. In the present work, we investigated cellulase production by *Trichoderma* sp. using agroindustrial by-products as substrates. In a preliminary experiment, 54 fungal strains isolated from Guaibim sand dunes were grown in submerged fermentation in Mandel's medium supplemented with Whatman N°1 Filter Paper as the sole carbon source (data not show). The *Trichoderma* sp. strain IS-05 was selected as a cellulolytic-promising strain for further fermentation studies. Table 1 presents the observed and predicted results, obtained after cultivation for 2 days. Cellulase activity varied from 8.0 to 564.0 $U\,L^{-1}$. The best result was obtained on Run 4, with WB and peptone concentrations of 4.0% (*w/v*) and 0.25% (*w/v*), respectively. When the concentrations of WB were 4.0% and 3.0% (*w/v*), the cellulase activity was 447.0 and 465.1 $U\,L^{-1}$, respectively, for Runs 2 and 7. Generally, best results of enzyme production were obtained for high concentrations of WB (Runs 2, 4, 6 and 7) together with low concentrations of the N source.

The model was tested for adequacy by the analysis of variance (ANOVA). The computed *F*-value (14.43) indicates that the model was significant at a high confidence level. The probability *P* value was also very low (<0.01) indicating the significance of the model (Table 2). The coefficient of variation ($R^2 = 0.86$) also indicates a very good correlation

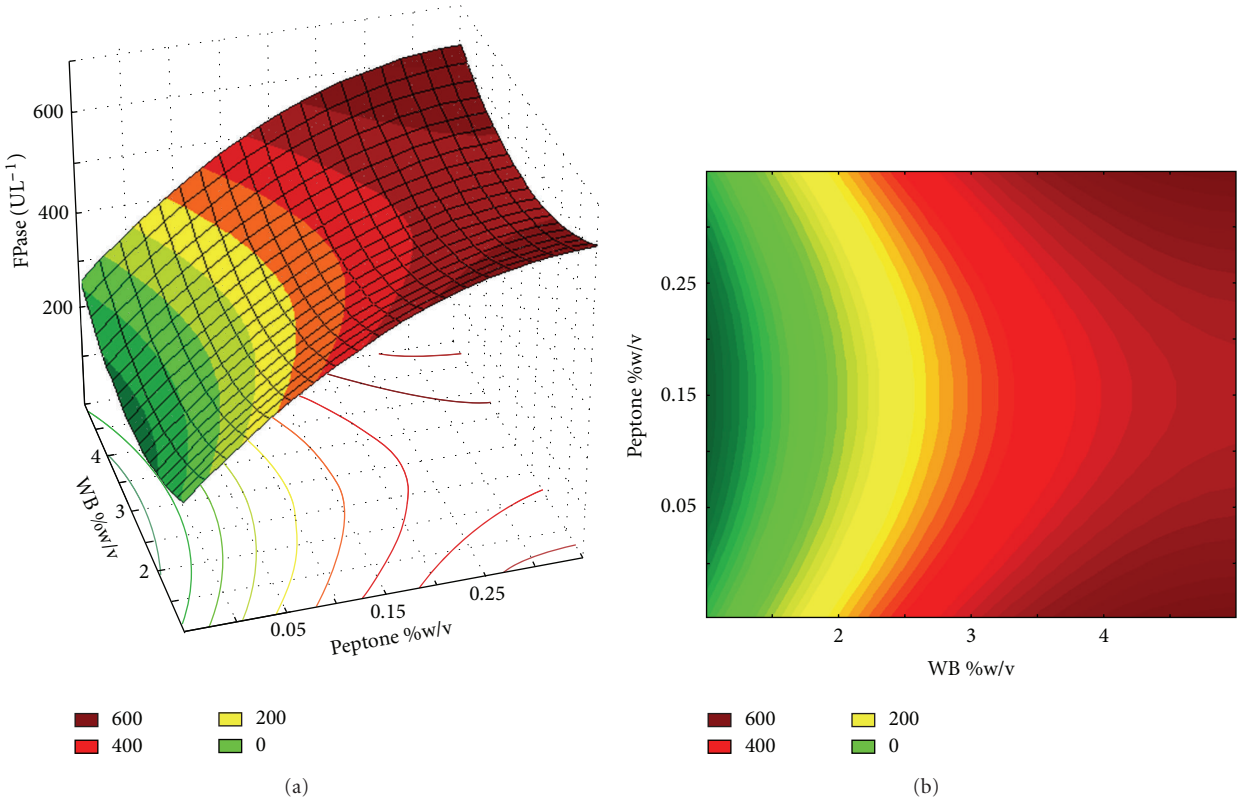

Figure 1: Response surface (a) and contour curve (b) on cellulase production by *Trichoderma* sp. IS-05 using WB and peptone concentrations as independent variables. The full factorial central composite design (2^2) used the response surface methodology to predict the best point for cellulase production.

between the experimentally observed and predicted values. The mathematical model representing the cellulase activity (Y) for the combination WB (X_1) + peptone (X_2) in the experimental region studied can be expressed by

$$Y = 285.14 + 140.41X_1 - 43.51X_1^2 + 40.97X_2^2. \quad (3)$$

Although the concentration of the independent variable WB had a significant effect on the cellulase production, interactions between WB and peptone did not ($P > 0.1$). The regression analysis for the experiment using the combination WB + peptone, (3) shows the significant coefficients of the full second-order polynomial model of cellulase production, determined by Student's t-test and P values. The resulting surface response plots and contour curve showing the effect of substrate concentration (WB and peptone) on the cellulase production by *Trichoderma* sp. IS-05 are presented in Figures 1(a) and 1(b).

The validation of the mathematical model used based on the CCRD experiments confirmed the maximal values for cellulase obtained, from 592.5 to 1224.0 U L^{-1} (4.8% and 4.4% (*w/v*) WB, resp.) supplemented with 0.25% (*w/v*) peptone, after 2 days fermentation.

According to the literature, it is well known that fungi, especially *Trichoderma* and *Aspergillus*, are able to degrade agroindustrial residues through lignocellulolytic enzymes, including cellulases [18–20]. Kovács et al. [19] studied the production of cellulase, among other enzymes by *Trichoderma reesei* RUT-C30 and other *Trichoderma* sp. mutant strains grown with pretreated willow (15 g L^{-1}) and cellulose powder Sigmacell type 20 (10 g L^{-1}). The highest cellulase activities observed were 620.0 U L^{-1} (pretreated willow) and 1090.0 U L^{-1} (cellulose powder Sigmacell type 20) after 3 days fermentation. Wen et al. [20] reported the effect of different dairy manure concentrations on cellulase production by *Trichoderma reesei* RUT-C30. The best result was 1200 U L^{-1} when 13 g L^{-1} of dairy manure was used. Jiang et al. [21] observed a quite similar cellulase activity (880 U L^{-1}) with a new isolate of *Trichoderma viride* strain using phosphoric acid swollen cellulose as carbon source. Our group has investigated various *Trichoderma* and *Aspergillus* strains using agroindustrial residues in order to produce lignocellulose degradation enzymes, including endoglucanases. The cellulase titers obtained, using WB (1224.0 U L^{-1}) as the carbon source and peptone as the nitrogen source, under submerged culture conditions, in this study were higher than the cellulase titers of 46.0 U L^{-1} obtained by Grigorevski-Lima et al. [6] also using WB with *A. fumigatus* FBSPE-05. Pothiraj et al. [22] observed maximum values for carboxymethylcellulase (CMCase) and filter paper activity (cellulase) of 120 U L^{-1} and 40 U L^{-1}, respectively, after 8 days fermentation for *A. niger*, using cassava waste as the carbon source. For *A. terreus*, for the same fermentation period, Pothiraj et al. [22] observed lower

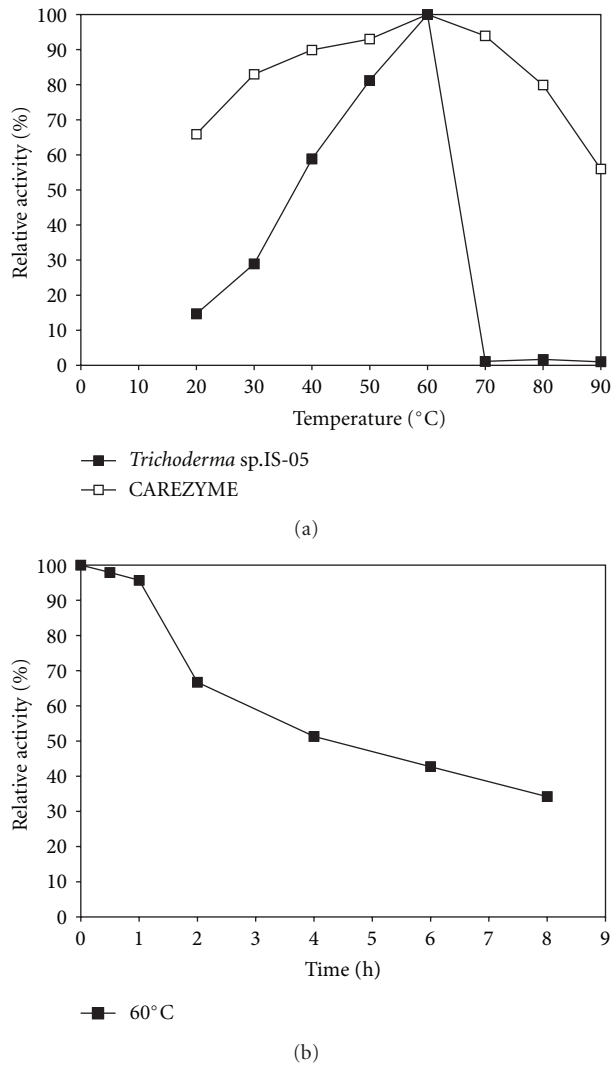

Figure 2: Effect of temperature (a) (■) and thermal stability at 60°C (b) on cellulase activity (pH 4.8) produced by *Trichoderma* sp. IS-05 grown on 4.4% (*w/v*) WB, 0.25% (*w/v*) peptone, and commercial enzyme CAREZYME (□). Residual activity is expressed as a percentage of the original activity. Error bars represent the standard deviation of each experimental point ($n = 2$) (100% residual activity = 1036.9 U L^{-1}).

values for CMCase (100 U L^{-1}) but the same values for cellulase (40 U L^{-1}). Considering two days fermentation, the *Trichoderma* sp. strain IS-05 (1224.0 U L^{-1}) produces 26.61 times more cellulase than *A. fumigatus* (46 U L^{-1}) [6] and 1.39 times more than *T. viride* (880 U L^{-1}) [21].

Cellulases present in the crude supernatant obtained from *Trichoderma* sp. strain IS-05 grown in 4.2% (*w/v*) WB and 0.25% (*w/v*) peptone in submerged fermentation showed maximal activity at 60°C (Figure 2(a)), and activity values of approximately 81% were still detected at 50°C. Other studies using *Aspergillus niger* [18] have shown a residual activity of around 100% for cellulase activity at temperatures between 50° and 60°C, very similar to our results. Crude enzyme from *Trichoderma* sp. strain IS-05 was able to retain 59.6% residual activity at 60°C for 4 h; the half-life of crude enzyme being 5 h at 60°C (Figure 2(b)). Half-lives of 8 h at 60°C or 1 h at 70°C have been cited in the literature for some *Aspergillus niger* [18]. Our results strongly suggest that the cellulases in this supernatant seem to be thermophilic, which are considered ideal for many biotechnological processes.

CAREZYME is a commercial enzyme preparation produced by submerged fermentation of a genetically modified *Aspergillus* microorganism. Optimum temperature for CAREZYME and *Trichoderma* sp. IS-05 enzyme preparations were the same (60°C). However, CAREZYME was able to retain over 70% of relative activity in the range between 30 and 80°C and retain 50% of the maximum activity even at 100°C, while the crude supernatant of *Trichoderma* sp. IS-05 was unable to retain any enzyme activity at temperatures above 70°C (Figure 2(a)).

The pH profiles (Figure 3) have shown more than 80% activity in the acidic pH range (2.0 to 4.0), with optimal activity occurring at pH 3.0. Values in the neutral range (6.5 to 7.5) of pH were very low, around 9% of residual activity at these pH values. However, in the alkali range, a new peak (21%) of cellulase activity at pH 10.0 was observed, suggesting the possibility of two cellulases. This biochemical characteristic could be very interesting for processes that require acidic conditions. There are few reports in the literature about cellulase activity in an acidic pH range. Nascimento et al. [23] have shown a pH activity profile within the range 2.0–5.0, with maximum activity observed at pH 4.0. CMCase activity in the acid pH range was also detected by Grigorevski-Lima et al. [6] for *Aspergillus fumigatus*.

The pH studies comparing two preparations have shown different pH profile patterns with major differences in optimal activity, which were pH 3.0 for *Trichoderma* sp. IS-05 supernatant (Figure 3(a)) and pH 6.0 for CAREZYME (Figure 3(b)). Differences in the results concerning some of the pH tested using different buffers were observed, especially in pH 3.0 for *Trichoderma* sp. IS-05 and pH 6.0 in CAREZYME. In fact, according to the buffers used to maintain each pH value, different activities were observed in same pH value. At pH 3.0, for *Trichoderma* sp. IS-05 the differences suggest a greater affinity of the crude enzymatic extract in sodium citrate buffer. The same can be said about results at pH 6.0 for the commercial enzyme. In fact, a similar result has been reported in the literature [5, 6, 23, 24].

Results of cellulase activity in the presence of metal ions are shown in Table 3. All ions tested had significant effect on cellulase activity. A considerable decrease (>80% inhibition) in activity was observed in the presence of Co^{+}, Cu^{2+}, and Mn^{2+}. These ions are commonly cited in the literature as inhibitors for several microbial cellulases [25–27]. Activity is probably inhibited through the attack of certain groups at the active site of the enzyme, for example, the thiol groups, leading to inactivation [25]. According to these results, these ions must be avoided in future cultivations for a high cellulase production.

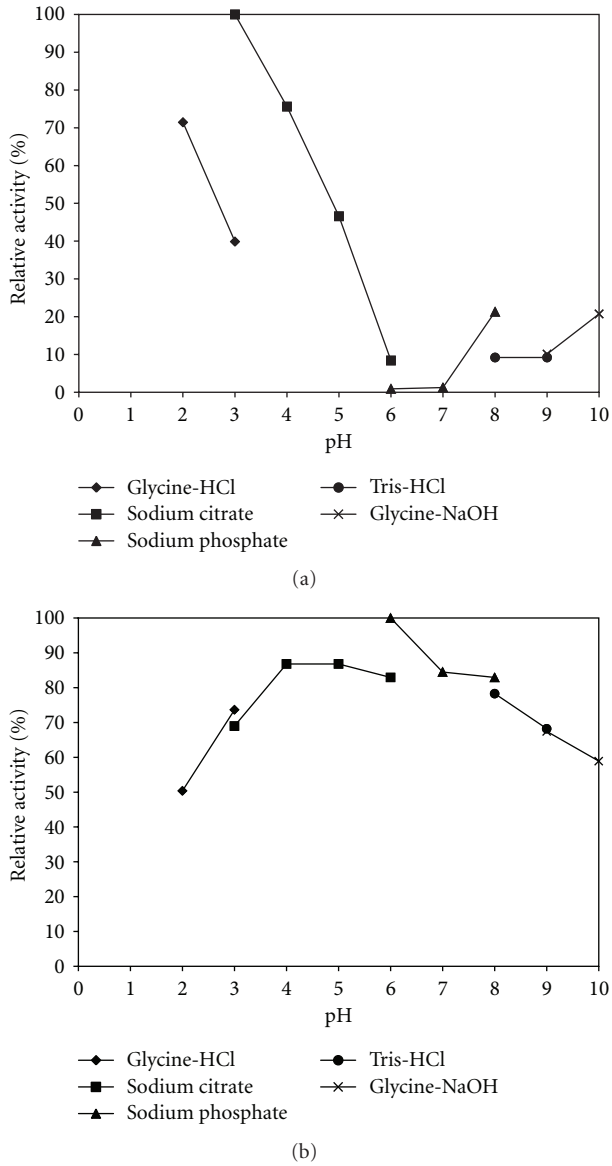

Figure 3: Effect of pH on cellulase activity at 60°C produced by *Trichoderma* sp. IS-05 (a) grown on 4.4% (*w/v*) WB, 0.25% (*w/v*) peptone, and commercial enzyme CAREZYME (b). The ionic strength for all buffers was 50 mM: glycine-HCl (♦); sodium citrate (■); sodium phosphate (▲); Tris-HCl (•); glycine-NaOH (-x-). Residual activity is expressed as a percentage of the original activity. Error bars represent the standard deviation of each experimental point ($n = 2$) (100% residual activity = 1079.7 U L^{-1}).

4. Conclusions

The fungi strain *Trichoderma* sp. IS-05 used in this study was able to grow and produce good levels of cellulase using wheat bran and peptone as the sole sources of C and N. The maximum cellulase activity detected was of 1224 U L^{-1}, on the second day of cultivation, when a mineral medium was supplemented with peptone 0.25% (*w/v*) and WB 4.4% (*w/v*). These results were obtained after using the validation of factorial experimental design for optimization.

Table 3: Effect of different ions on cellulase activity. Enzyme was produced by *Trichoderma* sp. IS-05 grown on 4.4% (*w/v*) WB and 0.25% (*w/v*) peptone.

Ion[a]	Relative activity (%)[b]
Control (no addition)	100.0
EDTA	42.4
Mg^{2+}	36.6
Zn^{2+}	48.4
Co^{+}	14.1
K^{+}	63.7
Cu^{2+}	11.7
Na^{+}	46.6
Ba^{2+}	33.9
Mn^{2+}	18.4
Ca^{2+}	44.0
Hg^{+}	31.4
Fe^{2+}	30.7

[a] The final concentration in the reaction mixture was 10 mM.
[b] Relative activity is expressed as a percentage of control (100% of enzyme activity = 1224.0 U L^{-1}).

The validation of experimental design resulted in a 2.17-fold improvement on cellulase production when compared with first results on cellulase matrix optimization in CCRD. The optimum pH and temperature of the crude extract were 3.0 and 60°C, respectively. Considering the design of the medium, and the high titers obtained for enzymatic activity, the results obtained indicate a possible use for these crude enzymatic extracts in biotechnology processes, especially for lignocellulosic biomass hydrolysis without contamination (pH 3.0/60°C) for generating reducing sugars for bioethanol production.

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Research Article

Multiresponse Optimization of Inoculum Conditions for the Production of Amylases and Proteases by *Aspergillus awamori* in Solid-State Fermentation of Babassu Cake

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This work aimed at investigating the simultaneous production of amylases and proteases by solid-state fermentation (SSF) of babassu cake using *Aspergillus awamori* IOC-3914. By means of experimental design techniques and the desirability function, optimum inoculum conditions (C/N ratio of propagation medium, inoculum age, and concentration of inoculum added to SSF medium) for the production of both groups of enzymes were found to be 25.8, 28.4 h, and 9.1 mg g^{-1}, respectively. Significant influence of both initial C/N ratio and inoculum concentration was observed. Optimum amylolytic activities predicted by this multiresponse analysis were validated by independent experiments, thus indicating the efficacy of this approach.

1. Introduction

Amylases comprise a group of hydrolases used in the breakdown of starchy homopolysaccharides, that is, amylose, a linear α-1,4-linked glucose-based polymer, and amylopectin, a glucose-based polymer with linear chains and α-1,6-linked branches [1]. In the production of ethanol, two major groups of amylases are important [2–4]: endoamylases (liquefying enzymes), which are composed mainly of α-amylases (EC 3.2.1.1) and release oligosaccharides of various lengths by the random attack of internal α-1,4 linkages and exoamylases (saccharifying enzymes), composed mostly by glucoamylases (EC 3.2.1.3), which release glucose as the main product by cleaving terminal α-1,4 bonds.

In previous works, alternative feedstocks, such as babassu (*Orbygnia phalerata*) [5] and castor bean (*Ricinus communis*) [6] were considered for the production of ethanol in Brazil due to their abundance. However, these materials, in addition to starch, contain considerable amounts of other fractions (proteins and other polysaccharides) [5, 6], which hinder the exposure of starch to amylases. An approach that has been proposed by other authors to improve ethanol yield consists of using proteases (exopeptidases, EC 3.4.11.x–3.4.18.x, and endopeptidases, EC 3.4.21.x–3.4.25.x) in combination with amylases [7, 8].

For the production of several bioproducts, especially enzymes, solid-state fermentation (SSF) processes are preferred over submerged fermentation (SmF) due to a number of advantages, including higher product concentration, lower catabolite repression [9], and lower operational costs [10, 11]. However, SSF is not the most widely used large-scale fermentation technology, and this can be attributed to engineering bottlenecks for its scaleup [11]. The challenges include difficulties in bioreactor design to avoid undesirable effects of overheating [12] and limitations related to inoculum propagation, particularly in the case of fungal processes based on inoculum using spores [13].

The impact of spore generation on the economic aspects of SSF processes was previously studied by de Castro et

al. [14], who compared five culture media for inoculum propagation and observed up to 7.5-fold cost differences to generate the amount of spores (10^{10}) required to inoculate a large-scale process. Moreover, Gutarra et al. [13] searched for new alternatives to spore inoculum, including the use of fungal pellets propagated by SmF, as well as the use of fermented solids obtained by small-scale SSF employing the same feedstock as in the main fermentation. Generally, the larger the scale, the more important the selection of a proper inoculum propagation strategy for an SSF process. Therefore, considering the production of amylases, which major application is in the hydrolysis of starch for the production of a biofuel, the optimization of inoculum propagation conditions is of paramount importance in the process economics.

Different authors have used statistical designs to optimize amylases production. However, the focus was largely on fermentation conditions [15–17]. From the several works that investigated culture media composition for the production of amylases [17–27], the influence of carbon and nitrogen sources became evident, and malt extract and peptone were pointed out as some of the nutrients which enhanced the best amylases production. Thus, the use of these compounds also in inoculum propagation steps could preadapt cells and so decrease lag time in the main fermentation and increase enzyme productivity.

Thus, in this work, the use of different inocula obtained by SmF as well as the optimization of inoculum propagation conditions were investigated. Inoculum age and concentration, and the C/N ratio of inoculum propagation media composed solely by malt extract and/or peptone, were evaluated in order to maximize the simultaneous production of amylases and proteases by *Aspergillus awamori*, using as raw material an agroindustrial byproduct largely available in Brazil (babassu cake). To our knowledge, this is the first report on the multiresponse analysis of the production of amylases and proteases using this feedstock.

2. Experimental

2.1. Raw Material. Babassu cake (mean particle size of 923 μm), which is an agroindustrial byproduct generated during the extraction of oil from babassu seeds, was kindly provided by TOBASA Bioindustrial de Babaçu S.A. (Tocantinópolis, Brazil). For the solid-state fermentation studies, the cake was dried, ground, and sieved to obtain particles in the range of 210 to 297 μm (65 and 48 mesh Tyler, respectively).

2.2. Microorganism Maintenance. A. awamori IOC-3914 was obtained from Instituto Oswaldo Cruz (IOC) culture collection. Before propagation in the liquid propagation media, cells were kept for 7 days at 30°C in starch agar medium, as described by Castro et al. [14].

2.3. Inoculum Propagation in Liquid Media. A. awamori was grown in different conditions in SmF at 30°C and 200 rpm in 250-mL shaken flasks containing 100 mL of culture medium. Propagation time (inoculum age), C/N ratio of the medium, and inoculum concentration were studied at the levels shown in Table 1 according to a central composite rotatable design (CCRD). The influence of the C/N ratio of the medium was evaluated by combining different proportions of peptone and malt extract but maintaining the sum of both concentrations fixed at 35 g L^{-1}. Thus, the culture medium with the lowest C/N ratio (3.0) contained only peptone as carbon source, whereas the medium with the highest one (C/N = 25.8) contained only malt extract as nutrient. The central points (CP) of the experimental matrix were carried out in quadruplicate.

The growth kinetics of the fungus in each of the culture media with the C/N ratios shown in Table 1 was studied for each inoculum condition. With this purpose, dry biomass was determined after filtration through 0.22 μm membranes and drying until constant mass. These biomass data were adjusted according to a logistic model for microbial growth (1) [28]

$$X = \frac{X_m}{1 + ((X_m/X_0) - 1) \times e^{-\mu t}}, \quad (1)$$

where X, X_0, and X_m represent biomass concentration at a propagation time t, biomass concentration upon inoculation and the predicted maximum biomass concentration, respectively. Equation (1) is also useful for predicting the specific growth rate (μ) and, as consequence, the doubling time (t_d) of the cells.

2.4. SSF Experiments. A. awamori cells propagated under different conditions were inoculated at different concentrations in lab-scale tray bioreactors containing 2.5 g of babassu cake, according to Table 1. The initial moisture content of all experiments was adjusted to 70%. The trays were incubated for up to 120 h at 30°C. Regularly, whole trays were taken as samples and submitted to enzyme extraction, as previously described [14].

For the final validation of results under the optimized conditions, in addition to the standard scale, an experiment in a 4-fold larger scale was performed, that is, with 10 g of babassu cake. All conditions were kept the same as in the standard fermentations, including bed characteristics (e.g., mass to area ratio equal to 2.46 $kg_{cake}\ m^{-2}{}_{tray}$).

2.5. Experimental Analyses. C/N ratio of malt extract and peptone was calculated based on analyses (in duplicate) on an Elemental Analyzer 2400 CNH (Perkin Elmer).

Microbial biomass samples collected after growth in different liquid media were coated with gold and observed using a scanning electron microscope (model INCAPentaFETx3, Oxford Instruments, Oxford, UK). The accelerating voltage was set to 20 kV for all images.

Regarding enzyme assays, endoamylase, exoamylase, and proteases activities were determined using 0.5% (m/v) soluble starch, 1.0% (m/v) soluble starch, and 0.5% (m/v) azocasein (Sigma Aldrich, St Louis, USA) as substrates,

Table 1: CCRD matrix for optimization of inoculum conditions of *A. awamori* IOC-3914 for amylases and proteases production by SSF.

Run	Factor levels		
	Inoculum age (h)	Initial C/N ratio	Inoculum concentration ($mg_{biomass}\ g^{-1}{}_{cake}$)
1	16.0	7.6	3.5
2	16.0	7.6	8.2
3	16.0	21.2	3.5
4	16.0	21.2	8.2
5	30.0	7.6	3.5
6	30.0	7.6	8.2
7	30.0	21.2	3.5
8	30.0	21.2	8.2
9	11.2	14.4	5.8
10	34.8	14.4	5.8
11	23.0	3.0	5.8
12	23.0	25.8	5.8
13	23.0	14.4	1.9
14	23.0	14.4	9.7
15–18 (PC)	23.0	14.4	5.8

respectively, as described by de Castro et al. [29]. In all cases, results are expressed as mean 1± standard deviation (SD).

2.6. Statistical Analyses. Amylolytic and proteolytic activities detected in the SSF experiments were analyzed using the software Statistica 8.0 (Statsoft Inc, Tulsa, OK, USA). After analysis of variance (ANOVA) and normality test of the results, nonlinear regression was performed to obtain models to predict the responses, as shown in (2). In this equation, Y represents the dependent variables or responses ($k = 3$: endoamylase, exoamylase, and proteases activities); ξ_0, ξ_i, ξ_{ii}, and ξ_{ij} represent the regression coefficients for the central, linear, quadratic, and interaction terms of the model, respectively, and X_i and X_j represent the independent variables or factors (1: C/N ratio, 2: inoculum age, and 3: inoculum concentration) in terms of their original (nonnormalized) levels

$$Y_k = \xi_0 + \sum_{i=1}^{n} \xi_i X_i + \sum_{i=1}^{n} \xi_{ii} X_i^2 + \sum_{i,j=1}^{n} \xi_{ij} X_i X_j. \quad (2)$$

After determining the models for the three responses, their combined behavior as a function of the different inoculum conditions was analyzed using the global desirability function [30] as the objective function. For both the simultaneous analysis of exoamylase and endoamylase production ($k = 2$), and the simultaneous analysis of the production of the three groups of enzymes ($k = 3$), (3) was adopted. In this equation, D represents the global desirability value and d_k stands for the individual desirability values of the responses, calculated as the ratio of the response under given conditions to the maximum value obtained for that response considering the whole experimental space investigated

$$D = \left(\prod_{i=1}^{k} d_k \right)^{1/k}. \quad (3)$$

3. Results and Discussion

3.1. Inoculum Propagation of A. awamori in Liquid Media. After initial spore propagation in starch agar medium, *A. awamori* was grown in a liquid propagation medium containing malt extract and peptone at a C/N ratio of 22 in order to investigate growth kinetics and to establish the levels of inoculum age to be evaluated in the CCRD experiments. According to the growth curve obtained (data not shown), at 11.2 h the cells were in the acceleration phase, at 16, 23, and 30 h they were in the beginning, middle, and end of the exponential phase, respectively, whereas at 34.8 h, cells were in stationary phase.

A. awamori was then cultivated in culture media with different C/N ratios (Table 1), and the growth curves for each condition were determined. It was observed that within the ranges evaluated, the higher the C/N ratio, the faster the kinetics, evidenced by the specific growth rate (μ) and the doubling time (t_d), as shown in Table 2. The specific growth rates observed (0.02–0.15 h^{-1}) were lower than those reported by Hellendoorn et al. [31] (0.28–0.40 h^{-1}) for cultivation of *A. awamori* in an airlift reactor, and this could be possibly due to oxygen transfer limitations in the shaken-flask cultures.

For the CCRD experiments, the dry weights of *A. awamori* cells after growth in the different media for different propagation times were at first determined (Table 3), in order to calculate the necessary amount of cell suspension to inoculate in babassu cake so as to meet the levels of inoculum concentration previously established (Table 1). Aiming at to simplify and integrate steps and to decrease costs when the process is scaledup, the liquid media from the submerged inoculum propagation stage (containing residual nutrients) were completely and solely used for moisture adjustment in the SSF. In the experiments where higher C/N ratios and inoculum ages were adopted, the culture supernatants were more viscous, possibly due the production of exopolysaccharides. According to Barbosa et al. [32], such condition favors the production of these molecules.

In the liquid propagation media, cells grew as mycelial biomass. Hyphae diameters were in the range of 1.8–3.4 μm, but in some cases, larger hyphae measuring 10.5–11.5 μm were observed. The hyphal organization pattern and absence of asexual reproductive structures detected in the present work are similar to those observed by Gutarra et al. [33] when *Penicillium simplicissimum* was propagated in a semisynthetic liquid medium.

3.2. Experimental Results of Amylases and Proteases Production by SSF. The cells propagated in the liquid media with different C/N ratios for different times were then transferred to a solid medium composed of babassu cake, adjusting the

Table 2: Specific growth rates and doubling times, calculated according to the logistic model, presented by *A. awamori* when grown in culture media with different C/N ratios.

Initial C/N ratio	Specific growth rate (h^{-1})	Doubling time (h)	R^2
3.0	0.015	46.3	0.919
7.6	0.031	22.4	0.774
14.4	0.063	11.0	0.851
21.2	0.112	6.2	0.846
25.8	0.152	4.6	0.798

Table 3: Biomass concentration after propagation of *A. awamori* in liquid culture media with different C/N ratios or different times.

Culture conditions		Biomass concentration ($g\,L^{-1}$)
Initial C/N ratio	Inoculum age (h)	
3.0	23	1.16 ± 0.03
7.6	16	0.31 ± 0.02
7.6	30	4.50 ± 1.27
14.4	11	0.83 ± 0.08
14.4	23	7.58 ± 0.18
14.4	34	7.69 ± 1.32
21.2	16	1.44 ± 0.03
21.2	30	7.59 ± 1.13
25.8	23	7.80 ± 1.70

initial moisture content to 70%. According to previous SSF experiments, fermentation time was studied in the range of 72–120 h. The activities of endoamylases, exoamylases, and proteases obtained for each experimental condition of the CCRD are presented in Figure 1.

It can be observed that in general terms, in the media with high C/N ratios both the production of amylases and proteases were favored. In run 12, where initial C/N ratio, inoculum age, and concentration were highest, the maximum exoamylolytic and endoamylolytic activities and the second highest proteolytic activity were observed although in different times of SSF. Under these conditions, the maximum productivities for these three groups of enzymes, found after 72 h, 96 h, and 120 h of fermentation, were $(1.49 \pm 0.54)\,U\,g^{-1}\,h^{-1}$, $(0.91 \pm 0.02)\,U\,g^{-1}\,h^{-1}$ and $(0.227 \pm 0.004)\,U\,g^{-1}\,h^{-1}$, respectively. The observation that in run 12 endoamylases were produced prior to exoamylases is in accordance with the role of each of these enzyme groups in the hydrolysis of starch, that is, with endoenzymes contributing to a rapid depolymerization of the polysaccharides and higher oligosaccharides [4] and exoenzymes to the final release of glucose [3].

It should also be noticed that in the samples from runs 12, 13, and central point replicates, where the highest proteolytic activities were observed, amylolytic activities were not decreased during the onset of proteases activity. This indicates that possibly the proteases were preferentially attacking the proteins from the feedstock (which is desired) or that the amylases produced are glycosylated and thus more stable in the presence of proteases [34, 35].

3.3. Statistical Analysis of Amylases and Proteases Production in SSF of Babassu Cake. The experimental results for amylases and proteases shown in Figure 1 were analyzed in terms of their statistical significance, mainly by means of ANOVA and normality of residues, based on the tests of Kolmogorov-Smirnov and Lilliefors [36] and Shapiro and Wilk [37]. All nine data groups (three enzyme activities measured at 72, 96, and 120 h of SSF) showed adequate ANOVA and residues data, thus validating the statistical analyses.

Regression models were generated, considering the statistically significant terms, as well as those that were not significant, but that, when removed, would worsen model adjustment. Equations (4)–(6) show the adjusted models for the production of exoamylases (Y_1), endoamylases (Y_2) and proteases (Y_3) by *A. awamori* IOC-3914 in babassu cake

72 h:

$$\begin{aligned} Y_1 &= 31.7851 - 3.4265X_1 + 0.0835X_1^2 + 1.5676X_2 \\ &\quad + 0.8936X_3, \\ Y_2 &= 24.5007 + 5.7361X_1 - 0.1354X_1^2 - 6.3310X_2 \\ &\quad + 0.2490X_2^2, \\ Y_3 &= 0.3876 - 0.0337X_1 - 0.0289X_2 + 0.2557X_3 \\ &\quad + 0.0044X_1X_2 - 0.0043X_1X_2 - 0.0115X_2X_3, \end{aligned} \tag{4}$$

96 h:

$$\begin{aligned} Y_1 &= -62.3051 + 6.7032X_1 - 0.1481X_1^2 + 0.3990X_2 \\ &\quad + 0.0651X_1X_2, \\ Y_2 &= 133.5655 - 0.7615X_1 - 2.4532X_2 - 30.1873X_3 \\ &\quad + 2.4476X_3^2 + 0.5541X_2X_3, \\ Y_3 &= -18.1781 + 1.1669X_1 - 0.0210X_1^2 + 0.7683X_2 \\ &\quad - 0.0109X_2^2 + 0.9718X_3 - 0.0925X_3^2 - 0.0177X_1X_2 \\ &\quad + 0.0190X_1X_3 - 0.0241X_2X_3, \end{aligned} \tag{5}$$

120 h:

$$\begin{aligned} Y_1 &= -107.615 + 9.7760X_1 - 0.1870X_1^2 + 5.1800X_2 \\ &\quad - 0.059X_2^2 - 0.7500X_3 - 0.0940X_1X_2, \\ Y_2 &= 58.4672 - 1.6609X_1 + 4.5235X_2 - 4.1081X_3 \\ &\quad + 1.2008X_3^2 - 0.6322X_2X_3, \\ Y_3 &= -145.0540 + 10.2570X_1 - 0.2230X_1^2 + 4.298X_2 \\ &\quad - 0.136X_2^2 + 8.508X_3 - 0.836X_3^2. \end{aligned} \tag{6}$$

For each fermentation time (72 h, 96 h, and 120 h), the models describing the production of the three groups of

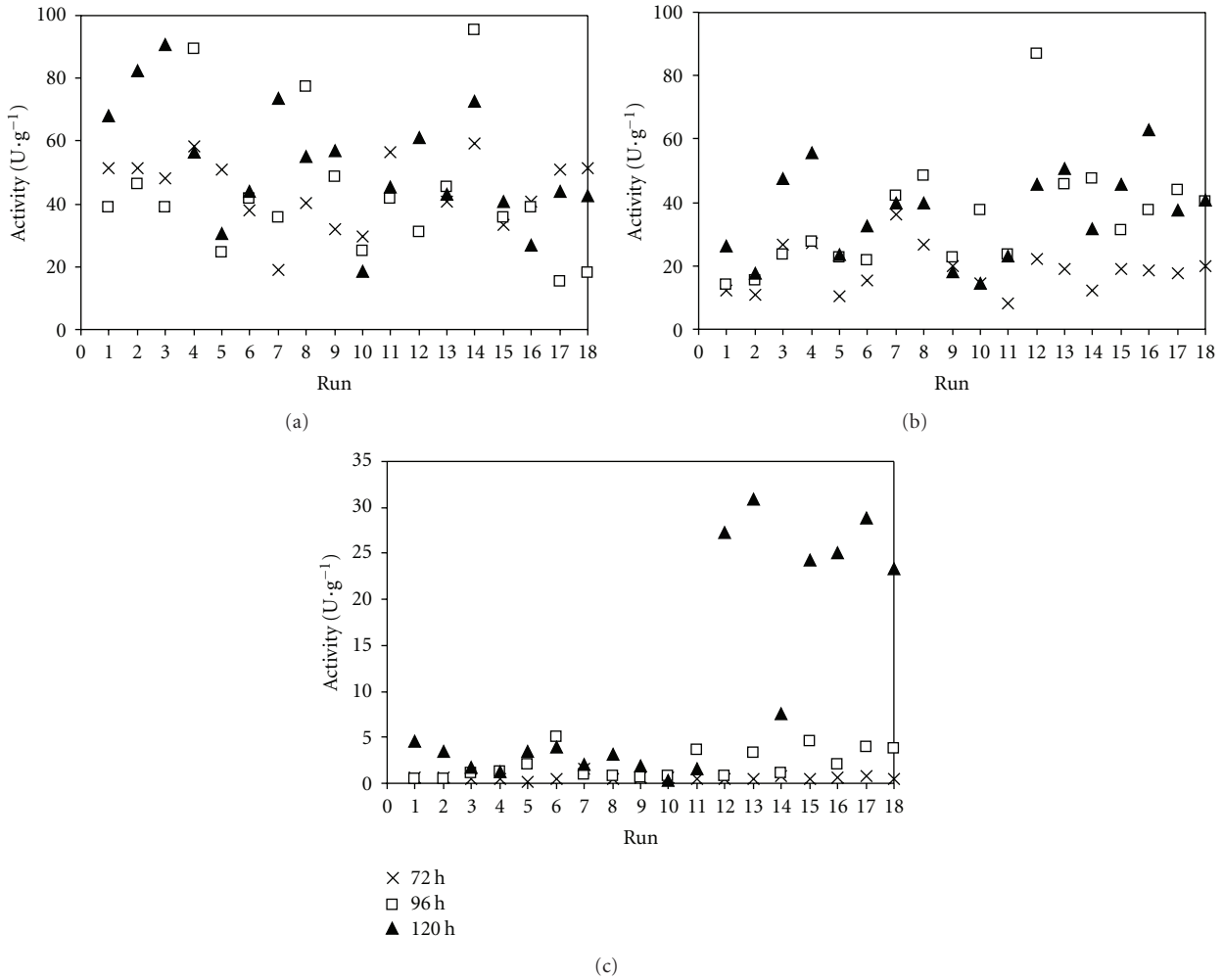

Figure 1: CCRD results for (a) endoamylases, (b) exoamylases, and (c) proteases production by *A. awamori* in SSF of babassu cake. Runs 15–18 correspond to central points.

Table 4: Optimum inoculation conditions and enzyme activities predicted by means of the global desirability function, applied to two scenarios: considering only the amylolytic activities and considering also the proteases activity.

Predicted parameters	SSF time		
	72 h	96 h	120 h
Simultaneous analysis of endoamylase, and exoamylase production			
D value	0.439	0.902	0.763
Optimum inoculum age (h)	21.1	28.4	20.2
Optimum C/N ratio	25.8	25.8	25.8
Optimum inoculum concentration (mg g^{-1})	7.8	9.1	1.9
Exoamylolytic activity in optimum condition (U g^{-1})	27.1	73.6	52.5
Endoamylolytic activity in optimum condition (U g^{-1})	67.8	115.6	99.2
Simultaneous analysis of endoamylase, exoamylase, and proteases production			
D value	0.549	0.524	0.613
Optimum inoculum age (h)	33.7	25.8	21.8
Optimum C/N ratio	25.8	14.6	21.0
Optimum inoculum concentration (mg g^{-1})	3.5	9.7	2.8
Exoamylolytic activity in optimum condition (U g^{-1})	42.3	40.3	53.0
Endoamylolytic activity in optimum condition (U g^{-1})	45.4	92.0	66.1
Proteolytic activity in optimum condition (U g^{-1})	1.7	2.3	20.3

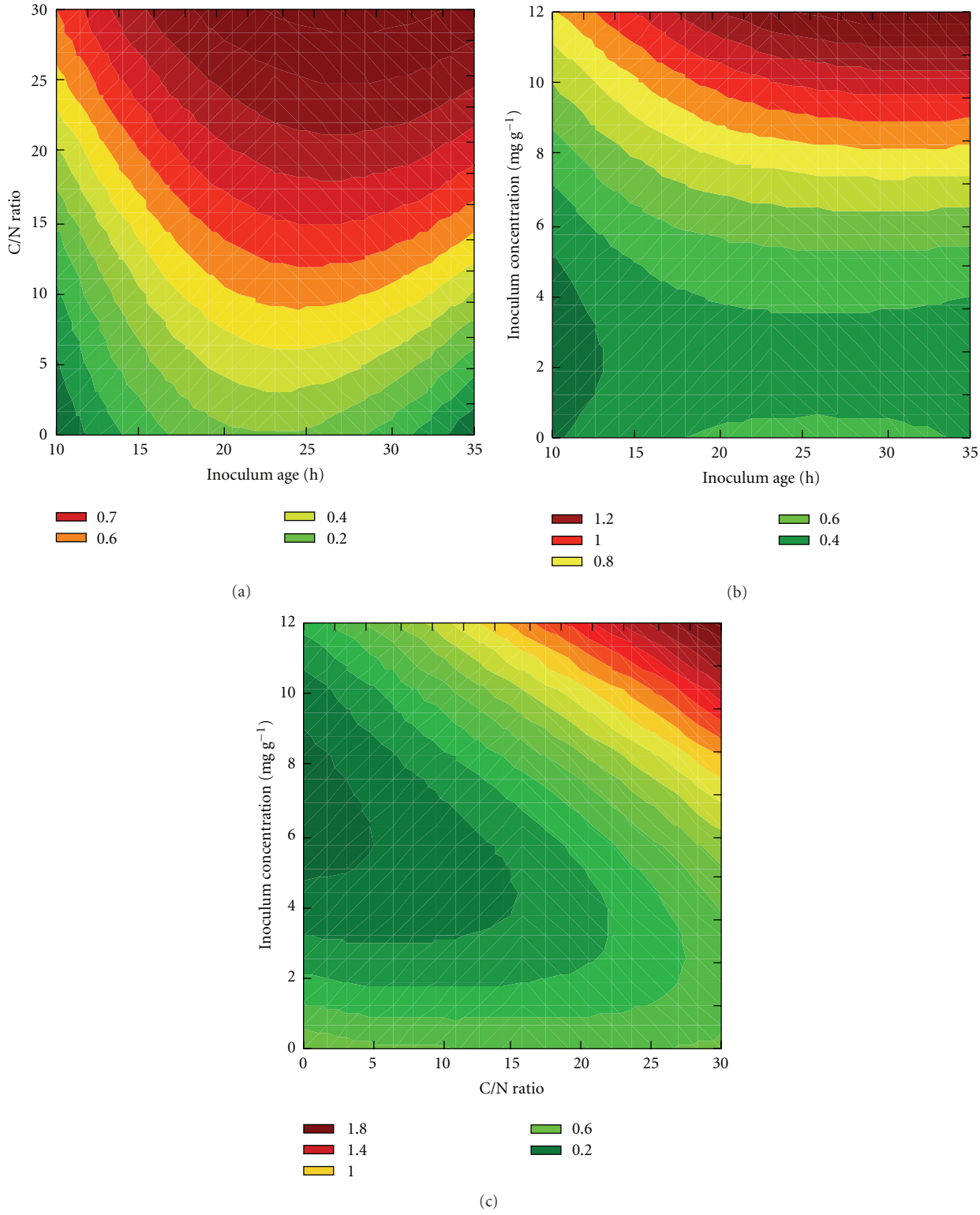

Figure 2: Response surfaces for the global desirability function (D) for simultaneous analysis of exoamylases and endoamylases after 96 h of fermentation of babassu cake by *A. awamori*.

enzymes were analyzed jointly through the use of the global desirability function, D [38], which represents a geometric mean of the desirabilities of each response (d_i). Since the experimental results (Figure 1) showed that the maximum activities of the different enzyme groups were achieved under distinct conditions, the multiresponse optimization was done in view of two scenarios: (1) considering only the production of exoamylases and endoamylases and (2) considering the production of all the three groups of enzymes. Results of these analyses are presented in Table 4.

Higher D values were obtained in the first scenario, when only the amylolytic activities were taken into consideration. This is in agreement with the fact that the production of these enzymes was favored under more similar conditions than when proteases were also considered. The maximum D value possible is 1, and it would represent a perfect combination of the experimental conditions for the production of all enzymes.

Besides giving the highest D values, the simultaneous analysis of the production of exoamylases and endoamylases after 96 h of SSF predicted also the highest enzyme activities, thus indicating that optimizing the inoculum propagation conditions could effectively enhance the production of amylolytic enzymes. Thus, further analyses were concentrated on this fermentation time. The response surfaces of the desirability function (considering only the activities of amylases) for each pair of factors are presented in Figure 2. The surfaces indicate that the three factors exert a significant influence on the combination of the responses (represented by D) and that at least inoculum concentration and C/N ratio should be maximized to enhance the production of the amylolytic enzymes. This is in agreement with the results reported by Djekrif-Dakhmouche et al. [39], who studied the production of amylases by *A. niger*, concluding that the C/N ratio of the medium should be at least 20.

3.4. Experimental Validation of the Predicted Results. Experimental runs were carried out in order to validate if the best inoculum conditions (inoculum age of 28.4 h, C/N ratio of 25.8, and inoculum concentration of 9.1 mg g^{-1}), as predicted by the desirability function analysis considering both amylolytic activities after 96 h of SSF, were really optimal. These runs were carried out in two scales: at the same scale as the CCRD runs and at a 4-fold larger scale. The results of replicates carried out at both scales are shown in Table 5. Considering a 95% confidence interval (1.96*SD) for both the predicted and the experimental results, the enzyme activities obtained experimentally did validate the optimum conditions determined by the desirability analysis.

The desirability function has been used for the multiresponse optimization of the production of enzymes, such as proteases and catalases [40] and cellulases [41] as well as for the application of enzymes, as reported by Castro et al. [29] regarding the use of multienzyme complexes containing amylases and proteases for the hydrolysis of babassu case.

4. Conclusions

Inoculum conditions for the simultaneous production of amylolytic and proteolytic enzymes were optimized using a multiresponse approach based on the desirability function. Kinetic profiles for the growth of *Aspergillus awamori* IOC-3914 in liquid medium containing malt extract and peptone were studied and used to select the variables that were subsequently investigated using design of experiments. Models were obtained to describe the production of each of the three enzyme groups under study (exoamylases, endoamylases and proteases) at different SSF process times (72, 96, and 120 h).

Table 5: Enzyme activities obtained in fermentations carried out for 96 h to validate the inoculum conditions predicted by desirability analysis to be optimal (inoculum age of 28.4 h, C/N ratio of 25.8, and inoculum concentration of 9.1 mg g^{-1}).

Replicate	Activity (U g^{-1})		
	Exoamylase	Endoamylase	Protease
Smaller scale-1	45.0	135.4	7.6
Smaller scale-2	44.2	113.9	21.1
Smaller scale-3	53.4	90.3	19.8
Smaller scale-4	63.9	100.3	23.3
Smaller scale-5	59.5	71.7	13.1
Larger scale	60.7	119.3	17.3
Overall mean	55.4	104.3	17.0
Overall SD	7.6	24.1	5.5
Values predicted by means of the desirability function	73.6	115.6	—

A statistical analysis using the global desirability function indicated the inoculum conditions that would optimize enzyme production (inoculum age of 28.4 h, C/N ratio of 25.8, and inoculum concentration of 9.1 mg g^{-1}), and these optimum conditions were validated experimentally, yielding exoamylases, endoamylases, and proteases activities of 55.4, 104.3, and 17.0 U g^{-1}, respectively. The use of the desirability function showed to be a useful tool for the optimization of enzyme production.

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Review Article

Production and Use of Lipases in Bioenergy: A Review from the Feedstocks to Biodiesel Production

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Lipases represent one of the most reported groups of enzymes for the production of biofuels. They are used for the processing of glycerides and fatty acids for biodiesel (fatty acid alkyl esters) production. This paper presents the main topics of the enzyme-based production of biodiesel, from the feedstocks to the production of enzymes and their application in esterification and transesterification reactions. Growing technologies, such as the use of whole cells as catalysts, are addressed, and as concluding remarks, the advantages, concerns, and future prospects of enzymatic biodiesel are presented.

1. Lipid Feedstocks

The main feedstocks which present paramount importance for the application of lipases are fats and oils. Such materials are primarily composed of triglycerides, which are glycerol esters with saturated and unsaturated fatty acids, from vegetable, animal, or microbial origins. One of the distinguishable characteristics between fats and oils is the occurrence of unsaturated and saturated fatty acids in the triglycerides: higher saturated fatty acids content (as examples in Figure 1), higher melting point, and the presence of remaining solids at room temperature are characteristics of a fat; on the other hand, oils usually present higher occurrence of unsaturated fatty acids, remaining in liquid state at room temperature. In addition to triglycerides, vegetable oils can present di- and monoglycerides, free fatty acids (FFAs), phosphatides, and unsaponifiable matter, such as carotenoids, phytosterols, tocochromanols, chlorophyll, triterpenic alcohols, and hydrocarbons [1–4].

The role of fats and oils in plants is related to energy reserve, regarding their occurrence in seeds, and protection against water loss (by wax formation) and against mechanical injuries (by hormone generation), when such components appear in the leaves and fruits [2, 5].

Worldwide production of fats and oils was estimated in 174.6 million tons for the season 2010/2011. From that, 86% represent vegetable oils (Table 1), with soybean, palm, rapeseed, and sunflower seed as the major resources [6, http://lipidlibrary.aocs.org/, 2011]. In Brazil, some oilcrops, such as castor bean (*Ricinus communis*), jatropha (*Jatropha curcas*), crambe (*Crambe abyssinica*), macaw palm (*Acrocomia aculeata*), and oiticica (*Licania rigida*), have been explored as alternatives for biodiesel production due to their high tolerance to drought and frost, higher productivity on low-fertility soils, and great potential for the sustainable development of Brazilian Northeast [7, http://www.ruralbioenergia.com.br/, 2009]. Moreover, the use of raw materials with appropriated physicochemical characteristics and widely available enables cost reduction for the production of the biofuel, since the feedstock cost represents 70–88% of the final price of biodiesel [8].

For the selection of a proper raw material for use as substrate for the production of biodiesel, some aspects should be observed, such as the following.

Table 1: World oilcrops distribution [6].

Fats and oils	World production (million tons)	Five major producers
Animal fat	24.4	USA, China, Brazil, Germany, and France
Coconut oil	3.7	Philippines, Indonesia, India, Vietnam, and Mexico
Cottonseed oil	4.8	China, India, Pakistan, Uzbekistan, and USA
Groundnut oil	5.3	China, India, Nigeria, Myanmar, and Sudan
Linseed oil	0.6	China, Belgium, USA, Ethiopia, and India
Maize oil	2.3	USA, China, Japan, Brazil, and South Africa
Olive oil	2.9	Spain, Italy, Greece, Syrian Arab Republic, and Tunisia
Palm kernel oil	5.6	Indonesia, Malaysia, Nigeria, Thailand, and Colombia
Palm oil	23.9	Malaysia, Nigeria, Thailand, Colombia, and Côte d'Ivoire
Rapeseed oil	21.2	China, Germany, India, Canada, and France
Safflower oil	0.1	India, USA, and Argentina
Sesame oil	0.9	Myanmar, China, India, Sudan, and Japan
Soybean oil	36.0	USA, China, Brazil, Argentine and India
Sunflower oil	13.0	Russian Federation, Ukraine, Argentine, Turkey, and France

Table 2: Nomenclature of fatty acids [1, 9].

Common name	Systematic name	Chemical structure[1]	Melting point (°C)
Lauric acid	Dodecanoic acid	12 : 0	44.2
Miristic acid	Tetradecanoic acid	14 : 0	54.4
Palmitic acid	Hexadecanoic acid	16 : 0	62.9
Palmitoleic acid	9-Hexadecenoic acid	16 : 1	−0.1
Stearic acid	Octadecanoic acid	18 : 0	70.1
Oleic acid	9-Octadecenoic acid	18 : 1	16.3
Elaidic acid	9-Octadecenoic acid	18 : 1	43.7
Vaccenic acid	11-Octadecenoic acid	18 : 1	44.0
Linoleic acid	9, 12-Octadecadienoic acid	18 : 2	−6.5
γ-Linolenic acid	6, 9, 12-Octadecatrienoic acid	18 : 3	−11.0
α-Linolenic acid	9, 12, 15-Octadecatrienoic acid	18 : 3	−12.8
Arachidic acid	Eicosanoic acid	20 : 0	76.1
Gadoleic acid	9-Eicosenoic acid	20 : 1	25.0
Arachidonic acid	5, 8, 11, 14-Eicosatetraenoic acid	20 : 4	−49.5
Behenic acid	Docosanoic acid	22 : 0	80.0
Erucic acid	13-Docosenoic acid	22 : 1	33.4

[1] $x : y$ nomenclature, where x represents the total number of carbon atoms and y represents the number of unsaturated bonds.

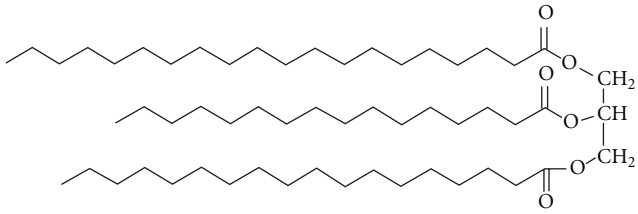

Figure 1: Schematic representation of a triglyceride with saturated fatty acids.

1.1. Fatty Acids Profile. The fatty acids profile varies greatly between fats and oils and can be referred by distinct nomenclatures (Table 2). It can comprise from high concentration of saturated fatty acids, like in palm seeds, such as coconut (*Cocos nucifera*), palm kernel (*Elaeis guineensis*), and babassu (*Orbignya oleifera*) (Table 3), as well as animal fats (Table 4), to high content of monounsaturated fatty acids, commonly in oleaginous fruits (Table 5). Certainly, there are some exceptions of typical profiles, such as castor bean oil, which has a high content of ricinoleic acid; crambe, with high quantity of erucic acid; palm, with similar quantities of saturated and unsaturated fatty acids (Table 3) [9].

Additionally to the vegetal and animal sources of lipids and fats presented in Tables 3–5, fatty acids can also come from microbial origin. As recently revised by Li et al. [21], yeasts from *Cryptococcus*, *Lipomyces*, *Rhodosporidium*, *Rhodotorula*, *Trichosporon,* and *Yarrowia* genera, as well as filamentous fungi and bacteria, can reach 53% of lipids content in its dry mass, with evidence for major appearance of palmitic and oleic acids. In another work, the profiles of fatty acids of the microalgaes *Spirulina* sp., *Scenedesmus*

Table 3: Fatty acids profile of oilcrops [1, 9].

Fatty acids[1]	Palm kernel	Soybean	*Jatropha curcas*	Crambe	Rapeseed	Sunflower	Castor bean	Babassu
12 : 0	41–55	NR	NR	NR	NR	NR	NR	40–55
14 : 0	14–18	NR	NR	NR	<0.2	<0.5	NR	11–27
16 : 0	6.5–10.0	7–14	10–17	1.8–2.0	2.5–6.5	3.0–10.0	1.1	5.2–11.0
16 : 1	NR	<0.5	NR	NR	<0.6	<0.1	0.2	NR
18 : 0	1.3–3.0	1.4–5.5	5–10	0.7–1.0	0.8–3	1–10	1	1.8–7.4
18 : 1	12–19	19–30	36–64	16.0–17.2	53–70	14–35	3.3[2]	9–20
18 : 2	1–3.5	44–62	18–45	8.0–8.7	15–30	55–75	3.6	1.4–6.6
18 : 3	NR	4–11	NR	5.2–7	5–13	<0.3	0.32	NR
20 : 0	NR	<1.0	NR	3.4	0.1–1.2	<1.5	0.4	NR
20 : 1	NR	<1.0	NR	NR	0.1–4.3	<0.5	NR	NR
22 : 0	NR	<0.5	NR	NR	<0.6	<1.0	NR	NR
22 : 1	NR	NR	NR	56–66	0.7	NR	NR	NR
% *oil*	45–50	18–20	26–35	35–60	40–50	22–36	35–55	65–68

[1] $x : y$ nomenclature, where x represents the total number of carbon atoms and y represents the number of unsaturated bonds; [2]80–90% ricinoleic acid (similar to oleic acid plus a hydroxyl group in position 12R). NR: not reported.

Table 4: Fatty acid profile from animal origin [9].

Fatty acids	Butter	Lard	Tallow
<14 : 0	11.0–23.8	0.5	0.9
14 : 0	8.2–12.0	1.3	3.0–3.7
16 : 0	21.3–29.0	23.8–25.0	24.9–27.0
18 : 0	9.8–13.0	12.0–13.5	7.0–18.9
16 : 1	1.8–2.0	2.7–3.0	4.2–11.0
18 : 1	20.4–28.0	41.2–45.0	36.0–48.0
18 : 2	1.8	10.0–10.2	3.1
18 : 3	1.2	1.0	0.6
% *fat*	2–5	70–95	70–95

Table 5: Fatty acids profile of oleaginous fruits.

Fatty acids[1]	Buriti	Olive	Avocado	Palm
12 : 0	NR	NR	NR	0.1–1.0
14 : 0	0.1	0.7	<0.13	0.9–1.5
16 : 0	17.3–19.3	10–11.7	19.8–22.7	41.8–46.8
18 : 0	1.9–2.0	2.1	0.5–1.0	4.2–5.1
20 : 0	NR	0.48	NR	0.2–0.7
16 : 1	NR	1.45	3.9–5.6	0.1–0.3
18 : 1	73.3–78.7	73.8–78	60–71	37.3–40.8
18 : 2	2.4–3.9	7.0–9.8	7.1–15.3	9.1–11.0
18 : 3	2.2	NR	0.4–1.0	<0.6
% *oil*	8–18	15–40	4–25	20–24
References	[10]	[1]	[11]	[12]

[1] $x : y$ nomenclature, where x represents the total number of carbon atoms and y represents the number of unsaturated bonds; NR: Not reported.

obliquus, *Chlorella vulgaris,* and *C. kessleri* were determined [22]. The authors observed the prevalence of saturated fatty acids (lauric, miristic, palmitic, and stearic acids), with contributions of up to 46% for the total fatty acids content.

These diversified profiles of fatty acids from different origins contribute for the generation of biofuels with different properties. For example, the higher the size of fatty acid hydrocarbon chain, the higher the cloud point and the cold filter plugging point. Therefore, due to the necessity of heating before ignition, it becomes difficult the use of a biodiesel with such characteristic in regions with low environment temperature.

Another factor concerning the use of unsaturated fatty acids for the production of biodiesel is that the fewer the double bonds in the molecules, the higher the cetane number of the biofuels (which, in turn, means a better quality of their combustion). Moreover, larger quantities of unsaturated bonds turn molecules more chemically unstable. This can cause some inconvenience due to the biofuel oxidation, degradation, and polymerization (resulting in low cetane number or formation of solid residues), if improperly stocked or transported. Then, in general, a biodiesel with high quantities of esters derived from monounsaturated fatty acids (e.g., oleic or ricinoleic acids) presents better results as a fuel [23].

1.2. Fats and Oils Processing. Animal fat processing is named rendering, where carcasses with fatty material are heated with hot water or steam to release fats, with subsequent separation by centrifugation or by surface removal. The vegetable oil processing is comprised of some steps, including mechanical pretreatment (cleaning, sorting, and comminution), heating, dehydration, mechanical pressing and/or solvent extraction, miscella distillation, meal desolventization, and refining [1, 9].

For biodiesel production, the oil refining processes play an important role in the yield of the conversion steps, since oil impurities, such as water, phosphatides, and pigments, can affect the conversion of triglycerides to esters due to excessive emulsification of the reaction mixture and difficulties in biodiesel separation, amongst others [1, 9].

Table 6: Insight into recent literature on microbial lipase production.

Microorganism	Raw material	Type of fermentation	Maximum activity (time of fermentation)	Reference
A. niger 11T53A14	Wheat bran	SSF	62.7 $U \cdot g^{-1}$ (48 h)	[13]
Penicillium sp.	Olive oil	SmF	21.0 $U \cdot mL^{-1}$ (120 h)	[14]
Rhizopus oryzae NRRL 3562	Coconut oil	SSF	96.2 $U \cdot g^{-1}$ (115 h)	[15]
Bacillus subtilis OCR-4	Ground nut oil cake	SSF	4.5 $U \cdot g^{-1}$ (48 h)	[16]
Burkholderia cepacia LTEB11	Sugarcane bagasse and sunflower seed meal	SSF	234 $U \cdot g^{-1}$ (96 h)	[17]
Rhizopus chinensis	Wheat bran, wheat flour, and olive oil	SSF	24.4 $U \cdot g^{-1}$ (72 h)	[18]
Pseudozyma hubeiensis HB85A	Soybean oil	SmF	5.3 $U \cdot mL^{-1}$ (18 h)	[19]
P. chrysogenum	Grease waste and wheat bran	SSF	46 $U \cdot mL^{-1}$ (168 h)	[20]

SSF: Solid-state fermentation; SmF: Submerged fermentation.

Another important factor during feedstock processing is the valorization of coproducts. Such approach can contribute to the profits of an industrial plant, thus bettering the viability of biodiesel. As a classical example, soybean meal generated during soybean oil extraction is already used for protein and isoflavones extraction [24], and its main phospholipid, lecithin, separated in the degumming step, is used as natural emulsifier [25].

2. Enzyme Production and Characteristics

Lipases are enzymes classified as hydrolases (glycerol ester hydrolase, E.C. 3.1.1.3) and act on ester bonds of several compounds, with acylglycerols being the most proper substrates, catalyzing reactions of hydrolysis, synthesis, and trans- and interesterification (Figure 2). Lipases are more active in insoluble substrates, especially triglycerides made of long-chain fatty acids with over 10 carbon atoms, while esterases are active in soluble substrates, especially simple esters, such as ethyl acetate and triglycerides made of short-chain fatty acids with less than six carbon atoms. Esterases follow Michaelis-Menten kinetics, while lipases need a minimum substrate concentration to show high activity levels [26].

Due to the similarity of the catalytic triad found in lipases compared to those observed in serine proteases, the most widely accepted hypothesis is that the mechanism of lipase catalysis is similar to that of serine protease catalysis [27]. It is believed that the kinetic mechanism of lipases does not depend on the type of reaction being catalyzed (hydrolysis, acidolysis, transesterification, etc.).

The reaction begins with a nucleophilic attack on the carbon from the ester bond of the susceptible substrate by hydroxyl group in the serine residue of the active site, forming an acyl-enzyme complex and releasing alcohol from the lipid. Later, the acyl-enzyme complex is hydrolyzed, releasing the lipase regenerated. Figure 3 shows the stages of the reaction catalyzed by the lipase and its intermediates.

Furthermore, characteristics such as stability in the presence of organic solvents, no necessity of cofactors for their action and high enantioselectivity, turn lipases into a group of enzymes with one of the major technological interests [28–30].

Lipases occur widely in nature and can be produced by many microorganisms and higher eukaryotes. In animals, lipases obtained from pig and human pancreas are best known and more investigated than all other lipases. In these organisms, they are engaged in several lipid metabolism steps, including fat digestion, adsorption, reconstitution, and in lipoproteins metabolism. In plants, lipases are present in higher plants seeds, as castor bean and canola (*Brassica napus*). They are also found in several plants' energy reserve tissues [28, 31–33]. However, for the production of industrial enzymes, microorganisms are the preferred source, once they have shortest generation time, high yield of conversion of substrate into product, great versatility to environmental conditions and, simplicity in genetic manipulation and in cultivation conditions. Due to habitats' multiplicity, microorganisms usually produce various lipases types, with distinct specificity regarding to substrate utilization and also to optimum pH and temperature range. Lipases can be produced by bacteria, filamentous fungi, and yeasts, allowing these microorganisms to use lipids from animal or vegetable origin as carbon and energy sources for their growth. Though many microorganisms have been reported in literature as lipase producers, the genera *Candida*, *Rhizopus,* and *Pseudomonas* are considered the main industrial sources of lipases. The yeast *Candida rugosa* is the most employed microorganism for lipase production [30]. Table 6 gives an overview on recent literature regarding lipases production.

The use of lipases in industry is still limited by the cost of commercial enzymes, especially when large quantities of enzyme are required and when the final product is of low added value. There is therefore a considerable interest in reducing the cost of producing these biocatalysts. The use of solid-state fermentation (SSF) as a production system is one way of reducing enzyme production costs, especially because agroindustrial waste can be used as a culture medium.

A comparative economic analysis showed that the production of lipase from *Penicillium restrictum* by SSF is more economically feasible than its production by submerged

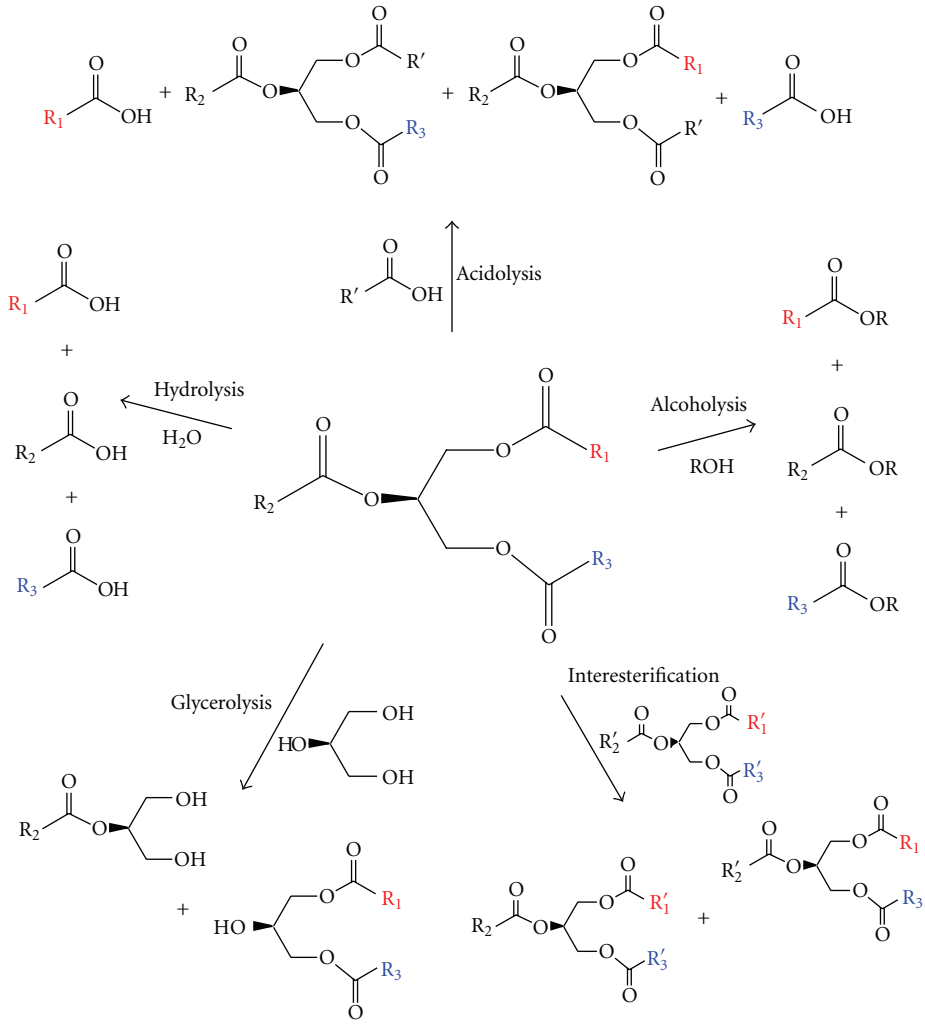

Figure 2: Reactions catalyzed by lipases.

Table 7: Sources of lipases and optimal conditions for their action.

Sources	pH	T (°C)	Reference
Candida rugosa	5–8	35–50	[34]
Pseudozyma antarctica A	6–10	35–70	[35]
Thermomyces lanuginosus	6–9	30–50	[36]
Aspergillus niger	6–8	40–55	[37]
Pseudomonas aeruginosa	5.5–7.5	35–45	[38]
Bacillus subtilis	8–10	30–40	[39]
Geotrichum candidum	6.5–8.0	32–42	[40, 41]
Streptomyces rimosus	8.5–10.0	45–60	[42]
Yarrowia lipolytica	4–7	30–45	[43–45]
Rhizopus niveus	5–7	30–45	[46]
Rhizomucor miehei	6.5–7.5	30–40	[47]
Porcine pancreatin	6–9	40–55	[48]
Castor bean (*Ricinus communis*)	4.0–4.5	30–35	[49, 50]

fermentation (SmF), with a production cost for the former being found to be 68% lower and a payback time of 1.5 years [54].

Other advantages of producing enzymes by SSF have been highlighted alongside the reduced production costs. In studies of lipase production by the fungus *Penicillium restrictum* using SSF and SmF, different significant physiologies were observed between the two systems when simple (oleic acid and glucose) and complex (olive oil and starch) sources of carbon were used, with a reduction in catabolite repression being observed for SSF [55].

Lipases from different microorganisms have been produced using SSF with different solid wastes, such as lipase from *Penicillium restrictum* in babassu cake [55, 56]; lipase from *P. simplicissimum* in babassu cake, soybean cake, and castor bean cake [57–61]; lipase from *Candida rugosa* in rice flour [62]; lipase from *Rhizopus homothallicus* in sugarcane bagasse [63, 64]; lipase from *Aspergillus niger* in wheat bran and sesame seed cake [65, 66]; lipase from *Rhizopus rhizopodiformis* and *Rhizomucor pusillus* in olive oil cake and sugarcane bagasse [67]; lipase from *Rhizopus oligosporus* in a variety of cakes [68]. These lipases were produced by SSF on a bench scale, mostly using tray bioreactors, and yielded high productivity rates.

Table 8: Reported conditions for enzymatic transesterification of some fats and oils [51–53].

Alcohol	Lipase source	Feedstock	Solvent	Yield (%)
Methanol	*C. antarctica*	Rapeseed oil	Hexane	98
Methanol	*C. antarctica*	Cottonseed oil	—	92
Methanol	*C. antarctica*	Cottonseed oil	t-Butanol	97
Methanol	*C. antarctica*	Degummed Soybean oil	—	94
Methanol	*T. lanuginosus*	Soybean oil	—	90
Ethanol	*P. cepacia*	Tallow fat	—	95
Propanol	*P. fluorescens*	Sunflower oil	1,4-Dioxane	>95
2-Ethyl-1-hexanol	*C. rugosa*	Rapeseed oil	—	97
Methanol	*P. cepacia*	Palm kernel oil	—	15
Ethanol	*P. cepacia*	Palm kernel oil	—	72
Methanol	*Mucor miehei*	Soybean oil	Hexane	75
Ethanol	*M. miehei*	Soybean oil	Hexane	97
Methanol	*M. miehei*	Tallow fat	Hexane	95
Ethanol (96%)	*M. miehei*	Tallow fat	Hexane	98
Anhydrous ethanol	*M. miehei*	Tallow fat	Hexane	68
Propanol	*M. miehei*	Tallow fat	Hexane	24
Butanol	*M. miehei*	Tallow fat	Hexane	20
Propanol	*C. antarctica*	Tallow fat	Hexane	61
Butanol	*C. antarctica*	Tallow fat	Hexane	84

There are no pre-established procedures in the literature for predicting the performance and design of SSF bioreactors. For this reason, large-scale systems have generally been developed from the results obtained from bench-scale or pilot systems. Ideally, a large-scale system should operate in the same way and with the same performance as a bench-scale system although this is often not the case for SSF processes [69]. The main limiting factor on scaling up such processes is heat transfer, which depends on the stage of fermentation, and the design and operation mode of the bioreactor [70–72]. Some mathematical models have been developed to describe the growth kinetics of the microorganisms under different operating conditions and to describe heat and mass transfer in tray bioreactors [73], fixed-bed bioreactors [70, 74], rotating drum bioreactors [75, 76], shaking reactors [77], and fluidized bed reactors. These models could be used as inputs for designing the scale-up of such systems.

In addition to the reduction of the costs related to fermentation step for industrial-scale production of lipases, the strategies to recover and purify lipases must also be as low as possible and should also be rapid, give high yields, and ideally be easy to scale up.

Lipases are enzymes that are known to be strongly hydrophobic, because of the presence of alkyl groups on the surface of their structure [30]. Generally, a good first step for lipase purification is the use of hydrophobic-interaction chromatography. Normally, prepurification involves precipitation with ammonium sulphate, and ion-exchange chromatography and gel filtration are also widely used [78–80].

Rua et al. [79] studied the production and purification of a thermostable alkaline lipase from *Bacillus thermocatenulatus* in *Escherichia coli*. The purification stages were done in butyl sepharose (hydrophobic bed) and TSK G3000 (gel filtration), giving a purification factor of 125 and a yield of 32%.

A lipase from *Aspergillus niger* F044 was purified by precipitation with ammonium sulphate, DEAE-Sepharose FF (ion exchange), and Sephadex G-75 (gel filtration). A yield of 33% was obtained, while the purification factor was 73 [37].

A lipase from *Penicillium simplicissimum* produced by submerged fermentation was purified in a five-step process [81]. First, the culture was concentrated using a 10 kDa membrane, then it was precipitated with ammonium sulphate. After concentration and prepurification, the sample was injected in sequential chromatography steps on phenyl sepharose CL-4B (hydrophobic interaction), Ultrogel AcA-54 (gel filtration), and hydroxyapatite (ion exchange). The resulting purification factor was 788, and the yield was 20%.

In order to purify a lipase from *Penicillium camembertii* U-159, Isobe et al. [82] used ethanol precipitation and ammonium sulphate precipitation as the first and second steps. A sequence of chromatography steps followed, using DEAE-sepharose (ion exchange), amino octyl sepharose (hydrophobic interaction), hydroxyapatite (ion exchange), and concanavalin-A sepharose (affinity). The yield obtained was 27%, and the purification factor was 213.

Cunha et al. [83] studied the purification/immobilization of a "pool" of lipases from *P. simplicissimum* produced by SSF using babassu cake as a culture medium. The process undertaken by means of sequential immobilization in hydrophobic supports (butyl, phenyl, and octyl agarose) resulted in three fractions with distinct thermal stability, specificity, and enantioselectivity properties.

Depending on the source, lipases can present molar mass ranging from 20 to 75 kDa, enzymatic activity at pH between 4 and 9 and at temperatures since 27 until 70°C. Lipases

are usually stable in neutral aqueous solutions at room temperature, presenting, in most cases, an optimal activity at 30–40°C. However, its thermostability varies considerably depending on the origin, and, according to Castro et al. [29], microbial lipases present the best thermostability.

Most commercial lipolytic preparations are composed by a mixture of various isozymes, in different proportions, such as those obtained from *Candida rugosa*, *Pseudozyma* (formerly *Candida*) *antarctica, Rhizopus niveus*, *and Chromobacterium viscosum*, among others. Each isoform has different properties (e.g., molar mass, specificity, stereoselectivity, glycosylation extension, and substrate preference) [28, 84, 85]. The main sources of lipases and their properties are described in Table 7.

For industrial applications, the specificity of lipase is a crucial factor. This enzyme can present specificity regarding the substrate (fatty acid or alcohol), including the differentiation of isomers. Lipases can be divided into three groups based on their specificity.

(i) Nonspecific lipases (such as those produced by *Candida rugosa*, *Staphylococcus aureus*, *Chromobacterium viscosum, Thermomyces lanuginosus,* and *Pseudomonas* sp.): They cleave acylglycerol molecules randomly generating FFAs and glycerol, as well as mono- and diglycerides as intermediates. In this case, the products are similar to those produced by chemical catalysis, but with less thermodegradation, due to the lower temperature used for the reaction, when compared to chemical processes [29, 46, 86].

(ii) 1,3-specific lipases (e.g., from *Aspergillus niger*, *Mucor javanicus*, *Rhizopus delemar*, *Rhizopus oryzae*, *Yarrowia lipolytica*, *Rhizopus niveus,* and *Penicillium roquefortii*): They release fatty acids from positions 1 and 3 of a glyceride and from, for this reason, products with different compositions of those obtained by nonregioselective lipases, or even by chemical catalysts. Generally, the hydrolysis of triglycerides to diglycerides is much faster than those into monoglycerides [29, 46, 87].

(iii) Fatty acid-specific lipases: they act specifically on the hydrolysis of esters, which have long-chain fatty acids with double bonds in *cis* position on carbon 9. Esters with unsaturated fatty acids, or without double bond in carbon 9, are slowly hydrolyzed. This type of specificity is not common among the lipases and probably the most studied example of this case is the lipase from *Geotrichum candidum* [29, 46, 87–89].

The study of substrate specificity is also of great importance for the application of lipases in biodiesel production, since it is a valuable input for the selection of the proper enzyme based on the composition of the raw material. Gutarra et al. [58] evaluated the substrate specificity of an acidic lipase produced by *Penicillium simplicissimum*, observing the highest activities on tricaprin (C8 : 0) and tricapryçin (C10 : 0), which were 83 and 92% higher, respectively, than those detected in the model substrate (olive oil).

Lipases can also be stereospecific, where one of the isomers of a racemate is hydrolyzed preferentially over another, or even the formation of one isomer can be catalyzed selectively from prochiral precursors such as *meso*-diester or *meso*-diol compounds. Some examples are lipases from *Burkholderia cepacia*, *Pseudozyma antarctica*, *Candida rugosa,* and *Rhizopus delemar* [88, 89].

3. Enzyme-Catalyzed Processes for the Production of Biodiesel

The main technology for biodiesel production in Brazil and in the world is homogenous alkaline transesterification (or alcoholysis). In this reaction, an alcohol (usually methanol or ethanol), with a molar basis, is added to the oil or fat and, in the presence of a catalyst (Brønsted acids or bases), a mixture of glycerin and alkyl esters of fatty acids is generated, which is called biodiesel (Figure 4). However, alkaline catalysts, especially sodium hydroxide, became dominant for the production of biodiesel, due to their lower costs and faster kinetics [9, 23, 91, 92].

However, homogenous alkaline transesterification presents some disadvantages over enzyme-catalyzed processes, such as the need of raw materials (refined oils and alcohols) virtually free of fatty acids, phosphatides, and water; excess of alcohol and catalyst to avoid reversible reactions, which in turn makes difficult the separation of biodiesel and glycerin. Therefore, alternative catalysts have been studied, such as organic bases, metallic complexes, oxides, aluminosilicates, and enzymes. Their main characteristics are that they are easily recycled and the absence of soap formation, which facilitates the products separation at the end of alcoholysis [91–93].

The use of biocatalysis has, therefore, advantages over chemical processes, and these include esterification of both triglycerides and fatty acids; generation of a cleaner glycerol; reuse, mostly in the case of an immobilized lipase utilization. However, some problems still need to be resolved, as high cost of lipases and possible inhibition in the presence of short-chain alcohols, glycerol, and other impurities in the raw material [23, 91, 93, 94]. In the case of biocatalysis, the schematic flowsheet of Figure 4(a) can also be applied, but it can also be necessary to use immobilized enzymes, for the reasons shown above. Due to kinetic disadvantages, it can be necessary also to use more sequential reactors in order to achieve the residence time of the feedstock in the presence of the enzymes, for a desired conversion (Figure 4(b)). For biodiesel production by enzymatic catalysis, some factors should be considered and some topics should be covered, which can be divided into aspects for current and prospective approaches. These aspects are detailed as follows.

3.1. Current Aspects

3.1.1. Refining Step. In enzymatic transesterification, higher yields are achieved for biodiesel production when refined oil is used, compared to crude oils. This is due to the presence of

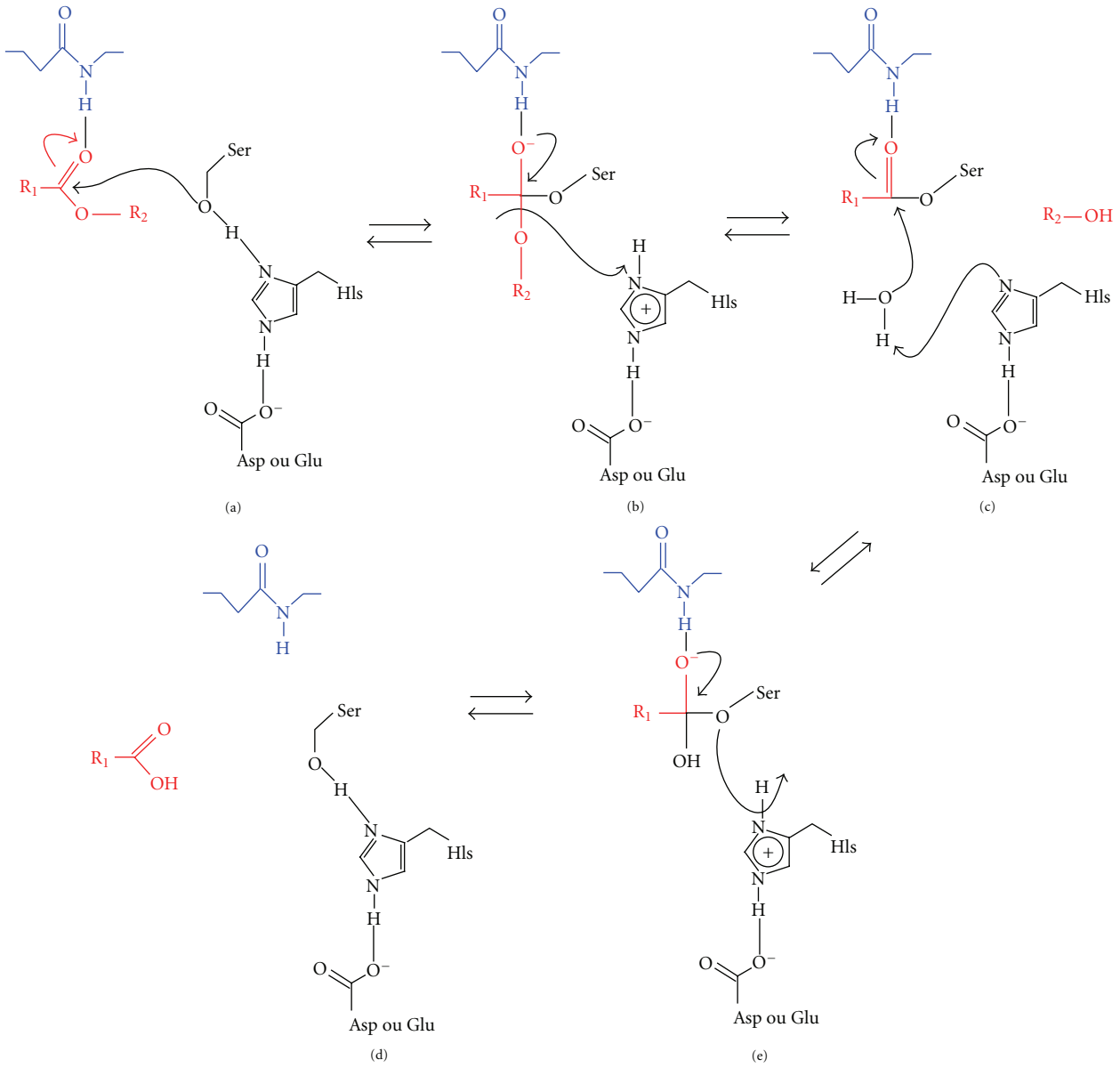

Figure 3: Mechanism of the hydrolysis reaction of ester bonds catalyzed by esterases and lipases. The catalytic triad and water are shown in black; the oxyanion hole residues are in blue; the substrate is in red. (a) Nucleophilic attack of the serine hydroxyl on the carbonyl carbon of the susceptible ester bond; (b) tetrahedral intermediate; (c) acyl-enzyme intermediate and nucleophilic attack by water; (d) tetrahedral intermediate; (e) free enzyme [90].

phospholipids in the nonrefined oil, which affect the interaction between lipase and substrate, since they possibly occlude the pores of the support, in the case of using an immobilized enzyme. Therefore, at least the oil degumming step should be conducted before transesterification reactions, in order to obtain a better production of biodiesel [23, 91].

The oil degumming is traditionally done using chemical and physical processes, such as water degumming, ultrafiltration, and mainly acid (phosphorous or citric) treatment [95, 96]. However, since the 1990s, enzyme-catalyzed degumming has been reported as a potential alternative to the conventional processes, and this comprises the use of phospholipases, which are classified into four groups [97]. Phospholipases types A1 (E.C. 3.1.1.32) and A2 (E.C. 3.1.1.4) catalyze the cleavage of ester bonds in phospholipids, thus releasing FFAs and contributing for the increase of the overall yield of biodiesel. There are also phospholipases types C (E.C. 3.1.4.3) and D (E.C. 3.1.4.4), but these are involved in the breakdown of phosphate bonds in phospholipids and do not contribute to the increase in FFAs content of the oil [98]. Enzymatic degumming is done at mild temperature (40–45°C) and pH of about 4.5–5.0, for a period of 2–4 h [97, 99].

3.1.2. Free and Immobilized Enzymes. The use of free enzymes for biodiesel production results in technical limitations, and it is practically unreliable, due to impossibility of their recovery and reuse, which in turn increases the

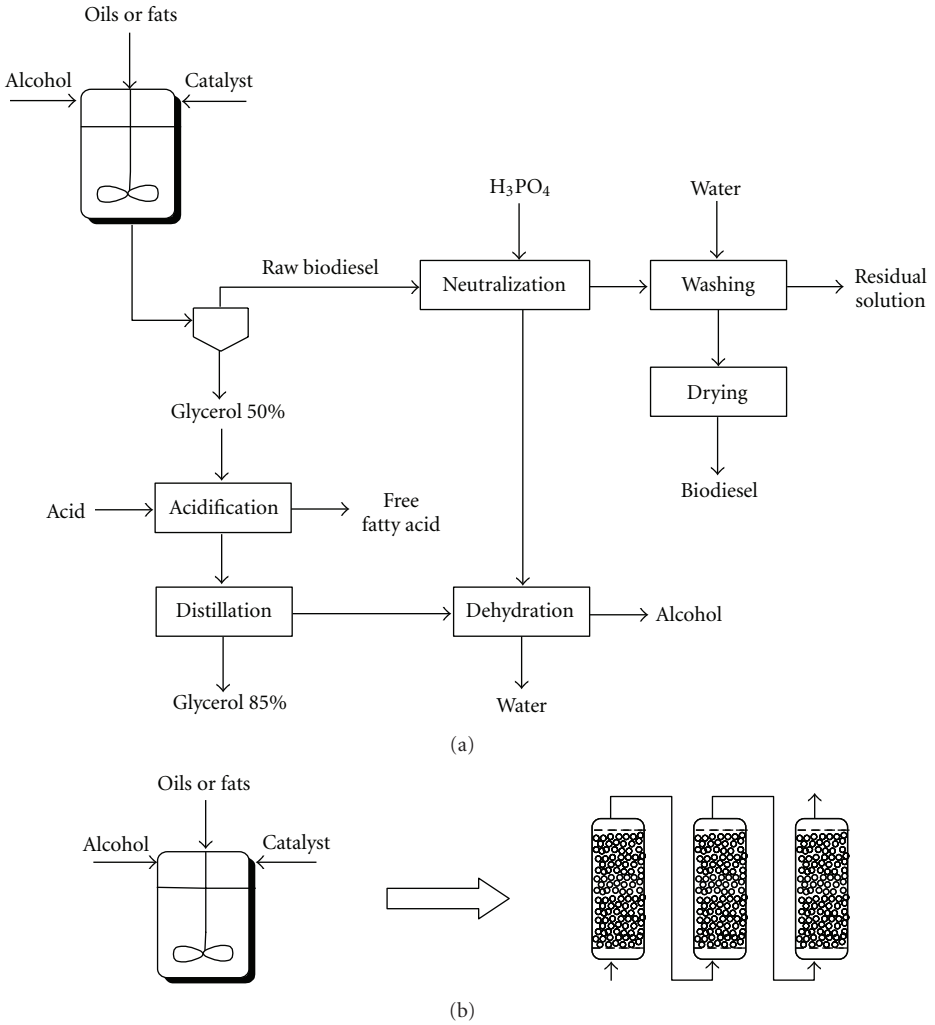

Figure 4: Simplified flowsheet for the production of biodiesel. (a) Classical industrial chemical process; (b) alteration in reactor design for biocatalysis.

production costs of the process, besides promoting the product contamination with residual enzyme. These difficulties can be overcome by the use of these enzymes in its immobilized form, allowing the reuse of biocatalyst several times, reducing costs, and further improving the quality of the product [100]. There are several techniques cited for lipases immobilization, such as adsorption, covalent bonding, entrapment, encapsulation, and cross-linking, but they will not be discussed in details in this paper, since there is a recently published review focusing on this issue [100]. In this context, Nielsen et al. [101] revised technical and economic aspects of biodiesel production, concluding that for enzyme-catalyzed biodiesel viability, using immobilized lipases, the enzymes must be stable for the production of 1200–7400 kg of biodiesel for each kg of enzyme preparation, depending on the substrate source and lipase used.

The confinement or physical location of an enzyme in a given region of a defined space, while maintaining their catalytic activity, which can be used repeatedly and continuously due to the ease of its separation from the reaction medium, comprises its immobilization [102].

The catalytic activities of enzymes and other features may change depending on the type of the immobilization technique (adsorption, covalent bound, entrapment, encapsulation, and cross-linking) and the interaction strength between enzyme and carrier used which may, in some cases, cause structural distortions in the protein. Still, the catalytic activity of the enzyme in a particular medium can be changed by increasing or decreasing stirring due to the support fragmentation by interaction between agitation system and support [100].

Thus, it is possible to occur some activity loss during transesterification reaction, even when immobilized lipases are used, and this is more probably due to enzyme leaching than to enzyme inactivation. On the other hand, if such leaching does not occur and the enzyme remains bound to the support, the increase of contact surface may help in raising mass transfer, thereby increasing the efficiency of the enzyme as a catalyst. Lipases from different sources have been immobilized and used in biodiesel production, but the most commonly reported were obtained from *Pseudozyma antarctica* and *Thermomyces lanuginosus* [8, 103].

The use of fixed bed reactors with immobilized lipases is a more suitable solution for continuous production of biodiesel, since the enzymes suffer lower shear stress compared with the batch process. Pandey [91] reported the methanolysis of a waste oil mixture (containing 1980 ppm of water and 2.5% FFAs), using immobilized lipase from *C. antarctica*, and considering 3 steps of substrate addition. The highest biodiesel yield observed was 90.4%. In another example, the same author reported the ethanolysis of a fat in a recirculating packed bed reactor (flow of $1.8\,L\cdot h^{-1}$) using a phyllosilicate sol-gel-immobilized lipase from *Burkholderia cepacia,* and in this case, a yield of 96% was observed. Over the subsequent four cycles evaluated, the yield was maintained in at least 90%.

3.1.3. Use of Solvents. The enzymatic production of biodiesel can be performed using organic solvents (usually hexane, heptanes, or petroleum ether) or simply using the mixture of substrates (lipids and alcohol) depending on the size of the chain of alcohol. If methanol or ethanol is used, a solvent can facilitate the oil solubility in alcohol and also decrease the viscosity of the reactional mixture, but there will be an additional cost for its removal (distillation or extraction) after the reaction [8, 94, 102].

Knothe et al. [23] reported the biodiesel production from sunflower oil using petroleum ether as solvent with immobilized lipase from *Pseudomonas fluorescens,* reaching 99% yield when the alcohol used was methanol, ethanol, or 1-butanol. In the absence of solvent, yields were reduced to 3%, 70, and 76%, respectively.

Soetaert and Vandamme [104] reported the use of the lipases from *Mucor miehei* and *C. antarctica* in the transesterification of various oils using hexane as solvent and found that the lipase from *M. miehei* is more efficient in converting primary alcohols (methanol, ethanol, propanol, and 1-butanol) with yields between 95 and 98%, whereas lipase from *C. antarctica* is more proper for the conversion of secondary alcohols (isopropanol and 2-butanol) with yields between 61 and 84%. In the absence of the solvent, the yields of methyl and ethyl esters decreased, particularly when methanol was used, with yield reduction up to 19%.

The use of solvents with intermediate polarity (such as t-butanol, 1,4-dioxane) has been suggested to achieve a better dissolution of the alcohol for transesterification (particularly methanol, due to its higher inhibitory effect over lipases) and oil, without affecting the lipase activity [102]. Ranganathan et al. [105] reported the use of t-butanol as a solvent for the transesterification of cottonseed oil using the commercial preparation Novozym 435 for 24 hours at 55°C and achieved a yield of 97%, maintaining the lipase activity by 95% of the initial activity during 500 h of continuous operation.

3.1.4. Type of Alcohol and Adsorption Agent. Many types of alcohol can be used in the reaction of enzymatic transesterification. Some examples are shown in Table 8.

The molar ratio between substrates is a variable with large influence on the biodiesel synthesis. Excess of alcohol, in relation to the stoichiometric ratio of 3/1, is used to ensure higher reaction rate and to minimize diffusional limitations. However, excessive levels of alcohol, mainly those with short chains, may inhibit the enzyme by increasing the polarity of the medium, which reduces the stabilization and removes the water layer associated with the immobilized enzymes [94]. This effect was observed by Brusamarelo et al. [106], for the transesterification of soybean oil. The optimal ethanol to oil ratio was 6/1, where 93% conversion was achieved after 480 min of reaction. When increased ethanol to oil ratios (9/1 and 12/1) were used, the conversion, considering the same time of catalysis, dropped to 51 and 55%, respectively.

Hence, the gradual addition of alcohol can maintain lipase stable for a longer period. Pandey [91] reported that the gradual addition of methanol (in 3 steps, every 16 h of reaction), maintaining the same enzyme in the bioreactor, resulted in a yield of 98% of biodiesel, and that the conversion was kept over 95% during 50 cycles. Another aspect that may accelerate the methanolysis is the preincubation of lipase in ester or oil [104].

The addition of silica to the reaction medium provides a positive effect on yield, due to the absorption of glycerol and water, thus reducing lipase inhibition [23]. One example cited by Robles-Medina et al. [102] is the use of 6% (w/w) of silica-gel in the reaction mixture along with the commercial preparation Lipozyme TL. The use of silica increased the yield from 66 to 90%. Another possibility would be to remove the glycerol by dialysis or its dissolution in isopropanol or t-butanol [102, 105].

3.1.5. Alternative Donors of Acyl Group. As the use of methanol and ethanol can promote lipase inhibition, the use of alternative donors of acyl group, such as methyl acetate, ethyl acetate, and propan-2-ol, is being studied, since their use avoids the production of glycerol as a by-product, which blocks the porous support and lipase active sites. Novozym 435 was tested for biodiesel production using several oils and the donors of acyl groups cited above, and the results observed were yields always above 90% [53, 91].

3.1.6. Water Addition. Alcoholysis reactions do not involve water as a reagent. However, the control of water content in the reaction system is important for some reasons: lipase requires a minimum amount of water to maintain its active conformation; an excess of water may promote the hydrolysis of the substrate and generate diffusion limitations of substrate, thus reducing the biodiesel yield; the water can influence negatively the reaction when methanol or ethanol is used but does not affect the reaction when higher-chain alcohols are considered [93, 94, 102].

Drapcho et al. [93] reported the use of lipase from *Pseudomonas cepacia* immobilized on polymeric sol-gel matrix in the transesterification of tallow oil at 40°C for 1 h using a mixture of 10 g of fat, 3 g of lipolytic preparation, 3 g of methanol or 5 g ethanol, and different amounts of water. The authors observed that when water concentrations below 0.2% were used, the conversion was significantly decreased and, after the reuse of lipase during 11 cycles, the activity was decreased by 10%.

Pandey [91] reported the use of lipase from *Chromobacterium viscosum* in the transesterification of jatropha oil with a 10% enzyme dosage. When the biocatalyst in a free form was used, it was observed a yield of 62%, whereas when the enzyme was immobilized on Celite 545, 71% of yield was achieved. By adding 1% of water to the free enzymatic preparation and 0.5% of water to the immobilized enzyme, the biodiesel yields raised to 73% and 92%, respectively.

3.1.7. Temperature. Generally, the higher the temperature, the higher the reaction rate of alcoholysis or transesterification, until reaching the temperature of inactivation of lipases (usually above 60°C). This approach is valid mainly for systems in which the enzyme is used just once or few times. When enzyme reuse is considered, high temperatures, which can be suitable for short-term use of the enzymes, may be not the most proper, since the half-life time of lipases decreases with increases in temperature.

Matassoli et al. [107] investigated the influence of temperature in ethanolysis of crude palm oil catalyzed by Lipozyme TL IM (3% w/w) with a molar ratio ethanol/oil of 3/1 and gradual addition of ethanol, observing the best result at 50°C. For the evaluation of the effect of temperature on lipase-catalyzed biodiesel production, a semiempirical model was proposed by Brusamarelo et al. [106]. The authors investigated soybean biodiesel production using the commercial product Novozym 435, within the range of 45–70°C, observing the highest yield (92%) when 65°C was used.

3.1.8. Enzyme Type and Dosage. The amount of enzyme added to reaction is also an important factor for biodiesel production, because it affects reaction rate (typically, the higher the enzyme dosage, the faster the reaction), but there is a limit in which the addition of enzyme does not alter anymore the rate of product formation or that the amount turns the process more economically prohibitive. In this context, enzyme-catalyzed biodiesel production was investigated using dosages of *C. antarctica* lipase B (Novozym 435) from 1 to 20% (w/w) [106]. The authors observed the highest conversion of triglycerides to ethyl esters (93%) when 10% (w/w) of the immobilized enzyme was used.

Regarding the effect of lipase specificity, Pandey [91] reported the use of some specific and non-specific lipases (from *C. rugosa, P. cepacia,* and *P. fluorescens*) in biodiesel production. The non-specific lipases promoted the highest yields of methyl esters when a molar ratio of 3/1 of methanol/oil was used. Specific lipases need gradual addition of methanol to achieve high yields (between 80 and 90%), and this is probably due to acyl migration of sn-2 to sn-1, which occurs spontaneously in glycerides [108].

3.2. Prospective Aspects

3.2.1. Whole Cells. For reduction of enzymatic processes costs, some researchers have studied microbial immobilization, such as fungal mycelia, bacteria, and yeasts cells, for their use as whole cell catalysts, taking advantage of functional proteins at the cell surface. From all whole cell support immobilization techniques, the most used is that named porous biomass support particles (BSPs) because it does not require chemical additives or cell preproduction; aseptic handling is unnecessary; higher enzyme production and rate of substrate mass transfer within BSP; the particles are reusable and resistant to mechanical shearing; the bioreactor scale-up is easy and presents lower costs compared to bioreactors used in other methods [8, 104].

The first example for biodiesel production using whole cell as biocatalysts was the *Rhizopus oryzae* mycelium immobilized in polyurethane foam [109]. The growth conditions were optimized regarding the production of intracellular lipase, as well as pretreatment methods and water content during methanolysis. The addition of substrates (olive oil or oleic acid) to the culture medium significantly improved lipase activity of the whole cell catalyst. The results for the obtainment of methyl esters of soybean oil using this catalyst at 32°C for 72 h (80–90% yield), when the addition of methanol to the system was implemented intermittently in the presence of 10–20% water, were very similar to those described with the use of extracellular lipases. Aiming to stabilize the *R. oryzae* cells, it was tested a cross-linking treatment with 0.1% glutaraldehyde, keeping the lipase dosage unaltered for 6 cycles. The yield of methyl ester varied between 70% and 83%, along 72 h of experiment. Without this treatment, the lipase activity decreased reaching a yield of 50% in the sixth batch [8, 91, 104].

In order to achieve higher yields of biodiesel using cells immobilized in BSP, Soetaert and Vandamme [104] used fixed bed systems. To increase the interfacial area between the reaction mixture and the whole cells, the former was emulsified by sonication before each batch cycle. When a gradual addition of methanol (ratio 4/1, methanol/oil) was conducted at a flow rate of $25\,L\cdot h^{-1}$, a yield of 90% was obtained and maintained at about 80% for 10 batches. The authors attributed this decrease to the cell removal from the BSP, since it was detected a decrease of 56% in the cell concentration in the BSP between the first and the tenth batches.

As further examples, other freeze-dried whole cells, such as from *R. chinensis* mycelium, *S. cerevisiae* (containing intracellular *R. oryzae* lipase, ROL), and recombinant *S. cerevisae* expressing the lipase gene of *R. oryzae* IFO 4697 (cell surface ROL), have been used as biocatalysts for the production of methyl biodiesel from soybean oil. In the absence of solvent, the yields observed for the cited examples were 86, 71, and 78%, respectively, after 165 h of reaction at 37°C [8, 102].

Salum et al. [110] showed that it is possible to decrease the costs associated to the synthesis of enzyme-catalyzed biodiesel, by using the fermented solids produced by cultivating *Burkholderia cepacia* LTEB11 on a mixture of sugarcane bagasse and sunflower seed meal. The authors used this fermented solids to catalyze the ethanolysis of soybean oil aiming to produce biodiesel in a fixed-bed reactor in a cosolvent-free system. They achieved 95% conversion after 46 h of reaction.

Although the use of whole cells does not require many of the steps related to the downstream process of biodiesel production, such as the isolation and purification of the enzyme

after fermentation, processes in which the transesterification reaction is done by using immobilized enzymes or cells present at least one notable difference, which is the reaction time. Fukuda et al. [111] reported the use of Novozym 435 in a continuous process, for 7 h, and they observed yields of 92–94% in terms of methyl esters. When a fed-batch operation was considered, the yield was 87%, after 3.5 h of reaction with methyl oleate. The authors also compared the process performance by using whole cells in a fed-batch operation mode, and observed the necessity of 70 h of reaction in order to obtain yields of 80–90% of biodiesel. In the last case, the utilization of t-butanol as solvent would possibly reduce the reaction time, due to higher efficiency of mass transfer in the system.

3.2.2. Use of Acid Oils. Some waste oils, by-products from vegetable oils processing, may also be suitable raw materials for biodiesel production. These oils usually present high contents of FFAs, and some examples are the sunflower oil and corn oil, which have 55.6% and 75.3% of FFAs, respectively. Pandey [91] reported the esterification and transesterification of these oils in the presence of hexane using immobilized lipase from *C. Antarctica* and observed yields of 64% and 50% of methyl esters, while maintaining the lipase stable for over 100 cycles.

Hou and Shaw [52] reported that the esterification of acid oils is much faster than the transesterification of nonacid oils. In the former case, it was necessary only 3 h of reaction and 1% of lipase for the esterification of FFAs, where a yield of 95% was achieved, whereas for the latter case, the same yield was observed only after 30 h of methanolysis and using a higher enzyme dosage (4%). One disadvantage of the esterification reaction is the formation of water as a by-product, which often inhibits the reaction of triglycerides. One possible solution to this is to conduct the reaction in two separate stages: first, esterification of the FFAs in the mixture, with the evaporation of the generated water; then the methanolysis of the triglycerides. In the first step, the molar ratio of methanol/FFA should be low, such as 1/2 and low quantity of enzyme (0.5% w/w) is needed. In the second step, on the other hand, the molar ratio between methanol and triglycerides should be changed to 1/1, and the enzyme quantity should be increased to about 6% (w/w).

3.2.3. Hydroesterification and Hybrid Catalysis. Hydroesterification is a process that combines two basic processes, the hydrolysis of triglycerides and the esterification of fatty acids, in sequential reactions, in order to produce biodiesel.

Talukder et al. [112] studied the use of residual cooking oil for biodiesel production by enzymatic hydrolysis accompanied by chemical esterification. The *C. rugosa* lipase used completely hydrolyzed the oil after 10 h of reaction. The FFAs were converted into biodiesel by chemical esterification using Amberlyst 15 (acidic styrene divinylbenzene), and a 99% conversion into biodiesel was obtained after 2 h. In this work, there was a loss of enzyme activity, and the hydrolysis yield was decreased to 92% after five batch cycles.

Cavalcanti-Oliveira et al. [113] studied the use of a lipase from *Thermomyces lanuginosus* (TL 100 L) in the hydrolysis of soybean oil in a hydroesterification process. The lipase hydrolyzed 89% of the oil after 48 h of reaction. This stage was followed by the esterification of the FFAs with methanol, which was catalyzed by niobic acid in pellets. They obtained 92% conversion of the FFAs into fatty acid methyl esters after only 1 h of incubation. Sousa et al. [114] studied the lipase from jatropha seeds for the hydrolysis of different raw materials for biodiesel production using hydroesterification strategy. The best conversions were obtained using soybean oil and jatropha oil, obtaining up to 98% of FFA after 2 h. The esterification of the FFAs from the jatropha oil with methanol was catalyzed by niobic acid in pellets, obtaining up to 97% conversion into biodiesel after 2 h. The biodiesel obtained from this process fulfilled all the legal requirements for its commercial use.

4. Concluding Remarks

The use of enzyme catalysts (lipases) in biodiesel production is being increasingly studied because of the advantages that these catalysts present over chemically catalyzed and noncatalytic processes. Some of the advantages offered by the use of lipases are lower energy consumption; lower thermal degradation of substrates and products; versatility in the use of raw materials, including possibility to use acid oils without the decrease of process efficiency; easier purification of the alkyl esters (biodiesel) and separation of the coproduct (glycerol), especially if immobilized enzymes or whole cells are used; environmental benefits, due to biodegradability of the catalyst.

Nevertheless, some process conditions should be taken into account in order to have a feasible enzyme-based technology for the production of biodiesel, and these include the establishment of descriptive correlations between the enzyme dosage and the substrate source, in order to rationalize enzyme usage and optimize costs [106]; deep study of reaction conditions and their optimization; the selection of a proper biocatalyst which can be reused and maintain its stability over several cycles; product recovery strategies, especially when a cosolvent is used in the reaction.

The enzyme-based production of biodiesel is still under development, and it seems that there is a tendency for the use of conventional technologies as a new application for lipases, such as their immobilization in magnetic nanoparticles [115], microwave and ultrasound-assisted transesterification [116], esterification in pressurized fluids [117], and transesterification in supercritical fluids [116]. Although technical aspects of such strategies may lead to conversion improvement, economical considerations must be investigated in more details.

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