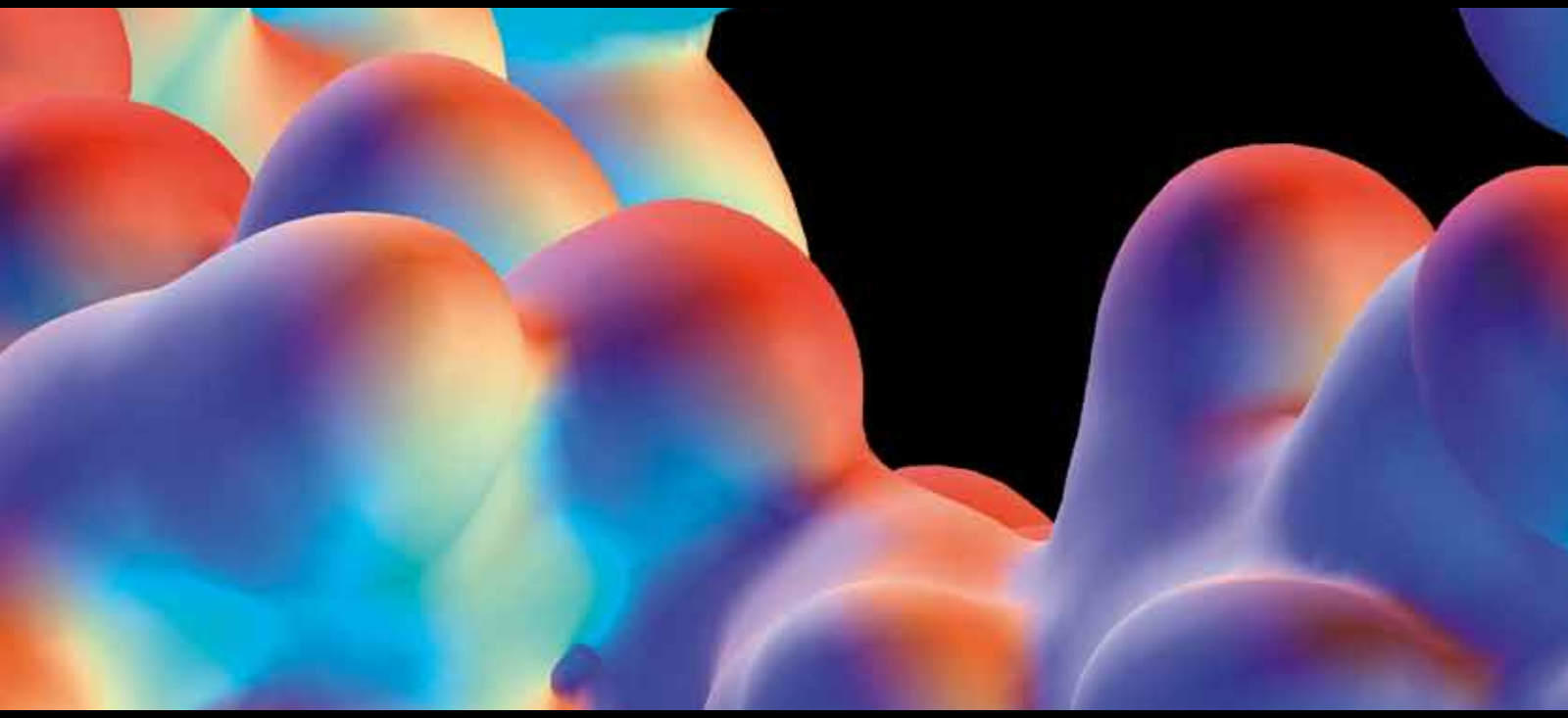
Enzyme Research

Enzymes in Biofuels Production

Guest Editors: Sulaiman Al-Zuhair, K. B. Ramachandran, Mohamed Farid, Mohamed Kheireddine Aroua, Praveen Vadlani, Subramanian Ramakrishnan, and Lucia Gardossi



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Editorial

Enzymes in Biofuels Production

Sulaiman Al-Zuhair,[1] K. B. Ramachandran,[2] Mohamed Farid,[3] Mohamed Kheireddine Aroua,[4] Praveen Vadlani,[5] Subramanian Ramakrishnan,[6] and Lucia Gardossi[7]

[1] *Department of Chemical and Petroleum Engineering, UAEU, Al-Ain 17555, UAE*
[2] *Department of Biotechnology, Indian Institute of Technology Madras, Chennai 600036, India*
[3] *Department of Chemical and Materials Engineering, The University of Auckland, Auckland 1142, New Zealand*
[4] *Department of Chemical Engineering, University of Malaysia, 50603 Kuala Lumpur, Malaysia*
[5] *Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA*
[6] *Department of Chemical and Biomedical Engineering, Florida A&M University, Tallahassee, FL 32310-6046, USA*
[7] *Dipartimento di Scienze Farmaceutiche, Universita' Degli Studi di Trieste, 34127 Trieste, Italy*

Correspondence should be addressed to Sulaiman Al-Zuhair, s.alzuhair@uaeu.ac.ae

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With the inevitable depletion of the nonrenewable resources of fossil fuels and due to their favorable environmental features, biofuels promise to be the preferred fuels of tomorrow. They can displace petroleum fuels and, in many countries, reduce the dependence on imported fuel. Biofuels, derived from biomass conversion, such as biodiesel, bioethanol, biohydrogen, and biogas, are sustainable and renewable sources of energy, which are also considered CO_2 neutral. In addition, burning biofuels results in reduced levels of particulates, carbon oxides and sulfur oxides, emissions compared to fissile fuels.

To respond to the increased demand for biofuels, advanced biochemical processes using enzymes are being developed, which are gaining increased global attention. Research in this field aims at improving efficiency, and reducing negative environmental impacts, of production processes, in addition to enhancing the quality of the produced biofuels. Enzymes have been employed to overcome the drawbacks associated with the use of conventional chemical catalysts. For example, biodiesel production by enzymatic catalyzed processes is less energy intensive and more environmental friendly compared to its production by conventional alkaline catalyzed processes. In addition, the biocatalyst allows using unrefined feedstock, including waste oil, readily without the need to separate the free fatty acids that may be present in large amounts in the feedstock. Another example is the use of enzymes for the hydrolysis of cellulose to produce fermentable sugars for bioethanol production. The utility cost of enzymatic hydrolysis is much lower compared to the alternative methods of acidic hydrolysis because it is carried out at mild conditions and does not require subsequent treatment step.

There are several obstacles, however, facing the use of enzymes as catalysts for biofuels production, most importantly is their high costs. Therefore, repeated use of the enzymes is essential from the economic point of view, which can be achieved by using them in immobilized form. In a continuous process using immobilized enzyme, the operational stability, the exhaustion of enzyme activity, and inhibition by reactants and/or products play vital roles. The use of membrane bioreactors for the enzymatic processing is increasingly becoming more attractive, as such systems allow continuous separation of products and prevent enzyme inhibition.

Research attention is also focused on genetic engineering in enzymes production. Recently, genes of various enzymes have successfully been cloned, and more genes are promised to be cloned rapidly in the coming years. The use of recombinant DNA technology to produce large quantities of recombinant enzymes will help lower the enzymes costs. In addition, protein engineering will help to create novel enzyme proteins that are more resistant and highly thermo-stable.

The introduction of a new generation of cheap enzymes, with enhanced activities and resilience, should change the economic balance in favor of enzyme use.

It gives me great pleasure to present to you this special issue. The issue covers both basic and applied aspects of using enzymes in the production of various types of biofuels. Articles published present different aspects of current and potential involvement of enzymes in biofuel production.

Acknowledgments

I would like to take this opportunity to express my sincere appreciation to the editorial team members, who have done a magnificent work. Without their cooperation and professionalism, this special issue would have not seen the light. I wish also to thank all the authors who have shown interest in participating in this special issue. Last but not least, I wish to extend my gratitude to SAGE-Hindawi Publishing Corporation for all their hard work and cooperation.

Sulaiman Al-Zuhair
K. B. Ramachandran
Mohamed Farid
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Lucia Gardossi

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Review Article

A Review of Enzymatic Transesterification of Microalgal Oil-Based Biodiesel Using Supercritical Technology

Hanifa Taher,[1] Sulaiman Al-Zuhair,[1] Ali H. Al-Marzouqi,[1] Yousef Haik,[2] and Mohammed M. Farid[3]

[1] *Chemical and Petroleum Engineering Department, UAE University, Al-Ain 17555, United Arab Emirates*
[2] *Mechanical Engineering Department, UAE University, Al-Ain 17555, United Arab Emirates*
[3] *Chemical and Materials Engineering Department, University of Auckland, 1142 Auckland, New Zealand*

Correspondence should be addressed to Sulaiman Al-Zuhair, s.alzuhair@uaeu.ac.ae

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Biodiesel is considered a promising replacement to petroleum-derived diesel. Using oils extracted from agricultural crops competes with their use as food and cannot realistically satisfy the global demand of diesel-fuel requirements. On the other hand, microalgae, which have a much higher oil yield per hectare, compared to oil crops, appear to be a source that has the potential to completely replace fossil diesel. Microalgae oil extraction is a major step in the overall biodiesel production process. Recently, supercritical carbon dioxide (SC-CO_2) has been proposed to replace conventional solvent extraction techniques because it is nontoxic, nonhazardous, chemically stable, and inexpensive. It uses environmentally acceptable solvent, which can easily be separated from the products. In addition, the use of SC-CO_2 as a reaction media has also been proposed to eliminate the inhibition limitations that encounter biodiesel production reaction using immobilized enzyme as a catalyst. Furthermore, using SC-CO_2 allows easy separation of the product. In this paper, conventional biodiesel production with first generation feedstock, using chemical catalysts and solvent-extraction, is compared to new technologies with an emphasis on using microalgae, immobilized lipase, and SC-CO_2 as an extraction solvent and reaction media.

1. Introduction

Continuous exploration and consumption of fossil fuels have led to a decline in worldwide oil reserves. As the world energy demand is continuously increasing, the most sufficient way to meet the growing demand is by finding alternative fuels. From the point of environment protection, finding alternative fuels that are sustainable and environment friendly is essential.

More than a century ago, Rudolf Diesel tested the suitability of using vegetable oils as fuel in his engine [1, 2]. In the 1930s and 1940s, vegetable oils were used as a diesel fuel for emergency situations. At that time, vegetable oil fuels were not competitive because they were more expensive than petroleum fuels, and therefore the idea was abandoned. With the worries about petroleum fuel availability and latest increases in petroleum prices, using vegetable oils in diesel engines has regained attention.

A number of studies have shown that triglycerides (TGs) hold promise as alternative diesel engine fuels [2, 3]. This has an advantage of being available, renewable with higher cetane number, and biodegradable [4–6]. However, the main disadvantage of oils is their high viscosity and low volatility [2, 7, 8]. Therefore, direct use of TGs is generally unacceptable and not practical since it causes engine coking, carbon depositing and gelling of the lubricating oil [8–10]. To overcome these problems, dilution, pyrolysis, cracking, and transesterification of the oil have been suggested [5, 11]. Among all these methods, transesterification has been used widely as a favorable method. Transesterification reaction of TGs, known as alcoholysis, is an important reaction that produces fatty acids alkyl esters (FAAE) [8, 12]. It was reported that replacing petroleum diesel with FAAE results in a reduction of unburned hydrocarbons, carbon monoxide (CO), and particular matter (PM) formation [13, 14]. Several methods of transesterification using alkali catalysts

[9, 14–18], acid catalysts [17, 19–23], and enzyme lipase in presence and absence of solvents have been reported [24–29]. Most of the commercial biodiesel processes require the use of a catalyst, which requires a recovery unit to separate reaction products and remove the catalyst. These disadvantages of using catalyst could be eliminated by carrying out noncatalytic reaction. Sake and Kusdiana [30] developed a method using supercritical methanol (SCM) where triglycerides fatty acids were converted to methyl esters without using any catalyst. Sake and Kusdiana [30] and Madras et al. [31] reported the advantage of using supercritical alcohols (SCA), especially methanol, whereas a process requires short reaction time and no need for reaction product separation from the solvent. However, this process is energy intensive as it is carried out at the supercritical conditions of methanol. Nevertheless, based on van Kasteren and Nisworo [32] economic assessment, this process appears to be feasible.

Enzymatic biodiesel approach showed promising results due to their high selectivity and mild operative conditions. Enzymatic transesterification reaction is similar to conventional transesterification, except that they are catalyzed by a variety of biological catalysts rather than chemical catalysts. In contrast to conventional processes, biocatalysts can transesterify TGs with a high free fatty acid (FFA) content [33]. Lipase-catalyzed transesterification of TG has been investigated by several investigators [33–37]. One common drawback with the use of enzyme-based processes is the high cost of the enzyme compared to conventional chemical catalysts; therefore, their recycle is required, which is possible through enzyme immobilization.

Immobilization of enzymes has generally been used to attain reusable enzyme with lower production cost [25, 38, 39]. Thus, immobilized form of lipase has been used in most of transesterification processes [25, 36, 40]. Besides enzyme reusability, other advantages of using immobilized lipase as a catalyst are enhanced activity and stability [41, 42].

Several researches have been carried out to produce biodiesel in solvent systems. Presently, industries are facing problems in using conventional solvents due to environmental worries. In the last couple of decades, enzyme-catalyzed reactions in supercritical carbon dioxide (SC-CO_2) has been studied. Previously, most of the studies were investigating the feasibility of using biocatalyst in SC-CO_2, whereas recent studies are focusing on obtaining good yield and conversions.

Vegetable oils consist of TG of straight chains of fatty acids. With the high cost of biodiesel produced from vegetable oils, researchers are looking for low-cost feedstocks. For that waste oils, cooking oils and fats from animal sources were proposed. The main drawback of using animal fats is their high melting points, which may require the use of organic solvents. However, organic solvent use requires a solvent recovery unit and energy needed for its separation. To overcome this, supercritical fluids (SCFs) were introduced.

During the past decades, SCFs have been investigated as alternative solvents for reactions rather than using conventional solvents. Among all supercritical fluids, SC-CO_2 is the most appropriate choice as a consequence of its availability. In general, CO_2 is nontoxic, nonflammable, environmentally friendly, and recyclable fluid [43]. Thus, reactions in SC-CO_2 media become the preferable route for chemical synthesis.

Conventionally, biodiesel is produced from vegetable oils, animal fats, and waste cooking oils [7, 30, 31, 44–46]. However, these feedstocks are inefficient and unsustainable [47]. Furthermore, using vegetable oil as a fuel source competes with its use as food and proposes for land development in order not to compete with food and land. On the other hand, animal fat cannot be considered as a continuous supply of feed stock [48]. Thus, biodiesel production using these feedstocks, realistically, cannot replace all world biodiesel requirements.

In contrast, microalgae have been recognized as a promising alternative source for biodiesel production. They are a group of organisms that can grow photosynthetically and accumulate large amounts of lipids [49, 50]. According to Sheehan et al. [50], if microalgal oil production could be scaled up, less than 6 million hectares would be required to meet current fuel demands.

Considering the above facts, this paper provides an overview on biodiesel production from microalgae with a particular emphasis on the use of microalgae as a promise feedstock, lipase as promise catalyst, and SC-CO_2 as a promise extraction solvent and reaction media.

2. Biodiesel

Biodiesel has arisen as a possible alternative for petroleum diesel because of the similarities that biodiesel has with petroleum diesel [39, 51]. Biodiesel fuel has many advantages over petroleum fuel such as being nontoxic, biodegradable, renewable, and do not contribute to net accumulation of the green house gases [52, 53]. Also, biodiesel has lower sulfur and aromatic content, higher cetane number, and flash point than petroleum diesel [5, 7, 54–56]. Other benefits of biodiesel include increased lubricity and lower emissions of certain harmful exhaust gases in comparison to petroleum diesel fuel [55].

Comparing petroleum diesel fuel to biodiesel, Schumacher et al. [14] reported that biodiesel results in a 45% reduction in total hydrocarbon emissions, 47% reduction in CO emissions, and 66% reduction in PM emissions, whereas, Demirbas [55] reported a 42% reduction in CO and 55% in PM emissions relative to standard diesel fuel. These effects are generally attributed to the higher cetane number and oxygen content of biodiesel fuel. Although the biodiesel environmental considerations are very positive, biodiesel increases nitrogen oxides (NO_x) emissions. However, reports show that reductions in NO_x emissions are possible with some modifications in combustion temperatures and injection timing [57, 58].

As mentioned earlier, direct use of vegetable oil has several negative aspects, such as their high viscosity and low volatility, which lead to incomplete combustion in diesel engines, therefore, carbon deposition [5, 9, 59, 60]. However, the direct use of vegetable oils as biodiesel may be possible by mixing them with conventional diesel in an appropriate ratio, but this mixing will be impractical for long-term

uses in the engine due to the high viscosity, low stability, acid composition, and FFA content [4, 61, 62]. Therefore, considerable efforts have been made to develop vegetable oil derivatives that have properties near those of the petroleum-based diesel fuels.

Pyrolysis (cracking), microemulsion, and transesterification are the possible methods to minimize problems associated with feedstock use [5, 8, 11]. The first two methods are costly and yield low quality biodiesel, whereas the latter, transesterification, is the most common method to transform oil into biodiesel, which is the focus of this paper.

2.1. Transesterification. Transesterification is the common method used to transform TG into biodiesel. This consists of the reaction between TG and an acyl-acceptor [11, 63]. Carboxylic acids, alcohols, or another ester can be used as acyl-acceptor. Transesterification produces glycerol when alcohol is used as acyl-acceptor or triacylglycerol when ester is used [8, 56, 60, 62, 64]. Transesterification process using a catalyst is called catalytic transesterification process, whereas that without catalyst is called noncatalytic transesterification process [8, 10, 65, 66]. Moreover, catalytic process is divided into two types: homogenous and heterogeneous processes depending on the catalyst used.

Transesterification is a chemical process of transforming large and branched TG into smaller and straight chain molecules, which is similar in size to the molecules of the species present in diesel fuel [67, 68]. Stoichiometrically, for each mole of TG three moles of alcohol are required. But in general, a higher molar ratio of alcohol is used in order to achieve maximum biodiesel production. This molar ratio depends on the type of used feedstock, type of catalyst, temperature, and reaction time. Methanol, ethanol, and propanol are the most commonly used alcohols. In fact, biodiesel yield is independent of the type of the alcohol used and the alcohol selection depends on cost [60]. In transesterification, ester bonds are broken first then followed by hydroxyl bond, whereas in esterification hydroxyl bonds are broken before ester bonds resulting in glycerol as byproduct in transesterification and water in esterification [54].

Transesterification can be carried in a number of ways, using different catalytic processes. For example, it can be carried out using alcohol and alkali catalyst, acid catalyst, and biocatalyst or using alcohols in their supercritical state [39, 69]. Overall, transesterification is a sequence of three reactions; TG is first converted to a diacylglycerol (DG) and one fatty acid ester, then the DG is converted to monoacylglycerol (MG) giving an additional fatty acid ester, and finally the MG is converted to glycerol giving the last fatty acid ester.

Catalyst promotes hydrolysis of the TGs to produce fatty acids and glycerol, with the last being a byproduct. By the end of the transesterification, produced biodiesel and glycerol have to be purified in order to remove the catalyst, which requires a separation step by washing with distilled water for several times. It is well understood that catalyst selection is an important criterion.

2.1.1. Chemical Catalytic Transesterification

Alkaline Catalyst Transesterification. A base catalyst is a chemical with a pH value greater than 7. It has the ability to give extra electrons. Sodium hydroxide (NaOH), potassium hydroxide (KOH), and sodium methoxide (CH_3ONa) are the most common homogeneous base catalysts employed during alkaline transesterification [11, 51, 60]. The base catalyzed process is the most commonly used because of its relative ease. It can be performed at low temperature and pressure and yields high conversion (98%) within a short time [5].

Most important limitation of the base catalysis method is the process sensitivity to both FFA and water contents. It works perfectly when the FFA and moisture contents are less than certain limits, usually below 0.5 wt% for FFA [70, 71]. In case of TGs where FFA contents exceed this limit, pretreatment step is required. The presence of FFA promotes soap formation, which consumes the catalyst, lowers the yield, and more importantly results in difficult downstream byproducts separation and product purification [7, 8]. About 60–90% of biodiesel cost comes from the high cost of the raw material [7]. In addition, alkali catalyst needs effluent treatment.

Most of the base catalyzed reactions were carried out at temperatures close to the alcohol boiling point with alcohol to oil molar ratio of 6 : 1. Akoh et al. [9] stated that to increase biodiesel yield, a stoichiometric excess of substrates (6 : 1 molar ratio of methanol to oil) is favored.

Homogeneous catalysts have been used industrially for biodiesel production where produced biodiesel and glycerol have to be purified to remove the catalyst. This purification process requires large quantities of water and energy. Thus, heterogeneous catalysts have been suggested to overcome this drawback. Heterogeneous catalysts can be separated easily from the system at the end by filtration and could be reused [60, 72]. Alkaline earth oxides [73], zeolites [3], calcined hydrotalcites [18, 74], and Magnesium and Calcium oxides [16, 72] have been suggested as heterogeneous catalysts and showed good results. However, the high cost of the purified feedstock remains the main problem facing the alkaline catalyzed process.

Acid Catalyst Transesterification. The reaction of TGs and alcohol may also be catalyzed with an acid instead of a base. Most commonly used acids are strong acids like sulphuric, sulphonic, phosphoric, and hydrochloric acids [5, 8, 10].

Acid-catalyzed transesterification processes are not as popular as the base-catalyzed processes, mainly because strong acids are corrosive and the processes are too slow. Several reactions may be required in order to achieve high conversion. It has been stated that acid-catalyzed reaction may be 4000 times slower than the base catalyst process [7, 9, 54, 66]. Above that, it requires high amount of alcohol and higher concentration of catalyst. Akoh et al. [9] stated that a molar methanol : oil ratio of 30 : 1 in a range of 55–80°C with 0.5 to 1 mol% catalyst concentration is required to achieve 99% conversion in 50 h. On the other hand, acid-catalyzed processes offer an important advantage for being independent of feedstock FFA content. That is because

feedstock FFA is not converted to soap using this kind of catalysts, and hence biodiesel can be produced from low cost feedstock [6, 54].

As mentioned before, feedstock of high FFA content requires a pretreatment step if a base catalyst is to be used. This pretreatment step can be achieved using acid catalysis and methanol, where the FFA is esterified to biodiesel. When equilibrium is reached, the acid catalyst and produced water are removed from the reaction vessel by centrifugation [11]. This is followed by adding fresh methanol and base catalyst to the oil in order to catalyze the transesterification reaction.

Heterogeneous acid catalysts have been also used. This is important to avoid problems associated with homogeneous catalysts. Sulphated tin oxide has been used as superacid catalysts to transesterified waste cooking oil [22]. Sulphated zirconia was also used as catalysts in the alcoholysis of soybean oil and in the esterification of oleic acid [21]. Heteropolyacid was used to transesterify yellow horn oil [75]. Anion and cation exchange resins were used for triolein transesterification reactions with ethanol to produce ethyl oleate [76].

2.1.2. Noncatalytic Transesterification. Although catalysts play a great role in reducing transesterification time, their presence promotes complications of final product purification. This results in increased production process cost.

To avoid catalyst drawbacks, supercritical alcohol (SCA) transesterification process was suggested [13, 51, 77]. SCA transesterification process is a catalyst free process, which provides high conversion of oil to ester in a short time. Tan et al. [78] compared SCM transesterification with conventional catalytic methods. They reported that conventional catalyst required 1 hr to convert palm oil to biodiesel, whereas SCM required only 20 min. As a result of catalyst absence, purification of the products of the transesterification reaction is much simpler and environmentally friendly compared to the previously mentioned processes.

In 2001, Saka and Kusdiana [30] conducted a research on biodiesel production from vegetable oils without any aid of catalysts. The oil-methanol mixture was heated above the supercritical temperature. Biodiesel was removed from the reaction mixture, and the excess methanol was removed by evaporation for 20 min at a temperature of 90°C. It was reported that 95% conversion was achieved in the first 4 min of reaction with optimum process parameters of alcohol : oil molar ratio of 42 : 1, pressure of 430 bar, and reaction temperature of 350°C. After one year (2002), Demirbaş [77] studied transesterification of six different vegetable oils in supercritical methanol and reported that increasing reaction temperature to supercritical condition had favorable influence on ester conversion.

Compared to catalytic reactions, SCM reactions are fast and can achieve high conversions in a very short time. However, the reaction requires higher temperatures, pressures, and alcohol to oil molar ratio in comparison to catalytic transesterification, which result in high production cost [67, 68].

It is clearly shown that the three transesterification processes presented have several drawbacks. They are energy intensive, recovery of byproduct is difficult, catalysts have to be removed, and waste treatment is required. To overcome these problems, enzymes have been proposed [7, 54, 79]. Most important advantage of using enzymes is their ability to convert FFA contained in the fat or oil to methyl esters completely. Additionally, glycerol, byproduct, can be easily recovered [26, 51, 80].

2.1.3. Enzymatic Transesterification. There is a great interest on using biocatalysts to catalyze TG transformation to biodiesel, which has the advantage of having low operating conditions and high product purity. Enzymatic transesterification can be carried out at 35 to 45°C [41, 42, 81]. Contrary to chemical catalysts, enzymes do not form soaps and catalyze esterification of FFA and TG in one step without any need of the washing step. On the other hand, the major disadvantages of the enzymatic transesterification are its slower reaction rate and possible enzyme inactivation by methanol [27, 62, 82]. Lipase is an enzyme capable of catalyzing methanolysis reactions. It can be obtained from microorganisms such as bacteria and fungi. Lipases from *Mucor miehei*, *Rhizopus oryzae*, *Candida antarctica*, and *Pseudomonas cepacia* are the most commonly used enzymes [39, 62]. Lipases belong to a group of hydrolytic enzymes called hydrolases. In biological systems, lipases hydrolyze TGs to fatty acids and glycerol [66]. They work in mild conditions and have an ability to work with TGs from different origins. They have the ability to catalyze transesterification of both TGs and FFAs to give esters.

Extracellular and intracellular lipases are the major biocatalyst [5, 56]. Extracellular lipases refer to the recovered enzymes from the microorganism which is then purified, whereas intracellular lipases, the enzyme remains inside the producing cell walls [62]. In term of regioselectivity, lipases have been divided into three types [81]:

(i) sn-1,3-specific: hydrolyze ester bonds in positions R_1 or R_3 of TG,

(ii) sn-2-specific: hydrolyze ester bond in position R_2 of TG,

(iii) nonspecific: do not distinguish between positions of ester.

Fjerbaek et al. [39] stated that for biodiesel production from TG, lipases should be nonstereospecific where all TG, DG, and MG can be converted to fatty acids methyl esters (FAME). In addition, they should also be able to catalyze FFA esterification.

Despite the lipases advantages over acid and base catalysts, lipases are costly which limit their industrial use [60, 83]. For that reason, reusability of the enzyme by using it in an immobilized form is essential from economic point of view.

Soluble enzyme acts as a solute in that they are dispersed in the solution and can move freely, but at the same time difficult to separate and to handle. One promising approach to overcome this difficulty is to immobilize the enzyme in a way that can be separated later by any simple separation method. Enzyme immobilization is a technique where free

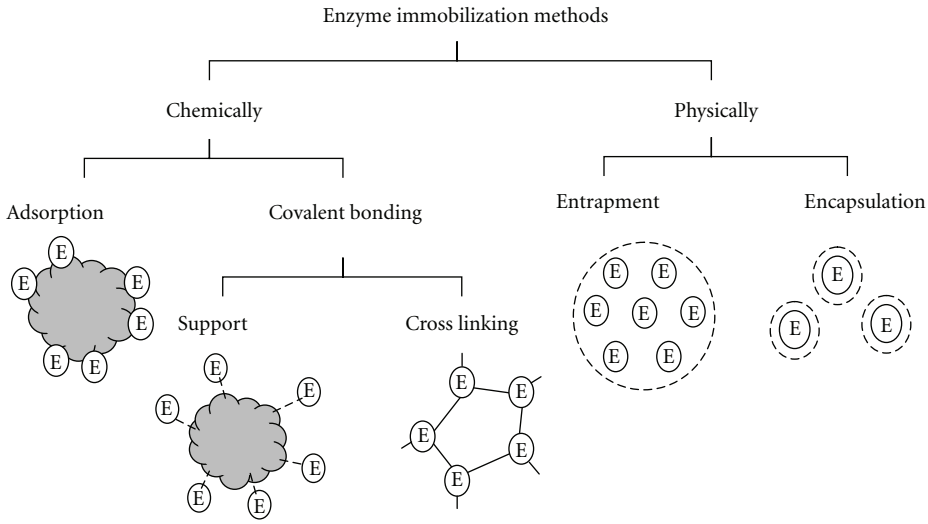

Figure 1: Enzyme immobilization methods.

movement of the enzyme is restricted and localized to an inert support or carrier. This technique has many advantages, the most important of which is that the immobilized enzyme can be reused [40, 84]. In addition, by immobilization, the operating temperature of the process can be increased [39]. Cao [84] mentioned that an immobilized enzyme has to perform two essential functions, namely, the noncatalytic functions that are designed to aid separation and the catalytic functions that are designed to convert the targeting substrates within a desired time. This is in addition to the fact that the process is environmentally friendly and more sustainable [82].

Enzyme immobilization can be carried out in different ways. It can be classified into chemical and physical methods as shown in Figure 1. In biodiesel enzymatic production, various immobilization techniques have been used. Du et al. [24] used adsorption on macroporous resin, Noureddini et al. [27] worked on hydrophobic sol-gel support by entrapment, and Orçaire et al. [28] worked on silica aerogel by encapsulation.

Amongst all possible immobilization methods, physical adsorption has been clearly selected by most researchers due to its ease, the absence of expensive and toxic chemicals, ability to retain the activity, and feasibility of regeneration [54]. But, immobilized enzymes are also subjected to diffusion limitation (internal and external) and inactivation (mostly by methanol) [85]. These problems have been studied and solved by different researchers.

To overcome immobilized lipase inactivation, the addition of an inert solvent has been suggested. However, solvent addition is not highly recommended since this will require using solvent recovery units, which will increase production cost.

Köse and coworkers [86] investigated lipase-catalyzed transesterification of cotton seed oil with methanol in solvent-free medium. Yield of 92% was achieved in the presence of the Novozym 435 in 24 h reaction. This was performed at 50°C, 4 : 1 alcohol to oil and 30% enzyme loading. In 2007, Royon et al. [87] performed comparable work for cotton seed oil using Novozym 435 at the same condition, but with using *tert*-butanol as solvent. They noted that *tert*-butanol dissolved both methanol and glycerol that might inhibit enzyme activity, and a higher conversion of 97% was observed after 24 h of reaction. Similarly, Nelson et al. [33] tested the effect of using solvent on biodiesel production yield. By using *Mucor miehei* lipase, yield of 94% was obtained in *n*-hexane system, whereas only 19% yield obtained in a solvent-free system after 8 h reaction with methanol. On the other hand, ethanol produced 65% yield in solvent-free system and 98% in n-hexane system within first 5 h of the reaction. In the same approach, using 80% *tert*-butanol (based on oil weight) improved biodiesel yield from soybean oil deodorizer distillate with 4% Novozym 435 from 80 to 84%. However, further increase in solvent use decreased the yield, which might result from the dilution effect on reactants [88]. Wang et al. [88] obtained same yield, 84%, when a combination of 2% Novozym 435 and 3% Lipozyme TL were used.

The importance of using solvents has been addressed well in the literature. However, as mentioned earlier solvents can be toxic, flammable, and have to be separated from the ester for reuse. Hence, efforts have been made to offer alternative solvents that are nontoxic and environmentally friendly. Candidate solvents that can replace previously mentioned solvents should have same advantages of dissolving both substrates and reduce excess alcohol inhibition and at the same time avoid the drawbacks of difficult separation of the solvent. In this regard, supercritical fluids (SCF) have been suggested as alternative solvents [89]. Further discussion on the use of SC-CO_2 as a reaction medium is found in Section 4.1.2.

2.2. Biodiesel Feedstocks. Biodiesel can be synthesized from a great variety of feedstocks. These feedstocks include most

vegetable oils (soybean oil, jatropha oil, rapeseed oil, palm oil, sunflower oil, corn oil, peanut oil, canola oil, and cottonseed oil) and animal fats (tallow and lard). They can also be produced from other sources like waste cooking oil, greases, and oleaginous microorganisms with excess microbial lipid such as microalgae [13].

2.2.1. Vegetable Oils. Since vegetable oil is a feedstock that is available in large quantities, it has been widely used for the conversion to biodiesel. Majority of vegetable oils have been employed for biodiesel production such as soybean oil [44, 90], rapeseed oil [30, 80], canola oil [64], palm oil [45, 54, 91], and sunflower oil [92, 93]. However, producing biodiesel from vegetable oils competes with their use as food and involves additional land use. Also, in industrial scale, biodiesel production requires considerable use of arable lands.

2.2.2. Waste Cooking Oils. Fried oils and fats are usually broken down after a period of use and become unsuitable for further cooking as a result of increasing of FFA content. Once this reached, they are discarded or recycled. This type of feedstocks is of low cost, making them attractive for fuel production [46]. Using waste cooking oil, especially those that cannot be treated, will reduce the environment pollution. Waste cooking oil conversion into biodiesel through the transesterification process reduces their molecular weight to approximately one-third, viscosity by about one-seventh, as well as reducing their flash point and volatility [4, 51]. High oil conversion (>90%) has been reported by many investigators [94–96] in spite of the high FFA contents that range from 5 to 15 wt%.

2.2.3. Animal Fat. Animal fats are received from cattle, hog, chicken, lamb, and fish. Tallow and animal meats which are not allowed to be used as food can be used as biodiesel feedstock. However, these two sources have discontinuity problem in their supply. It is possible that suddenly a high bulk of material is available followed by a period with no supply like in the case of animal disease [48]. Animal fats are characterized by the high amount of saturated fatty acids (SFA). They are solid at room temperature and cannot be used as fuel in a diesel engine in their original form [97]. Authors of this paper had investigated possibility of biodiesel production from lamb meat fat [98] and tallow [99, 100] as feedstock.

2.2.4. Oleaginous Microorganisms. As an alternative to vegetable oils and animal fats, oleaginous microorganisms have recently attracted great attention. It has been reported that such microorganisms accumulate oils and have microbial lipid content exceeding 20% [13]. The scope of this paper is on the use of microalgae in biodiesel production.

Using algae as a feedstock has been studied worldwide by several decades. However, for biodiesel production, this was started by an 18-year National Renewable Energy Laboratory (NREL) research project [50]. The potential of using algae for biodiesel production can be seen from their ability to produce large amount of biodiesel and reduce the production cost. Based on algae size, they are classified to macroalgae and microalgae. Macroalgae are large and multicellular, whereas microalgae are small and unicellular. Due to the simple cell structure, microalgae are widely used and have been accepted as promise feedstock. The following section gives more details about microalgae and their potential as feedstock for biodiesel production.

3. Microalgae

Algae that contain chlorophyll are photosynthetic microorganisms that convert inorganic carbon, such as carbon dioxide, in the presence of light, water and nutrients to algal biomass [101–106]. Majority of algae are living in aquatic (saline or freshwater) environments, whereas some of them can be found in other environments such as snow, desert soils, and hot springs [107]. They can be either autotrophic or heterotrophic. Autotrophic algae require only carbon dioxide, light, and salts to grow, whereas heterotrophic require an organic source of carbon, like glucose, as well as nutrients [105, 108–110]. However, heterotrophic algae are not as efficient as autotrophic algae for oil production [49, 111]. Autotrophic is more favorable as it does not require glucose which is a food source and at the same time fixes CO_2, which has positive effect on the environment. Microalgae also can be either phototrophic or chemotrophic. Phototrophic algae use light as an energy source, whereas chemotrophic type use oxidizing compounds [107]. Additionally, some algae are capable of behaving in both autotrophic and heterotrophic modes. These are called mixotrophic algae [104, 112].

Algae range from unicellular to multicellular forms [105]. Some algae are motile while others are nonmotile. Moreover, they may exist as colonies, filaments, or amoeboids [104]. Based on their internal structure, algae cells are generally categorized into eukaryotes and prokaryotes. Prokaryotic cells do not have nuclear membrane-bound DNA, organelles and other membranous structures as eukaryotic cells. As shown in Table 1, almost all the algae are eukaryotes. In eukaryotes, microalgae cells consist of cell wall, plasma membrane, cytoplasm, nucleus, and organelles such as mitochondria, lysosomes, and golgi.

As shown in Table 2, microalgae oil contents are usually between 20–50% of dry algae biomass weight. However, many microalgae oil content may exceed 80% of dry algae biomass weight [49, 101, 133, 134]. Besides, microalgae can grow very fast by doubling biomass in 24 hours, and during exponential growth phase they can double their biomass in about 3.5 hours [49, 111, 134, 135].

Algal species may change their composition, shape, and color based on growing culture and growth condition such as light, nutrients, temperature, and acidity, pH. It is well known that using stressful environment may cause algae to store more oil.

Unlike glycerolipids that are found in membranes under optimal conditions, many microalgae alter towards accumulations of neutral lipids in form TAG [136]. Microalgae

Table 1: Summary of different algal groups classification for different habitat types [113].

Kingdom	Division	Habitat			
		Marine	Freshwater	Terrestrial	Symbiotic
Prokaryota	Cyanophyta	Yes	yes	Yes	yes
	Prochlorophyta	Yes	yes	Not detected	yes
Eukaryota	Glaucophyta	Not detected	yes	Yes	yes
	Rhodophyta	yes	yes	Yes	yes
	Heterokontophyta	yes	yes	Yes	yes
	Haptophyta	yes	yes	Yes	yes
	Cryptophyta	yes	yes	Not detected	yes
	Chlorarachniophyta	yes	Not detected	Not detected	yes
	Dinophyta	yes	yes	Not detected	yes
	Euglenophyta	yes	yes	Yes	yes
	Chlorophyta	yes	yes	Yes	yes

Table 2: Oil content of some common microalgae [49, 114].

Microalgae	Oil content (% dry biomass weight)
Botryococcus braunii	25–80
Chlorella protothecoides	23–30
Chlorella vulgaris	14–40
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella salina	14–20
Neochloris oleoabundans	35–65
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Spirulina maxima	4–9
Tetraselmis suecia	15–23

composition is species specific and varies between different microalgae depending on nutrient, salinity, medium pH., temperature, light intensity, and growth phase. In all cells, lipid and fatty acids are constituents that act as membrane compounds, storage product, metabolites, and energy source. It is known that under stress condition, photosynthesis activity decreases; therefore, lipid synthesis occurs. Most of microalgae-produced oils having fatty acid constitutions similar to most common vegetable oils [137].

In general, lipids may include neutral lipids (nonpolar), polar lipids, wax esters, sterols, and hydrocarbons as well phenyl derivatives [138]. Major part of nonpolar lipids of microalgae is TGs and FFA. Typically, algae lipids have a carbon number range C_{12}–C_{22}. Most of fatty acids found in algae lipids are straight chain with even number of carbon atoms. They may be either saturated or unsaturated [115]. Table 3 gives a summary of the range of lipid reported in different algae species.

Microalgae are classified into four main classes according to their pigment components: diatoms (*Bacillariophyceae*), green algae (*Chlorophyceae*), blue-green algae (*Cyanophyceae*), and golden algae (*Chrysophyceae*) [102, 103, 108, 139]. Table 4 gives a brief description of each division.

Microalgae biomass contains three main components: protein, carbohydrates, and lipids and, therefore, can be used in different applications ranging from food products to biofuels. They are usually used as animal feed [140], human health food [104, 141], and as biofertilizer [49]. Additionally, microalgae can be used for atmospheric CO_2 mitigation. It was reported that there are over 40,000 species of algae [136], but only limited number of these have been studied and have commercial significance [142].

3.1. Potential of Using Microalgae as Feedstock for Biodiesel Production. Biodiesel production from microalgae oil is more promising and sustainable alternative to previously mentioned feedstocks (Section 2.2) [143]. Compared to plants, algae do not compete with food crops and have higher energy yields per area than terrestrial crops. They present a good source of renewable biofuels, which include methane via anaerobic digestion of algal biomass, biodiesel derived from microalgae oil, and biohydrogen via photobioproduction [133, 144, 145]. The focus of this paper is on biodiesel.

Using microalgae as a fuel source is not new; it was suggested more than 60 years ago by Meier for methane production [136]. However, only recently microalgae received noticeable attention due to increasing environmental concerns. Main advantage of using microalgae as feedstock is their rapid growth potential with short biomass doubling time (3.5 hours) during exponential growth and oil content ranging from 20 to 50% dry weight of biomass for numerous microalgae species, as shown in Table 2. Other major advantages and features of using microalgae are the following.

(i) Ability to grow in nonarable land [49, 126, 144, 146, 147] where they can be cultivated on lands that is unsuitable for agriculture, that is, waste land [103, 148]. Therefore, biodiesel production would not be in any way competing with food production [106, 149].

Table 3: Fatty acid composition of lipids of different microalgae [115].

Fatty acid	*Spirulina platensis*	*S. maxima*	*Scenedesmus obliquus*	*C. vulgaris*	*Dunaliella bardawil*
$C_{12:0}$	0.04	traces	0.3	—	—
$C_{14:0}$	0.7	0.3	0.6	0.9	—
$C_{14:1}$	0.2	0.1	0.1	2	—
$C_{15:0}$	traces	traces	—	1.6	—
$C_{16:0}$	45.5	45.1	16.0	20.4	41.7
$C_{16:1}$	9.6	6.8	8.0	5.8	7.3
$C_{16:2}$	1.2	traces	1.0	1.7	—
$C_{16:4}$	—	—	26.0	—	3.7
$C_{17:0}$	0.3	0.2	—	2.5	—
$C_{18:0}$	1.3	1.4	0.3	15.3	2.9
$C_{18:1}$	3.8	1.9	8.0	6.6	8.8
$C_{18:2}$	14.5	14.6	6.0	1.5	15.1
α-$C_{18:3}$	0.3	0.3	28.0	—	20.5
γ-$C_{18:3}$	21.1	20.3	—	—	—
$C_{20:2}$	—	—	—	1.5	—
$C_{20:3}$	0.4	0.8	—	20.8	—
Others	—	—	2.5	19.6	—

(ii) Can be cultivated in saline and brackish environments leading to reduction in fresh water load [111].

(iii) Daily harvesting [49, 126, 150] and short harvesting cycle [106, 111] in comparison to crop plants.

(iv) High photosynthetic efficiency due to their simple structure [111, 147, 148].

(v) Reduction in major greenhouse gas contributor, by utilizing CO_2 from industrial flue gasses [49, 126, 146, 149–151]. Thereby, they are considered as CO_2 fixers [152].

Table 5 compares different sources of biodiesel and their oil yield per area. Chisti [49] mentioned that in order to satisfy with the US demand for transportation fuel, the biodiesel industry would need to produce 530 billion liters annually. As can be found from Table 5, the most feasible biodiesel source for the US is microalgae.

Consequently, biodiesel production from microalgae is considered to be the best efficient feedstock for biodiesel production to displace conventional feedstock's and meet global demand of fuel [153].

To use microalgae for the production of biodiesel, several processes have to be carried out. These consist of strain selection, cultivation, harvesting, extraction of the oil, and production of biodiesel from extracted oil, in which each step can be accomplished with various technologies. These steps are detailed in the following sections.

3.2. Microalgae Oil Production Systems

3.2.1. Microalgal Strain Selection. Microalgae come in a variety of strains; each has different proportions of lipid, protein, and carbohydrates contents. From over 3,000 collected, screened, and characterized algal strains in the National Renewable Energy laboratory (NREL) sponsored project [50], selection of the most suitable strain needs certain parameters evaluation. These parameters include oil content, growth rate and productivity, strain adaptableness, and withstanding to different weather conditions such as temperature, salinity and pH, and high CO_2 sinking capacity and provide valuable coproducts [108]. Thus, right strain selection is critical.

Numerous researches have been carried out on different species tolerance. Many of them were found to be suitable for biodiesel production. As shown in Table 6, some microalgae have high lipids content such as *Nannochloropsis sp.*, and others have high protein contents like *C. protothecoides* (autotrophic) while others have high carbohydrates content like *Oscillatoria limnetica* under normal conditions. Among possible microalgae strains for biodiesel productions are *Chlorella* species. As can be seen from Table 2, *C. protothecoides* oil content is roughly 15% (% dry weight) under control conditions, but this can reach around 44% [154, 155], 53% [156], and 55% [120] when grown heterotrophically.

On the other hand, generally, lower oil strains grow faster than high oil strains. This is due to slow reproduction rate as a result of storing energy as oil not as carbohydrates [157]. In addition, it should be taken into consideration that some microalgae contain high levels of unsaturated fatty acids, which reduce the oxidative stability of the biodiesel produced [158–160].

Rodolfi et al. [126] have screened variety of microalgal strains by evaluating biomass productivity and lipid content in 250-mL flask laboratory cultures (Table 7). Strains that have shown some promise lipid productivity can be further

Table 4: Summary of different microalgae divisions [104, 113, 116].

Division	Examples	Occurrence	Photosynthesis pigments	Reproduction
Diatoms	*Coscinodiscus granii* *Tabellaria* *Amphipleura* *Thalassiosira baltica* *Skeletonoma* *Chaetoceros* *Cyclotella* *Chlorella sp.*	(i) Oceans (ii) Freshwater (iii) Brackish water	(i) Chlorophylls *a* and *c* (ii) *B*-carotene	(i) Vegetative (binary fission or fragmentation) (ii) Asexual (akinete, exospores, endospores or homospores) (iii) Sexual (isogamous, anisogamous or oogamous)
Green algae	*C. vulgaris* *C. protothecoides* *S. obliquus* *Haematococcus pluvialis* *Nannochloris* *D. salina* *B. braunii*	(i) Oceans (ii) Freshwaters (iii) Moist (iv) Terrestrial habitats.	(i) Chlorophylls *a* and *b*	(i) Vegetative (binary fission or fragmentation) (ii) Asexual (akinete or exospores or endospores or homospores) (iii) Sexual (isogamous, anisogamous or oogamous)
Blue-green algae	*S. platensis* *Synechococcus* *Cyanidium* *Oscillatoria* *Anabaena cylindrical*	(i) Freshwater (ii) Marine (iii) Terrestrial (iv) Symbiotic (v) Associations	(i) Divided in to two groups (ii) Most species have chlorophyll *a* as only form of chlorophyll and phycobilins as pigments (iii) Some have two forms of chlorophyll *a* and *b* and lack phycobilins	(i) Vegetative (binary fission and fragmentation) (ii) Asexual (akinete or exospores or endospores and homospores)
Golden algae	*Isochrysis galbana* *Dinobryon balticum* *Uroglena americana*	(i) Fresh water (ii) Marine (iii) Terrestrial	(i) Chlorophylls *a* and *b* (ii) Some have chlorophylls *e* carotene *a* and *c*	(i) Asexual (zoospores or aplanospore, hypnospores) (ii) Sexual (isogamous or anisogamous or oogamous)

improved genetically. Genetic engineering can improve all aspects of algal production, harvesting, and processing for enhanced biodiesel capabilities.

3.2.2. Microalgal Biomass Production. The main way to produce microalgal biomass is the cultivation. For commercial biomass production, microalgal biomass must be easily cultivated in the required scale. Microalgae cultivation can be carried out either via photoautotrophic methods in open systems (open-ponds) [153, 161] or closed systems (photobioreactors) [102, 161, 162], or via heterotrophic methods [120, 154]. All methods have their advantages and disadvantages; therefore, investigators disagree about which of the methods and systems is more favorable. Choosing best

Table 5: Comparison between different biodiesel sources [49].

Crop	Oil yield (L/ha)	Land area needed (M ha)[a]	Percent of existing US cropping area[a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae[b]	136,900	2	1.1
Microalgae[c]	58,700	4.5	2.2

[a] For meeting 50% of all transport fuel needs of the United States.
[b] 70% oil by wt in biomass.
[c] 30% oil by wt in biomass.

Table 6: Chemical composition of various microalgae (% dry weight).

Microalgae	Carbohydrates	Protein	Lipids	Reference (s)
Chaetoceros muelleri	19.3	46.9	33.2	[117]
I. galbana	26.8	47.9	14.5	[118]
Chaetoceros calcitrans	27.4	36.4	15.5	[118]
Isochrysis sp.	12.9	50.8	20.7	[119]
Prymnesiophyte (NT19)	8.4	41.3	14.7	[119]
Rhodomonas (NT15)	6.0	57.2	12	[119]
Cryptomonas (CRF101)	4.4	44.2	21.4	[119]
Chaetoceros (CS256)	13.1	57.3	16.8	[119]
C. protothecoides[a]	10.6	52.6	14.6	[120–122]
C. protothecoides[b]	15.4	10.3	55.2	[120, 121]
Microcystis aeruginosa	11.6	30.8	12.5	[122]
Nannochloropsis sp	29.0	10.7	60.7	[123]
S. obliquus	15	50.0	9.0	[124]
Oscillatoria limnetica	50	44.0	5.0	[124]
B. braunii	18.9	17.8	61.4	[125]
Botryococcus protuberans	16.8	14.2	52.2	[125]

[a] Autotrophic cultivation.
[b] Heterotrophic cultivation.

biomass production method or system depends on the selected algal strain and its integration with appropriate downstream processing which is the means for affordability and scalability of biodiesel production.

Photoautotrophic. As mentioned previously, photoautotrophic microalgae need light and carbon dioxide as energy and carbon sources, respectively. Thus, photoautotrophic algae cultivation is carried out in the presence of light in open ponds and photobioreactors.

Open ponds are the most commonly used systems, and their structure has been well documented. Open ponds are made of a closed loop with recirculation channels. A paddlewheel that continuously operates is usually used to prevent sedimentation and provide mixing. During daylight, the culture is fed continuously in front of the paddlewheel where the flow begins and circulates through the loop to the harvesting point. On completion of the circulation loop, broth is harvested behind the paddlewheel [49, 102, 161, 163]. Inclined, circular, and raceway ponds are operated at large scale. On the other hand, photobioreactors are closed bioreactors, which are designed as tubular, plate, or bubble column reactors. Among these, the most common type is tubular photobioreactors. These consist of less than 0.1 m diameter transparent tubes made from plastic or glass. Tube diameter is a critical design criteria as light does not penetrate too deeply in dense culture broths [49]. This leads to O_2 accumulation and thus inhibits the photosynthesis process.

Typically, open ponds are the preferred large scale cultivation system [49]. This is due to their simplicity and low construction and capital costs [163]. However, these systems are open to the atmosphere, which lead to water

Table 7: Biomass productivity, lipid content, and lipid productivity of 30 microalgal strains cultivated in 250-mL flasks [126].

Algal Group	Microalgae strain	Habitat	Biomass productivity ($g\ l^{-1}\ d^{-1}$)	Lipid content (%)	Lipid productivity ($mg\ l^{-1}\ d^{-1}$)
Diatoms	*Chaetoceros muelleri* F&M-M43	Marine	0.07	33.6	21.8
	C. calcitrans CS 178	Marine	0.04	39.8	17.6
	P. tricornutum F&M-M 40	Marine	0.24	18.7	44.8
	Skeletonoma costatum CS 181	Marine	0.08	21.0	17.4
	Skeletonoma sp. CS 252	Marine	0.09	31.8	27.3
	Thalassiosira pseudonana CS 173	Marine	0.08	20.6	17.4
	Chlorella sp. F&M-M48	Freshwater	0.23	18.7	42.1
	Chlorella sorokiniana IAM-212	Freshwater	0.23	19.3	44.7
	C. vulgaris CCAP 211/11b	Freshwater	0.17	19.2	32.6
	C. vulgaris F&M-M49	Freshwater	0.20	18.4	36.9
Green algae	*Chlorococcum* sp. UMACC 112	Freshwater	0.28	19.3	53.7
	Scenedesmus quadricauda	Freshwater	0.19	18.4	35.1
	Scenedesmus F&M-M19	Freshwater	0.21	19.6	40.8
	Scenedesmus sp. DM	Freshwater	0.26	21.1	53.9
	Tetraselmis. suecica F&M-M33	Marine	0.32	8.5	27.0
	Tetraselmis sp. F&M-M34	Marine	0.30	14.7	43.4
	T. suecica F&M-M35	Marine	0.28	12.9	36.4
	Ellipsoidion sp. F&M-M31	Marine	0.17	27.4	47.3
	Monodus subterraneus UTEX 151	Freshwater	0.19	16.1	30.4
	Nannochloropsis sp. CS 246	Marine	0.17	29.2	49.7
Eustigmatophytes	*Nannochloropsis* sp. F&M-M26	Marine	0.21	29.6	61.0
	Nannochloropsis sp. F&M-M27	Marine	0.20	24.4	48.2
	Nannochloropsis sp. F&M-M24	Marine	0.18	30.9	54.8
	Nannochloropsis sp. F&M-M29	Marine	0.17	21.6	37.6
	Nannochloropsis sp. F&M-M28	Marine	0.17	35.7	60.9
	Isochrysis sp. (T-ISO) CS 177	Marine	0.17	22.4	37.7
	Isochrysis sp. F&M-M37	Marine	0.14	27.4	37.8
Prymnesiophytes	*Pavlova salina* CS 49	Marine	0.16	30.9	49.4
	Pavlova lutheri CS 182	Marine	0.14	35.5	50.2
Red algae	*Porphyridium cruentum*	Marine	0.37	9.5	34.8

evaporation and unwanted species contaminations. Besides, cell's poor utilization of light and low mass productivity, due to the low CO_2 deficiencies and inefficient mixing, are other limitations [151, 164]. Therefore, for water, energy, and chemicals saving purposes, photobioreactors have been proposed, but they are not yet commercialized.

Main advantages of using photobioreactors are better algal culture and environment controlling [163], large surface to volume ratio, less water evaporation, better isolation from outside contaminations, and higher mass productivity [158]. However, photobioreactors are usually made of plastic, and UV deterioration of plastic surface is the main disadvantage. In addition, biofilm formation will require periodic cleaning [165]. Table 8 shows a comparison between the two photoautotrophic cultivation methods.

For a cost-effective cultivation, a combination of the two previous mentioned systems, referred to as hybrid system, is the most logical choice [153]. In this type of systems, microalgal strain with high oil content is grown in photobioreactors in nutrient and CO_2-rich conditions firstly to promote rapid reproduction; then the microalgae enter an open system with limited nutrient to encourage oil production [166]. This process has been successfully verified by Huntley and Redalje [167].

In addition to microalgae strains influence on oil accumulation, cultivation parameters like temperature, light intensity, pH, water salinity, and nitrogen sources also influence oil production. It has been reported that the lipid content in various microalgae strains from *Chlorella* species increased when growing in low-nitrogen media compared to nitrogen-rich media [168, 169]. However, in these low-nitrogen media, a reduction in growth rate was reported. Similar results were also found by Widjaja et al. [170]. Since the cell needs sufficient nitrogen for growth, the cell

Table 8: Comparison between open ponds and photobioreactors.

Method	Advantages	Limitations
Open ponds	(i) Simple (ii) Cheap (iii) Easy to operate and maintain (iv) Low capital cost	(i) Poor light utilization (ii) Difficulties in light and temperature controlling (iii) Water evaporation (iv) Foreign species contaminations (v) Lower mass productivity
Photobioreactors	(i) High surface to volume ratio (ii) Higher mass productivity (iii) Less contaminations (iv) Less water losses (v) Better light utilization	(i) Scalability problem (ii) Costly (iii) Complex (iv) Cells damage cases (v) Biofilm formation

Table 9: Lipid content, biomass, and lipid productivities of *C. vulgaris* grown autotrophically and heterotrophically on different carbon sources.

	Heterotrophic cultivation			Autotrophic cultivation
	Acetate	Glucose	Glycerol	
Biomass productivity (mg l^{-1} d^{-1})	87	151	102	10
Lipid content (%)	31	23	22	38
Lipid productivity (mg L^{-1} d^{-1})	27	35	22	4

production and division may reduce in the low-nitrogen media. However, carbon metabolism continues leading to utilize more energy for oil production rather than biomass growth [50, 171].

Other factors like CO_2, light intensity, and temperature also significantly affect microalgae lipid content and composition. Renaud et al. [119] investigated the effect of temperature within the range of 25 to 35°C on *Rhodomonas* sp., *Chaetoceros* sp., *Cryptomonas* sp., and *Isochrysis* sp. growth rate and lipid content. Their results showed that optimum growth temperature was 25–27°C for *Rhodomonas* sp., and 27–30°C for *Cryptomonas* sp., *Chaetoceros* sp., and *Isochrysis* sp. Only *Chaetoceros* sp. was able to grow at 33 and 35°C.

With the intent of providing sufficient light to the cultivation systems, open ponds are usually made shallow, and tabular reactors are made small in diameters. Tang et al. [172] studied the influence of the above mentioned parameters on *Dunaliella tertiolecta* growth, lipid content, and fatty acid composition. It was reported that increasing light intensity increases cell growth rate regardless of the light source. On the other hand, as for the CO_2 effect, the highest growth rate was found when CO_2 concentration was in the range of 2 to 6%.

Heterotrophic Cultivation. Unlike photoautotrophic microalgae, heterotrophic species are cultivated in a dark environment by utilizing organic carbon as carbon and energy sources [109, 173]. Heterotrophic cultivation method depends on the microalgae ability to eliminate light requirement and assimilate organic carbon [174]. This solve light limitation problem that appears with photoautotrophic cultivation methods. However, not all microalgae are able to assimilate the organic carbon. Thus, this cultivation method has been studied in a limited number of microalgae species [120, 154, 175]. It has been reported that heterotrophic cultivation provides high oil content and high biomass productivity [120, 121, 154, 176, 177]. Liu et al. [178] compared lipid content of *Chlorella zofingiensis* cultivated under heterotrophic and photoautotrophic conditions. Lipid content of 51 wt% and 26 wt% were obtained, respectively. Liang et al. [174] compared *C. vulgaris* cell growth rate and lipid productivity under autotrophic and heterotrophic conditions, evaluated glucose, acetate, and glycerol carbon sources uptakes. Table 9 illustrates the results obtained.

3.2.3. Harvesting Technologies. After algal cultivation, biomass needs to be separated from the culture medium using one or more solid-liquid separation steps. Due to the microalgae small size (3–30 μm) [102, 179, 180] and cultures medium dilution (less than 1 g L^{-1}), microalgae need to be concentrated to simplify the lipid extraction step. Biomass recovery is difficult [181] and require dewatering using suitable harvesting method [49, 151, 182].

Usually, microalgae are harvested by centrifugation, filtration, or sedimentation. Sometimes these require a pretreatment, flocculation step to improve recovery efficiency [183, 184]. Table 10 summarizes the advantages and disadvantages of each method.

To conserve energy and reduce costs, algae are often harvested in a two-step process. In the first, algae are concentrated by flocculation where diluted culture is concentrated to about 2–7% total suspended solids [108, 183]. In the second step, cells are further concentrated using conventional

Table 10: Advantages and disadvantages of different microalgal harvesting methods.

Method	Advantage	Disadvantage
Flocculation	(i) High recovery yield (up to 22 TTS) (ii) Low energy requirement	(i) Flocculants may be expensive (ii) Contamination issue may occur (iii) Marine environment high salinity may inhibit the process (iv) Long process period
Centrifugation	(i) Reliable (ii) Corrosion resistance (iii) Easy cleaning (iv) Rapid	(i) Energy intensive (ii) Expensive (iii) High speed may deteriorate the cell (iv) Cannot be used for species $<30\,\mu$m
Filtration	(i) Reliable (ii) Able to collect species of low density	(i) Filters may need to be replaced periodically (ii) Membrane blockage (iii) High maintenance cost (iv) May be slow (v) Head loss

harvesting methods to get an algae paste of 15–25% total suspended solids [183]. Algae harvesting cost can be high due to their low mass fraction and algal cell negative charge [182]. It is reported that microalgal cell recovery accounts for at least 20–30% of total biomass production cost [184, 185]. Harvesting technique selection depends on microalgal cells size and density, biomass concentration, culture conditions, and value of target product [102, 186].

Flocculation. Flocculation is a process that collects dispersed cells into aggregate to form large particles that facilitate cell broth separation by addition of chemical additives (flocculants). It is considered as pretreatment stage preceding the main harvesting process [184]. The main problems facing the flocculation step are the high cost and toxicity of the flocculent [187].

To endorse flocculation, chemical additives that bind algae or affect interaction between algae have to be used. There are two main types of flocculants inorganic and organic polymer. A large number of chemicals have been tested as flocculants for microalgal flocculation where the most effective one was aluminum sulfate and certain cationic polymers [187]. Numerous reports have been published concerning the flocculation of algal biomass. Among them is the work of Tenney et al. [188] which looked into fresh water microalgae flocculation using organic polyelectrolytes where extent of microalgal flocculation was determined. Cationic polyelectrolyte polyamine was found to flocculate the algae successfully at an optimum dose of 2.5 mg/L.

Filtration. Filtration separation method makes use of a permeable medium that has an ability to retain the biomass and allows the liquid to pass through. Surface and depth filtration systems are the two known types of filtration. In surface filtration, solids are deposited on the filter medium whereas in the depth type solids are deposited within the filter medium [189]. This is satisfactory for recovering large microalgae and not for algae that size approach bacterial dimension.

Sedimentation. Sedimentation is a technique that separates microalgae biomass suspension into a concentrated slurry and clear liquid based on gravity action and particle diameter. If the biomass to be separated is small in size, settling rate will be low, and flocculants addition will be inevitable. This is a low cost process; however, its reliability is low.

Centrifugation. Almost all types of microalgae can be separated from the culture medium by centrifugation. A centrifuge is mainly a sedimentation tank with an enhanced gravitational force, by centrifugation, that increases the rate of sedimentation. Biomass recovery depends on biomass residence time within centrifugal field, settling rate, and distance [184]. Centrifugal recovery can be rapid, but it is energy intensive. Nevertheless, this process is a preferred method of recovering algal cells [183, 184]. Currently, there is no low cost harvesting method for all strains. Table 10 summarizes advantages and disadvantages of each harvesting method.

3.2.4. Drying. Following harvesting of the algal biomass, algal slurry moisture content has to be reduced to at least 10% by drying and dehydration. Numerous methods have been employed for drying. Most common methods are sun drying, spray drying, drum drying, and freeze drying. Again,

best drying method selection depends on required operation scale and desired product value.

In biodiesel production, lipid-rich feedstock with low water content is required; therefore, microalgae drying has to be carried out. However, drying step is energy intensive, which adds to the cost complexity of the overall production process. Various drying systems differ in both energy and cost requirements.

Sun drying is an old and cheap drying method that can be performed easily by exposure to a solar radiation source. However, it takes long drying time, requires large drying surface area, and might result in loss of products. In addition, sun drying, unlike drum drying, does not have any sterilization effect of the dried sample. On the other hand, spray drying is a method that can be used for high value products, but it has the disadvantage of being expensive and might cause significant deterioration of algae [184]. In contrast, freeze drying has been commonly used by many investigators. Freeze drying has the advantage of breaking up species cells and turning them into fine powder that makes homogenization unnecessary [190]. Belarbi et al. [191] reported that freeze-dried sample can be subjected easily for oil extraction without cell disruption. Freeze driers have been used in algae lipid extraction to extract lipid from *I. galbana* [192], *P. tricornutum* [193], *C. vulgaris* [194], *S. platensis* [195], and *Chlorella sp*. [196]. Table 11 summarizes advantages and disadvantages of each technique. However, freeze drying is a slow process and requires very high capital investment.

3.2.5. Oil Extraction. As stated previously, effective extraction requires concentrated algae solution. Thus, a high degree of algae concentration which takes place in the harvesting step is necessary before biomass lipid extraction. Typically, there are four well-known methods to extract oil from microalgae: (1) expeller/press, (2) solvent extraction with hexane, (3) subcritical water extraction, and (4) supercritical fluid extraction.

The recovering of intracellular products like oils from microalgae is usually difficult due to the cell wall robust structure [151, 197]. Therefore, prior to lipid extraction, algae cell has to be disrupted to a degree that facilitates extraction step [184, 198]. Several methods can be used to disrupt cell membrane. They include homogenizer, bed mill, ultrasound, autoclaving, freezing, and osmotic shock [151]. Among them, homogenizers and bed mills are often preferred because of their short residence time and lower operating costs [175]. Chisti and Moo-young [197] reviewed microbial cell disruption for intracellular products.

Mechanical Extraction. Mechanical oil extraction includes expeller press and ultrasonic extraction. In pressing technique, the presser crushes and pushes the oil out of the dry microalgal biomass. Despite expeller simplicity and lower investment cost, low oil recovery yield, high power consumption, and maintenance cost are the limitations [199].

In ultrasonic technique, shock waves break the walls and release oil to solvent. These waves are created when bubbles (created by ultrasonic wave associated from ultrasonic reactor) collapse near cell wall. Ultrasonic extraction has an advantage of being fast and efficient; at the same time it needs large amount of solvent, especially in case of low sample concentration. That's because at low concentrations, samples need to be extracted more than once using new fresh solvent [200].

Chemical Extraction. The well-known concept of "like dissolve like" is the basic of the Bligh and Dyer [201] solvent extraction method. This method is the most widely used for extracting lipids from microalgae, wherein hexane is one of the most widely used solvent due to its high extraction capability and low cost.

For successful lipid extraction using an organic solvent, the solvent must be able to penetrate through the matrix to contact and dissolve the lipid. When hexane is used as a solvent, it is mixed with the algal biomass and is then separated by filtration. The solvent has to be separated from the extracted oil using distillation which is energy intensive. Miao and Wu [120] reported that large amount of microalgal oil was efficiently extracted from *C. protothecoides* using *n*-hexane. Beside, cosolvent combinations have been used by many other investigators [192, 193, 202, 203]. Hexane/ethanol and hexane/isopropanol cosolvents have been commonly used in microalgal lipid extraction. The polar solvent, which is the alcohol, is first added to disrupt the algal cell membrane. This will enhance the ability of the hexane to extract almost all the lipids. The cosolvent is then removed by liquid-liquid extraction with water. The hexane solvent extraction method can also be used in combination with the oil press/expeller mechanical method. After extracting the oil from the algae using the expeller, the remaining pulp is then mixed with hexane in order to remove any remaining oil. In this combined method, more than 95% of the total oil present in the algae is extracted [204].

The selection of lipid extraction methods depends on the extraction efficiency. Therefore, a method of high performance, such as chemical extraction, is favored over the less efficient methods, such as mechanical extraction, despite the organic solvents negative environmental impacts.

To avoid the environmental impacts of using organic solvent, nontoxic solvents have been suggested, such as subcritical water (SCW) and SC-CO_2. The SCW extraction operates at temperatures just below the critical temperature, 374°C, and at high pressure, usually from 10 to 60 bar, that maintains the water in liquid form. At these conditions, water becomes less polar, and lipids can be solubilized easily. Additionally, using water at subcritical condition can eliminate the dewatering step, and high-quality product within short extraction time can be achieved [205]. However, reaching the above mentioned temperature requires large energy consumption.

On the other hand, supercritical fluids extraction makes use of fluid's salvation power enhancement when reached

Table 11: Comparison between common four microalgae drying methods.

Method	Advantages	Disadvantage
Sun drying	(i) Cheap (no running cost, low capital cost)	(i) Difficult (ii) Slow (iii) Weather dependent (iv) Require large surface (v) Contamination
Spray drying	(i) Fast (ii) Continuous (iii) Efficient	(i) Cost intensive (ii) Species deterioration (i.e. pigments)
Drum drying	(i) Fast (ii) Efficient (iii) Sterilization advantage	(i) Cost intensive
Freeze drying	(i) Gentle	(i) Slow process (ii) Cost intensive

above their critical point. Due to supercritical carbon dioxide's preferred critical properties, low toxicity, biodegradability, and availability, it has been used to extract many desired compounds from solid matrix. Other attractive point of using SC-CO_2 as extraction solvent is that after extraction, solvent and product can easily be separated once the temperature and pressure are lowered to atmospheric conditions.

3.3. Microalgae Oil Production Costs. The idea of producing biodiesel from microalgae was the main focus of NREL project [50]. It is not much different from other biodiesel produced from vegetable oils, animal fats, or waste cooking oils. It was reported that biodiesel from vegetable oil and waste grease roughly costs \$ 0.54 to \$ 0.62/L and \$ 0.34 to \$ 0.42/L, respectively [206]. Chisti [49] reported that biodiesel from palm oil almost costs \$ 0.66/L and in year 2006 petrodiesel average price was \$ 0.49/L, which added about \$ 0.14 to palm oil cost and 35% more than petrodiesel price. The objective is therefore to reduce the product cost to at least \$ 0.48/L ignoring the effect of tax on biodiesel. However, the estimated cost of biodiesel increases to \$ 0.72–1.4/L using microalgae with 70 wt% and 30 wt% (per dry-weight) oil content, respectively [49].

The high cost of biodiesel comes mainly from the high feedstock cost; 60–90% of biodiesel cost is estimated to be from the cost of the feedstock [7]. Therefore, looking for alternatives that are cheap became essential. From that point, microalgal oil production should be enhanced. Microalgae growth requires light, CO_2, water, and salt utilization. To minimize production cost, oil production must rely on maximum available mentioned requirements. Therefore, using water from waste water treatment units that contain required growth nutrients and salts and diverting CO_2 from power plants is desirable and beneficial. Other attributers are development of low harvesting process by genetic engineering, improvements in photobioreactors design, and coproduction of other high values products from residual biomass after lipid extraction [184].

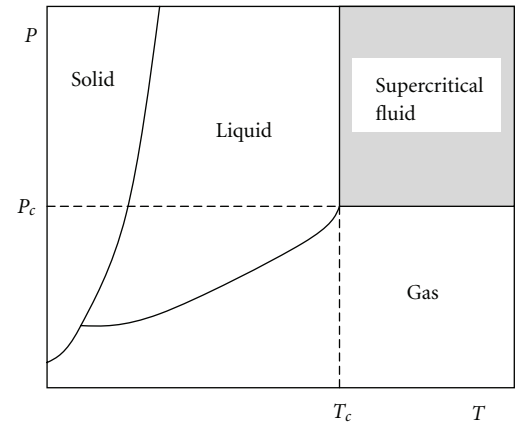

Figure 2: Pure component phase diagram.

4. Supercritical Fluids

Supercritical fluids (SCFs) are fluids at pressures and temperatures above their critical values. Critical values represent the highest temperature and pressure at which the substance exists as a vapor and liquid in equilibrium. This can be simply clarified from supercritical fluids phase diagram (Figure 2).

As shown in Figure 2, there are three single phases, solid, liquid, and gas, where a substance may occur. If a mixture of two, or more, phases exists in these regions, a separation between the phases is distinct, as a result of the difference in properties of the different phases. In Figure 2, the solid curves between phases indicate the coexistence of two phases. On the other hand, at a point beyond the critical point neither fusion, as a result of pressure increase, nor vaporization, as a result of temperature increase, will take place, which was defined earlier as a supercritical region. Table 12 presents the values of the critical temperatures and pressures of selected fluids most commonly used as extraction solvents [127].

In the SCF state, a solvent displays properties which are intermediate to those of liquid and gaseous states; SCFs have more desirable transport properties than liquids and better solvent properties than gases. The liquid-like density of a SCF gives high solvation power and facilitates solubility while the

Table 12: Critical properties of common solvents [127].

Fluid	Critical temperature (°C)	Critical pressure (bar)
Xenon	16.7	59.2
Carbon dioxide	31.1	72.8
Ethane	32.4	49.5
Nitrous oxide	36.6	73.4
Chlorodifluoromethane	96.3	50.3
Ammonia	132.4	115.0
Methanol	240.1	82.0
Water	374.4	224.1

gas-like diffusivity gives excellent transport properties, which increases the rates of transfer from the substrate matrix to the SCF solvent as compared to that of liquid organic solvents [207]. Moreover, the low viscosity of SCFs which is close to that of the gases is an additional advantage. This last property gives rapid solvent penetration into a solid matrix [208].

4.1. Supercritical Carbon Dioxide as a Candidate Solvent. The ability of supercritical carbon dioxide, SC-CO_2, to extract a solute depends on the compounds functional groups, molecular weight, and polarity. Near to its critical point, CO_2 is a good solvent for nonpolar to slightly polar solutes with low molecular weight. It is an inert at most conditions, inexpensive, nontoxic, and environmentally friendly [43, 209]. Moreover, when using SC-CO_2 as a solvent, no solvent residue remains in the extract since CO_2 is in a gas phase at the ambient conditions. The critical temperature and critical pressure of CO_2 are 31.1°C and 72.8 bar, respectively, which are not extremely high. SC-CO_2 has been identified as a good alternative solvent for a number of applications including separation and reaction.

4.1.1. SC-CO_2: Extraction Solvent. Extraction is the process of removal of a solute from a matrix using a solvent which is able to dissolve the desired solute. This involves contacting the matrix with the solvent either in a single stage or in multiple stages for certain period of time and then separating the solvent. During extraction period, the solute transfers from the matrix to the solvent. Required time to achieve successful extraction depends on the solubility. That depends on extraction temperature, contact area between the solute and solvent, solvent viscosity, and solvent flow rate.

Other conventional solvent extraction techniques suffer from several drawbacks such as long extraction time and high solvent consumption, in addition to being labor intensive, difficult to automate, and often require a postextraction cleanup [210]. With these drawbacks, supercritical fluid extraction, SFE, has been proposed using the extraction solvent in its supercritical state.

SCFs were first observed more than a century ago in 1822. However, it has been developed as a novel separation technique only in the past two decades. From an economical point of view and in order not to thermally alter the properties of the extracted materials, SCFs are mostly used as in the approximate range of temperature up to 1.2 times the critical temperature, T_c, and pressure up to 3.5 times the critical pressure P_c [211].

Although a number of substances could serve as solvents, CO_2 is the most common. SC-CO_2 has many applications, especially in food processing, which include decaffeination of coffee and tea, production of hops extracts, flavors extract from herbs, and extraction of edible oils. Friedrich and Pryde [212] extracted oil from soybeans using SC-CO_2 and achieved a yield almost to that using *n*-hexane. In order to extract polar compounds from a matrix, polar supercritical fluid should be used. Thus, using a nonpolar solvent, CO_2 sometimes faces difficulties to extract certain compounds from a sample matrix. To overcome this limitation, modifier fluids can be used to increase extraction efficiency. Among all modifiers tested, methanol was the most commonly used by investigators such as Tonthubthimthong et al. [213] who extracted nimbin from neem seeds. Brewer et al. [214] who extracted cocaine from human hair and Aghel et al. [215] who extracted pennyroyal essential oil using SC-CO_2.

Due to the attractive features of SC-CO_2, it has been used and assessed to extract lipids from different strains of microalgae [128–132, 216]. Maximum yields of 13, 9, 25, 8, 6, and 3% have been reported from *C. vulgaris* [128, 132], *C. cohnii* [216], *Nannochloropsis* sp. [129], *S. platensis* [130], *chlorococum* sp. [131], and *S. maxima* [132], respectively. The lipids were extracted from dried biomass in a temperature range of 40°C–80°C and pressure range of 100–550 bars. The lower extract yield was due to the low lipid content of grown biomass.

The extraction efficiency of SC-CO_2 was compared to conventional solvent extraction methods. Table 13 shows the extraction yields of lipids, defined as amount of extracted lipids per dry biomass weight, extracted from different strains of microalgae using SC-CO_2, as compared to that of conventional solvent extraction. As shown, similar yields were reported when using SC-CO_2 and *n*-hexane for extracting lipids from *S. platensis* [130] and *S. maxima* [132]. However, 25–40% lower yields were reported when comparing SC-CO_2 to *n*-hexane and acetone extractions, from *C. vulgaris* [128]. A lower yield was also reported when comparing SC-CO_2 and ethanol extractions from *S. maxima* [132]. However, other studies showed a better performance than *n*-hexane from *chlorococum* sp. and *Nannochloropsis* sp. [129, 131].

To further enhance the SC-CO_2 extraction yields, the use of a cosolvent has been suggested. SC-CO_2 with 10% ethanol as a cosolvent for lipid extraction from *S. maxima* has been reported [217]. By doing so, the extraction yield increased by 24% from 32% to reach 40%. This enhancement was explained by ethanol destruct effect on microalgal cellular walls.

4.1.2. SC-CO_2: Reaction Media. Majority of chemical processes are carried out in organic solvents, which in most cases are toxic and flammable. Furthermore, these organic solvents need to be separated from the desired product and recycled

Table 13: Comparison of SC-CO_2 performance and other conventional extraction solvents on lipids extraction yields from microalgae biomass.

Microalgae species	SC-CO_2	Other conventional solvents			Reference
		Acetone	Ethanol	*n*-Hexane	
C. vulgaris	13.3	16.8	—	18.5	[128]
Nannochloropsis sp	25	—	—	23	[129]
S. platensis	7.8	—	—	7.7	[130]
chlorococum sp	5.8	—	—	3.2	[131]
S. maxima	2.5	4.7	5.7	2.6	[132]

back. To avoid these drawbacks, SCFs are suggested as an alternative.

As mentioned earlier, SCFs have gas-like diffusivities and low viscosities, which reduce mass resistance between reaction mixture and the catalyst and therefore result in an increase of reaction rate. Among possible solvents that can be used in supercritical conditions to conduct transesterification reactions, CO_2 was chosen due to its low critical temperature which make the process less energy intensive and more importantly below the denaturation temperature of the biocatalyst.

Kumar et al. [41] esterified palmitic acid with ethanol in temperature range of 35 to 70°C in the presence of three different lipases in SC-CO_2. Their results showed that Novozym 435 was the best catalyst. In SC-CO_2, Lipolase 100T and hog pancreas lipase showed similar results. Yields of 74, 44, and 40% were reached using Novozym 435, Lipolase 100T, and hog pancreas lipase, respectively, which were comparable to yield in solvent free system. Romero et al. [89] esterified isoamyl alcohol in SC-CO_2 and *n*-hexane. They noted that similar esterification degree was obtained in both SC-CO_2 and *n*-hexane systems; however, initial reaction rate was higher in SC-CO_2. Laudani et al. [218] compared FFA esterification with 1-octanol over immobilized lipase from *R. miehei* (Lipozyme RM IM) using three different reaction media: SC-CO_2, *n*-hexane, and solvent free systems. SC-CO_2 showed the highest conversion followed by *n*-hexane then solvent-free system.

Although SC-CO_2 has been used as a reaction media for enzyme esterification of FFA, limited work has been done on transesterification. D. Oliveira and J. V. Oliveira [219] compared enzymatic alcoholysis of palm kernel oil using *n*-hexane and SC-CO_2 systems. In SC-CO_2, highest conversion of 63% was obtained using Novozym 435 as catalyst whereas in *n*-hexane Lipozyme IM provided the highest conversion of 77%. Rathore and Madras [61] produced biodiesel from Jatropha oil with Novozym 435 in SC-CO_2. Optimum conditions were found to be 45°C, alcohol : oil molar ratio of 5 : 1, 30% enzyme loading, and 8 h with conversions of 60–70%. Varma and Madras [220] produced biodiesel from caster and linseed oils with Novozym 435 in SC-CO_2, and 45% yield in methanol and 35% in ethanol were obtained from linseed oil, whereas a very low yield of less than 10% was obtained from castor oil. Varma et al. [221] synthesized biodiesel from mustard and sesame oils using different acyl acceptors at 50°C for 24 h reaction. Their results showed that using mustard oil, conversion of roughly 70% and 65% can be obtained using methanol and ethanol, respectively. On the other hand, using sesame oil, a conversion of round 55% was obtained from ethanol, whereas only 45% conversion was obtained with methanol.

Despite the advantages of using SC-CO_2 as a reaction media for the enzymatic production of biodiesel, it has not been reported in any previous work on microalgae oil.

5. Microalgae for Biodiesel Enzymatic Production Using SC-CO_2

5.1. Microalgae as a Feedstock in Conventional Process. Recently, feasibility of using microalgae to produce biodiesel, as an alternative to fossil fuels, received significant attention since they are rich in lipids. Species like *C. vulgaris*, *C. emersonii*, *Nannochloropsis* sp., *P. tricornutum*, and *T. suecica* have been reported in the literature for biodiesel production, where most of them were cultivated using glucose as a carbon source. However, glucose can be fermented directly to produce bioethanol.

Conventionally, microalgae have been used for biodiesel production using chemical catalytic reactions. Miao and Wu [120] studied biodiesel production from heterotrophic cultivated microalgae oil from *C. protothecoides* by 100% H_2SO_4 (based on oil weight) acidic transesterification. Biodiesel optimum conversion yield of 63% was obtained with 56 : 1 methanol : oil molar ratio at 30°C in 4 h reaction time. To overcome disadvantages of homogeneous catalysts, Carrero et al. [222] tested the ability of using hierarchical zeolites as heterogeneous catalyst.

With the target to reduce biodiesel production cost associated with oil extraction cost, *in-situ* transesterification, which is a direct conversion without solvent extraction, of the biomass oil to biodiesel has been performed [223, 224]. A conversion of 91% was achieved after 8 h of reaction at 60°C from Chlorella sp. [223] and 39, 40, 77, 78, and 82% were obtained from *Synechocystis* sp. PCC 6803 *Synechococcus elongates*, *Chlorella sorokiniana*, *T. suecica*, and *Chaetoceros gracilis* at 80°C [224]. The high conversion obtained with *Chlorella* species could be due to the use of a stirring

reactor that enhanced the mixing and reduced mass transfer resistances.

5.2. Enzymatic Biodiesel Production from Microalgae. Similar to conventional feedstocks conversion, microalgae oil can also be converted to biodiesel using lipase. In this perspective, *C. protothecoides* is the only species that has been tested so far. Xiong et al. [225] produced biodiesel with 98% conversion from *C. protothecoides* with 30 wt% of *Candida* sp. lipase. Reaction conditions were 3 : 1 methanol : oil molar ratio, 10% water content, 38°C, pH of 7, and 12 h reaction. Similar conversion was obtained by Li et al. [154] at similar conditions but using 75 wt% of the immobilized lipase rather than 30 wt%. *C. protothecoides* was cultivated heterotrophically in both studies using glucose.

5.3. Enzymatic Production with SC-CO_2 Reaction Medium. To overcome the lipase inhibition limitations, mainly by methanol and glycerol, chemical solvents that can dissolve both methanol and glycerol have the advantage of increasing conversion yield. However, the use of organic solvents is not recommended due to its harmful environmental input and the solvent extraction unit.

Using SC-CO_2 as a reaction media adds to the advantages of organic solvents in saving downstream processing cost where product purification is not necessary. Since solubility is greatly influenced by fluid temperature and pressure adjustments, separation can be easily achieved by a pressure reduction where the product and enzyme do not dissolve at room temperature.

Due to its advantages over conventional organic solvents, the application of the high cost SC-CO_2 process may be justified in oil extraction from microalgae. However, its justification for biodiesel production may not be evident, despite its positive effect on reducing inhibition effects and easy product separation. Nevertheless, a combined continuous process of extracting oil from microalgae using SC-CO_2 and the use of the extracted oil for biodiesel production using immobilized lipase in SC-CO_2 in a one integrated system would economically be feasible. In this continuous process, the oil that is extracted from microalgae is already dissolved in SC-CO_2 and can be fed directly to the enzymatic bioreactor to produce biodiesel without the need for further expensive pumping. In this way, the attractive advantages of performing the reaction in SC-CO_2 media will be gained, avoiding at the same time the disadvantage of high pumping cost. Besides, using high pressure CO_2 might not have significant negative effect of lipase stability. Lanza et al. [226] investigated the influence of SC-CO_2 pressure on lipase activity and reported that the residual activity of Novozym-435 was approximately 90%. Previous study of D. Oliveira and J. V. Oliveira [219] on converting palm kernel oil to biodiesel using Novozym 435 showed that the rise in pressure in the range 60–90 bar actually results in an enhancement of initial reaction rate and conversion. However, at pressures beyond but 200 bar, a change in lipase structure may occur, which has a negative effect on the reaction. Therefore, the application of SC-CO_2 in the enzymatic reaction system should not exceed 200 bar.

6. Conclusions

As verified in this paper, biodiesel produced from microalgae can realistically satisfy the global demand of diesel-fuel requirements. However, for cost effective production, this will not be applicable without microalgae biology and production processes enhancements. The potential of microalgal biodiesel production depends on selected microalgal strain and its ability to live in saline or use wastewaters and utilize CO_2 as a sole carbon source. In addition, biomass recovery that usually requires high energy and oil extraction has to be optimized for effective low cost overall process production. Another important point to be taken into consideration is the ability to use spent biomass, after oil extraction, for the production of other valuable coproducts such as animal feed or fertilizers.

The paper presents SC-CO_2 as a promising oil extraction technique from microalgae, and lipase as a biocatalyst for biodiesel production instead of the conventional chemical catalysts that require feed purification. The use of SC-CO_2 as a reaction media for the enzymatic production of biodiesel has also been discussed in the paper. Authors of this review, suggest future work to be done on designing an integrated SC-CO_2 extraction/reaction process, whereby a stream of extracted oil-rich SC-CO_2 from selected microalgae species is fed to a bioreactor containing lipase for enzymatic conversion of the oil into biodiesel.

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Review Article

Chemical and Physicochemical Pretreatment of Lignocellulosic Biomass: A Review

Gary Brodeur,[1] Elizabeth Yau,[1] Kimberly Badal,[1] John Collier,[1] K. B. Ramachandran,[2] and Subramanian Ramakrishnan[1]

[1] *Department of Chemical and Biomedical Engineering, FAMU-FSU College of Engineering, Tallahassee, FL 32312, USA*
[2] *Department of Biotechnology, Indian Institute of Technology, Chennai 600036, India*

Correspondence should be addressed to Subramanian Ramakrishnan, sramakrishnan@fsu.edu

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Overcoming the recalcitrance (resistance of plant cell walls to deconstruction) of lignocellulosic biomass is a key step in the production of fuels and chemicals. The recalcitrance is due to the highly crystalline structure of cellulose which is embedded in a matrix of polymers-lignin and hemicellulose. The main goal of pretreatment is to overcome this recalcitrance, to separate the cellulose from the matrix polymers, and to make it more accessible for enzymatic hydrolysis. Reports have shown that pretreatment can improve sugar yields to higher than 90% theoretical yield for biomass such as wood, grasses, and corn. This paper reviews different leading pretreatment technologies along with their latest developments and highlights their advantages and disadvantages with respect to subsequent hydrolysis and fermentation. The effects of different technologies on the components of biomass (cellulose, hemicellulose, and lignin) are also reviewed with a focus on how the treatment greatly enhances enzymatic cellulose digestibility.

1. Introduction

The goals stated in the recent roadmap published by the United States Department of Energy (US DOE) [1] is to accelerate biomass to energy conversion research, helping make biofuels practical and cost competitive by 2012 and offering the potential to displace up to 30% of the nation's current gasoline use by 2030. A major source of biomass which will form the focus of energy research is the lignocellulosic biomass which is particularly well suited for energy applications because of its large-scale availability, low cost, and environmentally benign production. In particular, many energy production and utilization cycles based on cellulosic biomass have near-zero greenhouse gas emissions on a life-cycle basis [2–4].

One of the key steps in the biochemical platform of the biomass to fuels or chemicals process being developed by the US DOE is depolymerization of cellulose to glucose by fungal cellulases before fermentation to ethanol or other products by microbial biocatalysts (Biomass Multiyear Program Plan, March 2008, Office of Biomass Program, EERE, DOE). Novozymes, an enzyme production company, estimated (2007 values) that the cost of enzymes to depolymerize cellulose and hemicellulose to sugars for fermentation would be about 40–100 times higher than the cost of enzymes for starch hydrolysis to glucose on a per gallon ethanol basis [5]. Major cellulase producers estimate (2010) the cost of fungal cellulases to be about $0.50 per gallon [6] of cellulosic ethanol produced. Since this price of enzymes is about 25% of the total cost of ethanol production, new strategies for reducing enzyme loading need to be identified to reduce the cost of enzymes in bioprocessing of biomass to fuels and chemicals.

The primary obstacle impeding the more widespread production of energy from biomass feedstocks is the general absence of low-cost technology for overcoming the recalcitrance of these materials [7–10]. Lignocelluloses are composed of cellulose, hemicellulose, lignin, extractives, and several inorganic materials. Cellulose is a linear syndiotactic (alternating spatial arrangement of the side chains) homopolymer composed of D-anhydroglucopyranose units which are linked together by β-(1$\rightarrow$4)-glycosidic bonds.

Taking the dimer cellobiose as the basic unit, cellulose can be considered as an isotactic (identical spatial arrangement of the side chains) polymer of cellobiose. The cellulose chains are packed into microfibrils which are stabilized by hydrogen bonds (Figure 1) [1]. These fibrils are attached to each other by hemicelluloses and amorphous polymers of different sugars as well as other polymers such as pectin and covered by lignin. Hemicellulose has a lower molecular weight than cellulose and is composed of mainly pentoses (like xylose and arabinose) and hexoses (like mannose, glucose, and galactose). It also has considerable side chain branching consisting of hydrolysable polymers. Lignin is an amorphous polymer whose attributes include providing rigidity to the plant cell wall and resistance against microbial attack. The cellulose microfibrils which are present in the hemicellulose-lignin matrix are often associated in the form of bundles or macrofibrils. The structure of these naturally occurring cellulose fibrils is mostly crystalline in nature and highly resistant to attack by enzymes (limited accessibility of cellulose chains). Cellulose is more susceptible to enzymatic degradation in its noncrystalline form. The presence of lignin also impedes enzymatic hydrolysis, as enzymes bind onto the surface of lignin and hence do not act on the cellulose chains [11]. If enzymatic hydrolysis of biomass is to proceed in typical processes, the crystalline structure of cellulose needs to be disrupted, accessible area increased, and the lignin and hemicellulose separated from the cellulose before treatment with enzymes (Figure 1). The process of pretreatment is considered to be one of the expensive steps in the conversion of lignocellulosic feedstock's to ethanol and accounts for nearly \$0.30/gallon of ethanol produced [1].

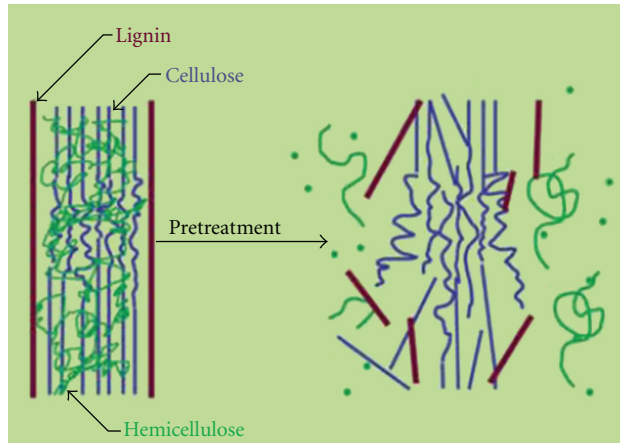

Figure 1: Schematic representation (adapted from [1, 12]) of the matrix of polymers in which cellulose exists. Pretreatment of biomass by different methods removes hemicellulose and lignin from this matrix before hydrolysis.

2. Production of Biofuels by Fermentation

Pretreatment of the biomass is followed by enzymatic hydrolysis to produce simple sugars, fermentation of sugars to produce biofuels, and then product separation [13, 14]. The pretreatment step is key for subsequent enzymatic hydrolysis and fermentation steps in order to maximize the volumetric productivity of the desired product. Cellulose and hemicellulose can be broken down into simple sugars either enzymatically or by acid hydrolysis. The hydrolysis product, six carbon sugars (hexoses), can easily be fermented to ethanol, while only a few microorganism strains can ferment the five carbon sugars (pentoses) [7, 8, 15, 16]. There has been considerable research done in genetically modifying organisms to produce strains that are capable of fermenting both glucose and xylose to useful chemicals (lactic acid) [13, 17]. The process in which the cellulose is broken down and fermented at the same time in the presence of the microorganism is called simultaneous saccharification and fermentation (SSF) [15, 18, 19]. SSF has been the preferred route to the production of biofuels and chemicals, since the operations of both hydrolysis and fermentation are done in the same reactor vessel thus reducing costs. In SSF, the fungal cellulases are most active at 50°C to 55°C, while the microbes ferment effectively at temperatures below 35°C. Because of the mismatch in the optima (cellulase activity and the microbial biocatalyst), simultaneous SSF of cellulose to fuels and chemicals is carried out at a compromise temperature that is close to that of the microbial biocatalyst than that for cellulase activity. This compromise temperature of SSF leads to higher than needed enzyme loading to depolymerize cellulose to glucose with an associated higher cost of cellulases. Recent work in the group of Shanmugam and coworkers [13, 17, 20] has shown that by genetically modifying microorganisms to operate (ferment) at a higher temperature of 50°C to 55°C results in a lower cellulase loading, thus reducing the cost of the process and the final product. Current efforts in the literature are focussed on how to combine pretreatment and SSF to maximize productivity and to make the overall process economically efficient. This paper will just focus on the first step of pretreatment and how this affects the subsequent processing steps.

3. Goals of Pretreatment

Numerous pretreatment strategies have been developed to enhance the reactivity of cellulose and to increase the yield of fermentable sugars. Typical goals of pretreatment include (1) production of highly digestible solids that enhances sugar yields during enzyme hydrolysis, (2) avoiding the degradation of sugars (mainly pentoses) including those derived from hemicellulose, (3) minimizing the formation of inhibitors for subsequent fermentation steps, (4) recovery of lignin for conversion into valuable coproducts, and (5) to be cost effective by operating in reactors of moderate size and by minimizing heat and power requirements. The goal of this paper is to review promising pretreatment technologies and to discuss recent developments which have greatly aided the production of biofuels. For each technology, a brief process description is first given with recent developments, and then the feedstocks on which these technologies are used are highlighted, followed by discussion of the technology's

advantages and disadvantages. Recent results from the corresponding author's laboratories in solvent pretreatment are given with a goal of trying to integrate pretreatment with SSF.

4. Pretreatment Categories

Pretreatment technologies are usually classified into physical, chemical, physicochemical, and biological. Physical pretreatment involves breakdown of biomass size and crystallinity by milling or grinding. Improved hydrolysis results due to the reduction in crystallinity and improved mass transfer characteristics from reduction in particle size. The energy requirements for physical pretreatments are dependent on the final particle size and reduction in crystallinity of the lignocellulosic material. In most cases where the only option available for pretreatment is physical, the required energy is higher than the theoretical energy content available in the biomass. This method is expensive and likely will not be used in a full-scale process. Biological pretreatment, as normally defined, involves the use of microorganisms (mainly fungi) to degrade lignin and hemicellulose but leave the cellulose intact [21–23]. Lignin degradation occurs through the action of lignin degrading enzymes secreted by the fungi. Even though biological pretreatments involve mild conditions and are of low cost, the disadvantages are the low rates of hydrolysis and long pretreatment times required compared to other technologies [19]. Current efforts in biological pretreatments are in combining this technology with other pretreatments and in developing novel microorganisms for rapid hydrolysis [21, 22, 24]. Since both physical and biological processes are not cost competitive compared to the chemical and physicochemical pretreatments, they will not be reviewed in this current paper. Table 1 highlights the advantages and disadvantages of the pretreatment technologies that would be discussed in this work. A brief description of these technologies is given below.

4.1. Chemical Pretreatments

4.1.1. Alkaline. Alkaline pretreatment involves the use of bases, such as sodium, potassium, calcium, and ammonium hydroxide, for the pretreatment of lignocellulosic biomass. The use of an alkali causes the degradation of ester and glycosidic side chains resulting in structural alteration of lignin, cellulose swelling, partial decrystallization of cellulose [25–27], and partial solvation of hemicellulose [27, 28]. Sodium hydroxide has been extensively studied for many years, and it has been shown to disrupt the lignin structure of the biomass, increasing the accessibility of enzymes to cellulose and hemicellulose [29–32]. Another alkali that has been used for the pretreatment of biomass is lime. Lignocellulosic feedstocks that have been shown to benefit from this method of pretreatment are corn stover, switchgrass, bagasse, wheat, and rice straw [19, 33–36].

Sun and coworkers [37] studied the effectiveness of different alkaline solutions by analyzing the delignification and dissolution of hemicellulose in wheat straw. They found that the optimal condition was using 1.5% sodium hydroxide for 144 hours at 20°C, which resulted in 60% release of lignin and 80% release of hemicellulose. Xylose was the major component of the hemicellulose fractions and the hydrolysates from pretreatment, while glucose and galactose were present in smaller amounts. Recently, Zhao and coworkers [31] showed the effectiveness of sodium hydroxide pretreatment for hardwoods, wheat straw, switchgrass, and softwoods with less than 26% lignin content. Sodium hydroxide pretreatment has also been shown to increase biogas production from corn stover by 37% compared to that of untreated cellulose [32]. The conditions for alkaline pretreatment are usually less severe than other pretreatments. It can be performed at ambient conditions, but longer pretreatment times are required than at higher temperatures. The alkaline process involves soaking the biomass in alkaline solutions and mixing it at a target temperature for a certain amount of time. A neutralizing step to remove lignin and inhibitors (salts, phenolic acids, furfural, and aldehydes) is required before enzymatic hydrolysis. A recent approach to lime pretreatment eliminates the solid-liquid separation step after neutralization by neutralizing the lime with carbon dioxide before hydrolysis resulting in 89 wt% glucose recovery from leafstar rice straw [34]. Park and coworkers [34] also used this method to test SSF which resulted in an ethanol yield that was 74% of the theoretical value using a mixture of *Saccharomyces cerevisae* and *Pichiastipitis* after 79 hours of fermentation at 30°C.

The advantage of lime pretreatment is that the cost of lime required to pretreat a given quantity of biomass is lowest among alkaline treatments. For example, in 2005, the estimated cost of materials was \$70/ton hydrated lime compared to \$270/ton fertilizer grade ammonia and \$320/ton for 50 wt% NaOH and 45 wt% KOH [19]. Though lime pretreatment is energy intensive, $CaCO_3$ can be recovered by precipitation with CO_2 after solid-liquid separation [38]. If the method developed by Park and coworkers is used, CO_2 for neutralizing lime can be supplied from a fermentor or a heater to reduce costs. However, Park's process makes it difficult to separate $CaCO_3$ from other solid particles after hydrolysis and fermentation. A number of studies have combined alkaline pretreatment with other pretreatment methods, such as the wet oxidation, steam explosion, ammonia fiber explosion, and ammonia recycled percolation, which are discussed in later sections. Recently, Zhao and coworkers [31] showed that the sugar yield from switchgrass could be improved by combining alkaline pretreatment with radiofrequency-based dielectric heating, which allows pretreatment of high solid content and uniform temperature profile in the pretreated biomass. It is hypothesized that the use of radiofrequency dielectric heating accelerates the disruption of the lignocelluloses structure by causing an explosion effect among the particles, thus resulting in higher xylose and glucose yields compared to the pretreatment with alkali and conventional heating.

4.1.2. Wet Oxidation. Wet oxidation utilizes oxygen as an oxidizer for compounds dissolved in water. There are two reactions that occur during this process. One is a low

Table 1: Advantages and disadvantages of different pretreatment methods of lignocellulosic biomass.

Pretreatment method	Advantages	Disadvantages
Alkali	(i) Efficient removal of lignin	(i) High cost of alkaline catalyst
	(ii) Low inhibitor formation	(ii) Alteration of lignin structure
Acid	(i) High glucose yield	(i) High costs of acids and need for recovery
	(ii) Solubilizes hemicellulose	(ii) High costs of corrosive resistant equipment
		(iii) Formation of inhibitors
Green solvents	(i) Lignin and hemicellulose hydrolysis	(i) High solvent costs
	(ii) Ability to dissolve high loadings of different biomass types	(ii) Need for solvent recovery and recycle
	(iii) Mild processing conditions (low temperatures)	
Steam	(i) Cost effective	(i) Partial hemicellulose degradation
	(ii) Lignin transformation and hemicellulose solubilization	(ii) Acid catalyst needed to make process efficient with high lignin content material
	(iii) High yield of glucose and hemicellulose in two-step process	(iii) Toxic compound generation
LHW	(i) Separation of nearly pure hemicellulose from rest of feedstock	(i) High energy/water input
	(ii) No need for catalyst	(ii) Solid mass left over will need to be dealt with (cellulose/lignin)
	(iii) Hydrolysis of hemicellulose	
AFEX	(i) High effectiveness for herbaceous material and low lignin content biomass	(i) Recycling of ammonia is needed
	(ii) Cellulose becomes more accessible	(ii) Less effective process with increasing lignin content
	(iii) Causes inactivity between lignin and enzymes	(iii) Alters lignin structure
	(iv) Low formation of inhibitors	(iv) High cost of ammonia
ARP	(i) Removes majority of lignin	(i) High energy costs and liquid loading
	(ii) High cellulose content after pretreatment	
	(iii) Herbaceous materials are most affected	
Supercritical fluid	(i) Low degradation of sugars	(i) High pressure requirements
	(ii) Cost effective	(ii) Lignin and hemicelluloses unaffected
	(iii) Increases cellulose accessible area	

temperature hydrolysis reaction and the other is a high temperature oxidation reaction [39]. Typically, the procedure for wet oxidation consists of drying and milling lignocellulosic biomass to obtain particles that are 2 mm in length, to which water is added at a ratio of 1 L to 6 g biomass. A compound usually Na_2CO_3, is introduced to the mixture to reduce the formation of byproducts. Air is pumped into the vessel until a pressure of 12 bar is reached. This method of pretreatment is performed at 195°C for a range of 10 to 20 minutes [40–42].

Wet oxidation can be used to fractionate lignocellulosic material by solubilizing hemicellulose and removing lignin [39, 43]. It has been shown to be effective in pretreating a variety of biomass such as wheat straw, corn stover, sugarcane bagasse, cassava, peanuts, rye, canola, faba beans, and reed to obtain glucose and xylose after enzymatic hydrolysis [40, 41, 43–46]. Biomass such as straw, reed, and other cereal crop residues have a dense wax coating containing silica and protein which is removed by wet oxidation [47]. For pretreated wheat straw (pretreatment time of 10 minutes), Pederson and Meyer [42] obtained yields of 400 and 200 g/kg dry matter for glucose and xylose, respectively, after 24 hours at 50°C using an enzyme mixture of 36 FPU/g Celluclast-1.5 L and 37 CBU/g of Novozyme-188. Difficult biomass such as grape stalk (which contains tannins, a chemical that complicate delignification) has also been shown to benefit wet oxidation with up to 50% cellulose conversion compared to 25% conversion with sulphuric acid pretreatment [48].

During wet oxidation, lignin is decomposed to carbon dioxide, water, and carboxylic acids [40, 43]. The amount of lignin removed after pretreatment ranges from 50% to 70% depending on type of biomass pretreated and the conditions used. For bagasse with a pretreatment time of 15 minutes 50% of lignin was removed which resulted in 57.4% conversion of cellulose compared to only 35% lignin removal and 48.9% cellulose conversion for steam explosion, explained later, under the same conditions [41]. However, Martín and coworkers also found that the amount of byproducts formed was almost always higher for pretreatment by oxidation than by steam explosion. Byproducts obtained included

succinic acid, glycolic acid, formic acid, acetic acid, phenolic compounds, and furfural which would have negative effects on further downstream processing due to inhibition.

Wet oxidation can be combined with other pretreatment methods to further increase the yield of sugars after enzymatic hydrolysis [47, 49–52]. Combining wet oxidation with alkaline pretreatment has been shown to reduce the formation of byproducts, thereby decreasing inhibition [53]. In combination with steam explosion, in a process called wet explosion, the biomass not only undergoes the chemical reaction described above but also undergoes physical rupture [50]. The advantages to combining wet oxidation with steam explosion includes the ability to process larger particle sizes and to operate at higher substrate loadings, up to 50% substrate [51]. Georgieva and coworkers [51] were able to obtain a cellulose conversion of 70%, a hemicellulose conversion of 68% and an ethanol yield of 68% for SSF using wet explosion. Biomass conversion has also been shown to benefit from the addition of an acid soaking step prior to wet explosion, since acid pretreatment helps to hydrolyze the hemicelluloses, while wet explosion will expose more enzyme binding sites [50].

4.1.3. Acid. Acid pretreatment involves the use of concentrated and diluted acids to break the rigid structure of the lignocellulosic material. The most commonly used acid is dilute sulphuric acid (H_2SO_4), which has been commercially used to pretreat a wide variety of biomass types—switchgrass [54, 55], corn stover [56, 57], spruce (softwood) [58], and poplar [59, 60]. Dilute sulphuric acid has traditionally been used to manufacture furfural [61] by hydrolyzing the hemicellulose to simple sugars, such as xylose, which continues to convert into furfural. Other acids have also been studied, such as hydrochloric acid (HCl) [62], phosphoric acid (H_3PO_4) [63, 64], and nitric acid (HNO_3) [65]. Due to its ability to remove hemicellulose, acid pretreatments have been used as parts of overall processes in fractionating the components of lignocellulosic biomass [63]. Acid pretreatment (removal of hemicellulose) followed by alkali pretreatment (removal of lignin) results in relatively pure cellulose.

This chemical pretreatment usually consists of the addition of concentrated or diluted acids (usually between 0.2% to 2.5% w/w) to the biomass, followed by constant mixing at temperatures between 130°C and 210°C. Depending on the conditions of the pretreatment, the hydrolysis of the sugars could take from a few minutes to hours [66–72].

Recent articles [35, 73, 74] have reviewed the development of acid pretreatment of biomass over the years and highlighted the advantages and disadvantages and the optimum conditions of operation. A key advantage of acid pretreatment is that a subsequent enzymatic hydrolysis step is sometimes not required, as the acid itself hydrolyses the biomass to yield fermentable sugars. Hemicellulose and lignin are solubilized with minimal degradation [75], and the hemicellulose is converted to sugars with acid pretreatment. However, extensive washing and/or a detoxification step [66, 71] is required to remove the acid before a fermentation step [68, 69]. Due to the corrosive nature and toxicity of most acids, an adequate material for the reactor is required in order to withstand the required experimental conditions and corrosiveness of the acids. Another drawback is the production of fermentation inhibitors like furfural and HMF (hydroxymethyl furfural) that reduces the effectiveness of the pretreatment method and further processes [66, 70].

As mentioned before different types of biomass have proven to be effectively treated by the acid pretreatment. Softwoods were studied by Nguyen and coworkers [68], showing that the sugar yield could be maximized using a two-stage diluted sulphuric acid pretreatment process. Sun and Cheng [70] studied bermudagrass and rye straw. After a 48-hour enzymatic hydrolysis of pretreated bermudagrass and rye straw with 1.5% sulphuric acid, the total reducing sugars were found to be 197.1 mg/g and 229.3 mg/g of dry biomass. Other studies done by Saha and coworkers [66, 71] showed that the maximum sugar yield of wheat straw was 565 ± 10 mg/g (76% yield based on total carbohydrate content) and that of rice hull was 287 ± 3 mg/g (60% yield based on total carbohydrate content). Under these conditions, no furfural and hydroxymethyl furfural were produced. Marzialetti and coworkers [64] studied the acid hydrolysis of loblolly pine using a number of different acids (triflulororacetic acid—TFA, HCl, H_2SO_4, HNO_3, and H_3PO_4). The effect of the type of acid, pH, reaction temperature, and reaction time on hydrolysis products such as monosaccharides (mannose, glucose, galactose, xylose, and arabinose) and the subsequent degradation products, 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (furfural), is reported using a batch reactor. Trifluoroacetic acid (TFA) is found to yield the highest amount of overall soluble monosaccharides (similar to 70% yield from the hemicellulose fraction) at 150°C at pH 1.65. TFA was found to be the most "gentle" acid, leading to limited monosaccharide degradation among the acids used.

The optimum conditions for the acid pretreatment depend highly on the targeted sugars and the purpose of the pretreatment. Lloyd and Wyman [76] found that the optimal conditions for obtaining the maximum sugar yield depends on whether the goal is to maximize the yield after the pretreatment or after the enzymatic hydrolysis of the pretreated solids or if the goal is to obtain maximum yield after both steps. In addition, finding the optimal conditions is extremely important to reduce the formation of inhibitory products that will reduce the efficiency of the fermentation step.

4.1.4. Green Solvents. Processing of lignocellulosic biomass with ionic liquids (IL) and other solvents has gained importance in the last decade due to the tunability of the solvent chemistry and hence the ability to dissolve a wide variety of biomass types. Ionic liquids are salts, typically composed of a small anion and a large organic cation, which exist as liquids at room temperature and have very low vapor pressure. The chemistry of the anion and cation can be tuned to generate a wide variety of liquids which can dissolve a number of biomass types—corn stover [78], cotton [79], bagasse [80], switchgrass [81], wheat straw [82], and woods

Table 2: Solubility of cellulose dissolved in different ILs (adapted from [77]). For the cellulose column, the numbers in brackets correspond to the DP values, if known. MCC: microcrystalline cellulose with DP ca. 270–300. Key for abbreviations of IL cations: $[C_n\text{mim}]^+$: 1-alkyl-3-methylimidazolium (n = number of carbons in the alkyl chain); $[C_n\text{mmim}]^+$: 1-alkyl-2,3-dimethylimidazolium (n = number of carbons in the alkyl chain); $[\text{Amim}]^+$: 1-allyl-3-methylimidazolium; $[\text{Ammim}]^+$: 1-allyl-2,3-dimethylimidazolium; $[C_4\text{mP}_y]^+$: 1-butyl-3-methylpyridinium; $[\text{Bu}_4\text{P}]^+$: tetrabutylphosphonium.

IL	Cellulose	Method	Solubility %
$[C_2\text{mim}]\text{Cl}$	Avicel	Heat, 100°C	10
$[C_3\text{mim}]\text{Cl}$	Avicel	Heat, 100°C	0.5
$[C_4\text{mim}]\text{Cl}$	Avicel	Heat, 100°C	20
$[C_4\text{mim}]\text{Cl}$	Pulp (1000)	Heat	10
$[C_4\text{mim}]\text{Cl}$	Pulp (1000)	Microwave	25
$[C_5\text{mim}]\text{Cl}$	Avicel	Heat, 100°C	1.5
$[C_6\text{mim}]\text{Cl}$	Pulp (1000)	Microwave	5
$[C_6\text{mim}]\text{Cl}$	Avicel	Heat, 100°C	6.5
$[C_7\text{mim}]\text{Cl}$	Avicel	Heat, 100°C	5
$[C_8\text{mim}]\text{Cl}$	Avicel	Heat, 100°C	4
[Amim]Cl	Pulp (650)	Heat, 80°C	14.5
[Amim]Cl	MCC	Ultrasound	27
$[C_4\text{mmim}]\text{Cl}$	Pulp (569)	Heat, 90–130°C	12.8
$[C_4\text{mmim}]\text{Cl}$	Pulp (286)	Heat, 90°C	9
$[C_4\text{mmim}]\text{Cl}$	Pulp (593)	Heat, 90°C	6
$[C_4\text{mmim}]\text{Cl}$	Pulp (1198)	Heat, 90°C	4
$[C_4\text{mPy}]\text{Cl}$	Pulp (593)	Heat, 105°C	37
$[C_4\text{mim}]\text{Br}$	Pulp (1000)	Microwave	5–7
[Ammim]Br	Pulp (286)	Heat, 80°C	12
[Ammim]Br	Pulp (1198)	Heat, 80°C	4
$[C_4\text{mim}][\text{SCN}]$	Pulp (1000)	Microwave	5–7
$[C_2\text{mim}][\text{OAc}]$	Avicel	Heat, 100°C	8
$[C_2\text{mim}][\text{OAc}]$	Avicel (225)	Heat, 110°C	28
$[C_4\text{mim}][\text{OAc}]$	MCC	Heat, 70°C	28.5
$[C_4\text{mim}][\text{OAc}]$	Avicel	Heat, 100°C	12
[Amim][HCOO]	MCC	Heat, 85°C	22
$[C_4\text{mim}][\text{HCOO}]$	MCC	Heat, 70°C	12.5
$[C_4\text{mim}][\text{HCOO}]$	Avicel (225)	Heat, 110°C	8
$[C_4\text{mim}][(C_6H_5)\text{COO}]$	MCC	Heat, 70°C	12
$[C_4\text{mim}][(\text{NH}_2)\text{CH}_2\text{COO}]$	MCC	Heat, 70°C	12
$[C_4\text{mim}][\text{OHCH}_2\text{COO}]$	MCC	Heat, 70°C	12
$[\text{Bu}_4\text{P}][\text{HCOO}]$	Avicel (225)	Heat, 110°C	6
$[C_4\text{mim}][\text{HSCH}_2\text{COO}]$	MCC	Heat, 70°C	13.5
$[C_2\text{mim}][(\text{CH}_3\text{CH}_2\text{O})_2\text{PO}_2]$	Avicel	Heat, 100°C	12–14
$[C_1\text{mim}][(\text{CH}_3\text{O})_2\text{PO}_2]$	Avicel	Heat, 100°C	10
$[C_2\text{mim}][(\text{CH}_3\text{O})(\text{H})\text{PO}_2]$	MCC	Heat, 45°C	10

of different hardness [83] (pine, poplar, eucalyptus, and oak). The low vapor pressure of IL and similar solvents make them more than 99% recoverable in a number of operations, thus reducing costs of solvent usage. Since no toxic products are formed during the pretreatment operation and since IL are recoverable, they are termed green solvents. Table 2 (adapted from the work of Sun and coworkers [77]) lists the dissolving capacity of different celluloses by a variety of ILs. For an IL to be used in pretreatment of biomass, it should not only have high dissolution capacity, but also low melting point, low viscosity, low/no toxicity, and high stability. Sun and coworkers [77] found out that although the dissolution is greatly affected by the source of cellulose, different degrees of polymerization (DP), and the dissolution conditions (heating method, irradiation, heating temperature, time, etc.), generally, with the same cation, the solubility of cellulose in ILs decreases in the order

$$[(\text{CH}_3\text{CH}_2\text{O})_2\text{PO}_2]^- \approx [\text{OAc}]^- > [\text{SHCH}_2\text{COO}]^- > [\text{HCOO}]^- > Cl^- > Br^- \approx [\text{SCN}]^-. \quad (1)$$

Pretreatment with IL involve dissolution of biomass in the solvent at ambient pressures, temperatures of 90°C to 130°C for varying amounts of time (1 hour–24 hours) [81, 84]. The biomass is then reprecipitated by the addition of water and washed a number of times before enzymatic hydrolysis. The anion of the IL forms hydrogen bonds with cellulose (sugar hydroxyl protons) in a 1 : 1 ratio and breaks up the cellulose crystalline hydrogen bonded structure, thus making it more amorphous and accessible to enzymatic hydrolysis. In addition, the chemistry of the IL can also be tuned to dissolve the hemicellulose and lignin, thus making it suitable to dissolve the different components. It should be mentioned here that the IL dissolves cellulose and generally does not degrade the chains and reduce its degree of polymerization. Also, research studies [59, 81, 84] have proven that the structure of lignin and hemicellulose are unaltered after treatment with many ILs.

Dadi and coworkers [85, 86] studied the enzymatic hydrolysis of Avicel (microcrystalline cellulose formed by acid treatment) regenerated from two different ILs, 1-*n*-butyl-3-methylimidazolium chloride and 1-allyl-3-methylimidazoliumchloride. Hydrolysis kinetics of the IL-treated cellulose was significantly enhanced. With appropriate selection of IL treatment conditions and enzymes, the initial hydrolysis rates for IL-treated cellulose were up to 90 times greater than those of untreated cellulose. The enhanced hydrolysis rates were attributed to the amorphous nature of the cellulose on pretreatment. Wyman and coworkers [59] have recently shown the ability of 1-ethyl-3-methylimidazolium acetate [Emim]Ac to dissolve maple wood flour and that the IL has a high solubility for lignin and a low solubility for cellulose. Therefore, they were able to selectively extract the unaltered lignin from the lignocelluloses while simultaneously yielding a highly degradable cellulose fraction. High throughput screening methods to determine the effectiveness of the ILs to dissolve the cellulose have also been recently developed [87]. Work by Nguyen and coworkers [88] have combined ammonia and ionic liquid pretreatment of rice straw to result in 97% conversion of cellulose to glucose. The ionic liquid in their work was recycled more than 20 times, thus reducing costs.

A limitation in using ionic liquids is the fact they tend to inactivate cellulase. Turner and coworkers [90] studied the hydrolysis of cellulose by *T. reesei* cellulase in 1-butyl-3-methyl imidazolium chloride [Bmim]Cl and [Bmim]BF_4 that contained 5% of cellulose in 50 mM citrate buffer, pH 4.8, at 50°C. The hydrolytic rate in the ILs was poor, at least 10-fold less than that performed in aqueous buffer. Low activity in [Bmim]Cl was attributed to the high concentration of the Cl^- ion (and was also observed through the addition of NaCl) that leads to unfolding and inactivation of the enzymes. This inactivation was irreversible. Hence, one needs to completely regenerate the cellulose after pretreatment and remove all traces of this IL before hydrolysis by cellulases in order to preserve their activity. This introduces a regeneration and separation step into the process which increases the overall cost and precludes the development of a single stage continuous process for conversion of lignocellulosic biomass. Thus, selection of a solvent for pretreatment in which cellulases and microorganisms are active is a key step in the development of the "biorefinery concept" or "consolidated bioprocessing" schemes which try to develop a single-stage continuous process for biomass conversion.

A solvent which has been effective in dissolution of cellulose and has a low vapor pressure similar to that of the ionic liquids is N-methyl morpholine N-oxide (NMMO), also known as the Lyocell solvent used commercially to produce Tencel fibers. NMMO retains all the advantages of the ionic liquids ability to dissolve a variety of lignocellulosic substrates [91–93] without the need to chemically modify them and >99% of the solvent can be recovered due to its low vapor pressure [94]. It is also nontoxic and biodegradable as proven by the work of Lenzing and other researchers [95]. Cellulose regenerated from NMMO solutions has also yielded increased rates of hydrolysis by cellulose, thus highlighting its ability to disrupt the crystalline structure of cellulose [91, 92].

Recent work (Figure 2—[89]) in the corresponding authors' laboratories has shown that an in situ enzymatic saccharification process is possible with NMMO that eliminates the need to recover regenerated cellulose. Using dissolving pulp as a substrate and a commercially available cellulase (Accellerase 1000), it was shown that initial rates of hydrolysis of cellulose and yield of reducing sugars in the presence of NMMO water was initially higher (at times less than five hours) and overall at least comparable to that of regenerated cellulose when suspended in aqueous solutions (Figure 2(a)). The results shown in Figure 2(a) have also been compared with data from the literature in which cellulose that has been pretreated in different manners is resuspended in aqueous solutions and enzymatic reactions are carried out. The initial rates of hydrolysis and final yields of simple sugars for cellulose dissolved in NMMO are comparable and in most instances higher than values in the literature. Figure 2(b) is an *in situ* hydrolysis of dissolving pulp (DP of 1160—the same substrate used in Figure 2(a)) carried out in three different solvents—NMMO and two ionic liquids. The two ionic liquids studied are 1-ethyl-3-methylimidazolium acetate [Emim]Ac and 1-ethyl-3-methylimidazolium diethylphosphate [Emim]DEP. The reason for using these ionic liquids is that both are excellent solvents for dissolution of woody biomass [96, 97]. Our initial results indicate as shown in Figure 2(b) that the chemical structure of the cellulose solvent (NMMO/H_2O, [Emim]Ac and [Emim]DEP) has a definite effect on the conversion to sugars with NMMO having the highest conversion. Ultimate conversion of cellulose is 50–60% lower while using the ionic liquids. This is an indication that these highly polar species might be deactivating the enzymes. This would be especially troublesome for a process in which one would attempt to recycle the solvent and enzyme as is envisioned for the NMMO-based process. Another ionic liquid—1-butyl-3-methylimidazolium chloride [Bmim]Cl (used in spinning cellulose fibers and a good solvent for lignocellulosic biomass) was also tested for in-situ hydrolysis. However, there were no sugars released as a function of time indicating that the cellulases were inactivated. Thus, NMMO led to the best yields and high initial rates in comparison with the tested ionic liquids.

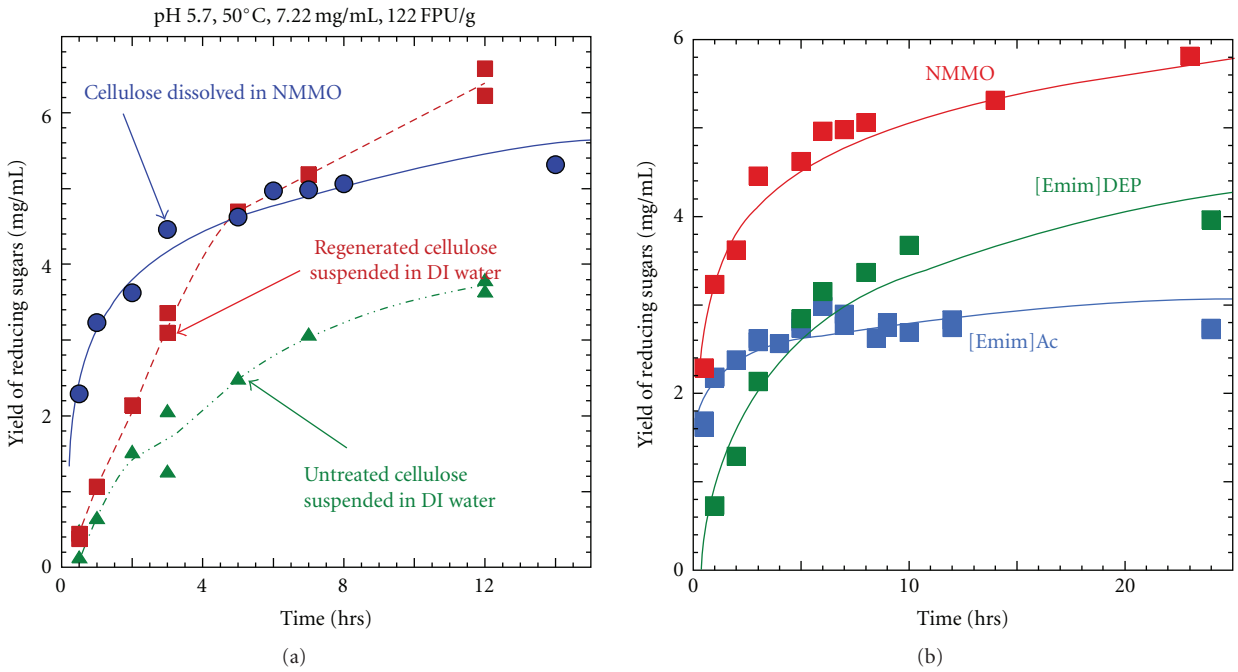

Figure 2: (a) [89]: Yield of reducing sugars as a function of time for four different samples—cellulose (dissolving pulp of degree of polymerization 1160) dissolved in NMMO (blue circle), regenerated cellulose suspended in DI water (red square), and untreated cellulose suspended in DI water (green triangle). The initial cellulose concentration is 7.22 mg/mL, pH is 5.7 and enzyme loading is 122 FPU/g. The lines are drawn to guide the eye. The cellulase used is Accellerase 1000 obtained from Genencor. (b) Yield of sugars as a function of time for in situ hydrolysis of cellulose (the same substrate as in Figure 2(a)) in NMMO and two ionic liquids—[Emim]Ac and [Emim]DEP. The initial cellulose concentration is 7.22 mg/mL, pH is 5.7, and enzyme loading is 122 FPU/g. The lines are drawn to guide the eye.

Further research is needed to evaluate and improve the economics of usage of ILs and NMMO for pretreatment of biomass and to integrate it with SSF. ILs are still expensive and need to be synthesized at lower cost and on a larger scale. The ability of microorganisms to ferment sugars in the presence of these solvents also needs to be tested to carry out a continuous process. Despite these current limitations, pretreatment of lignocellulosic biomass with both NMMO and IL offers a great potential for substantially reducing the costs of pretreatment and in developing a consolidated biorefinery process.

4.2. Physicochemical Pretreatments

4.2.1. Steam-Explosion. Steam-Explosion pretreatment is one of the most commonly used pretreatment options, as it uses both chemical and physical techniques in order to break the structure of the lignocellulosic material. This hydrothermal pretreatment method subjects the material to high pressures and temperatures for a short duration of time after which it rapidly depressurizes the system, disrupting the structure of the fibrils. The disruption of the fibrils increases the accessibility of the cellulose to the enzymes during hydrolysis. Particle size is a major contributing factor on the effectiveness of the process, and it has been seen that relatively large particle sizes have been able to yield maximum sugar concentrations. This is a promising finding, as decreasing the particle sizes of the material requires further mechanical processing of the raw material driving up the production costs [98]. Temperatures ranging from 190°C to 270°C have been used with residence times of 10 minutes and 1 minute, respectively. The starting material and particle sizes that will be processed will be the determining factor on the relationship between temperature and time [99, 100].

A two-step steam-explosion pretreatment option has also been looked at in order to solubilize the structure in stages creating an optimum cellulose fraction during hydrolysis. The first step involved temperatures of 180°C in order to solubilize and remove the hemicellulose fraction. The second stage used a high-temperature pressurized pretreatment with temperatures up to 210°C, not exceeding 240°C, in which the cellulose fraction was subject to breaking down of its carbohydrate linkage [101, 102]. This two-step process increased the downstream ethanol yield by increasing accessibility to cellulose structure because of the reduction of the hemicellulose fraction. Operation costs also decreased as less enzyme dosage was required due to increased accessibility of the cellulose fraction [102]. However, increased costs of equipment needed for processing and the additional energy usage of a second steam-explosion process is required.

Acid catalysts have been used within the steam explosion process in dilute quantities in order to improve hemicellulose hydrolysis during the pretreatment and cellulose digestibility further on in the process. Dilute acids have the ability to decrease retention time and temperature of current operating systems or allow for the use of softwoods in this

pretreatment technique, where it was seen to not be originally economical. By decreasing the retention time and temperature with the addition of this acid catalyst, a reduction of inhibitory compounds formed is seen, complete removal of hemicellulose is approached, and the improvement of hydrolysis later on in production is attained [103].

As stated, this process utilizes both chemical and physical interactions in order to effectively break down the lignocellulosic structure. The chemical pretreatment is present in the form of hydrolysis of the glycosidic bonds contained within the hemicellulose and cellulose structure along with the removal and/or redistribution of lignin. Breaking off of these acetyl groups into acetic acid as well as the acidic nature of water at high temperatures will promote further hydrolysis of the hemicellulose [104]. The physical pretreatment comes about during the rapid decompression of the system. This rapid expansion vaporizes the saturated water within the fibrils, breaks down the molecular linkages, and leads to an effective lignocellulosic matrix [105].

The steam-explosion pretreatment process has been a proven technique for the pretreatment of different biomass feedstocks. It is able to generate complete sugar recovery while utilizing a low capital investment and low environmental impacts concerning the chemicals and conditions being implemented and has a higher potential for optimization and efficiency [106]. The large particle sizes that were mentioned can greatly reduce the costs of the overall process. This is due to the fact that obtaining smaller chip sizes may make up to a third of the overall energy cost associated with a particular pretreatment process [107]. Poplar chips (90% glucose yield after enzymatic hydrolysis compared to 15% for untreated biomass) [108], olive tree residues (50% yield of total sugars) [109], herbaceous residues such as corn stover (73% theoretical sugar yield with dilute sulphuric acid addition) [110], wheat straw (80% theoretical conversion yield to ethanol with 0.9% H_2SO_4 addition) [103], and agricultural residues such as *B. carinata* straw (70–99% enzymatic hydrolysis yield depending on pretreatment parameters) [98] have all seen dramatic improvements in sugar yields when a steam pretreatment is used over nontreated materials. Softwoods, such as woody hemp (feedstocks considered to be less acetylated; higher lignin content), have even shown increased yields when dilute acids (1% sulphuric acid) are introduced into the system [111]. These dilute acids may also potentially reduce sugar degradation product formation, treatment temperatures, and reaction times or even increase sugar yields in the pretreatment of feedstocks with lower lignin content, that is, 30% improvement with 1% sulphuric acid addition with olive tree prunings or increase to 81% with 3% sulphuric acid addition to bark containing wood samples [111–113]. However, some disadvantages can be seen when using this process. As mentioned, dilute acids will need to be added during softwood pretreatment or even when increased yields are warranted for lower acetylated feedstocks. These costs will come about not only in the cost of the raw materials (acids) being used, but also in the equipment requirements and the higher formation of degradation products (by the way of monomeric sugar degradation into aldehydes) that would need to be neutralized [114]. These neutralization salts would then need to be separated from the system and disposed [12].

With the inefficient means by which to pretreat many of the different biomass materials into purified cellulose, steps are being taken to move in the direction of complete utilization of the entire lignocellulosic materials that are involved in these conversions. That is, to possibly use less severe or staged pretreatment processes in order to improve hemicellulose and lignin recovery on top of the cellulose results that are already being achieved. Some groups have used a two-stage pretreatment for the utilization of the entire feedstock of lespedeza stalks (alkali-ethanol 2nd treatment) or wheat straw (alkaline peroxide 2nd treatment) in which steam pretreatment is utilized during the first stage followed by extraction and isolation of the degraded hemicellulose fraction from the filtrate [115, 116]. The final products would then consist of a cellulose rich fraction (directly from steam explosion process), a lignin-rich filtrate (post-2nd stage filtrate treatment), and degraded hemicellulose pellets [115, 116]. Optimum steam pretreatment conditions of 20 or 22.5 kg/m^2 for 4 minutes were found to yield the highest isolation quantities of high molecular weight hemicelluloses, where this fraction could then be utilized as oligosaccharides.

4.2.2. Liquid Hot Water (LHW). Much like the steam-explosion process, liquid hot water (LHW) pretreatment uses water at elevated temperatures and high pressures to maintain its liquid form in order to promote disintegration and separation of the lignocellulosic matrix. Temperatures can range from 160°C to 240°C and over lengths of time ranging from a few minutes up to an hour with temperatures dominating the types of sugar formation and time dominating the amount of sugar formation [117]. This process uses many of the same features that steam explosion employs, primarily autohydrolysis, without the rapid decompression, utilizing flow through reactors of varying configurations or batch techniques with the latter being the primary emphasis at the laboratory scale [117–119]. One goal of this process is to completely solubilize hemicellulose and separate it from the rest of the solid material while reducing the formation of inhibitors. The generation of reactive cellulose fibers for the production of pentosans as well as disruption of the entire lignocellulosic matrix is achieved through the cell penetration of the biomass by the water, along with solubilisation of both hemicellulose and lignin by this liquid hot water acting as an acid [120, 121].

There are two products that are formed at the outlet of this process: the solubilized hemicellulose-rich slurry and the cellulose-rich solid fraction that are separated from each other [122]. The solubilized product, consisting primarily of oligosaccharides derived from hemicellulose (HDS) (nearly complete removal from solid fraction) and lignin (35–60% of total starting material) and a minor amount of cellulose (4–15%), is the primary focus for this particular pretreatment, while the solid fraction will need to be separated and dealt with enzymatically [12]. This HDS-rich fraction is primarily converted to oligomers, while limiting the formation of monomers as long as the pH is maintained between four

and seven [12]. Temperature has also been seen to play an important role in the liquid fraction of this pretreatment as the quantity of inhibitor product formation is seen to primarily rise with an increase in temperature. For example, 180°C (low end of temperature range) and 30 minutes (moderate-high time-scale range) have seen to be the most cost-effective pretreatment condition for rice straw conversion to glucose [117]. The notable inhibitors or byproducts that are seen to form within the liquid fraction of this process are due to the degradation of pentoses, hexoses, and the lignin present. These products can include furfural, acetic acid, 5-hydroxymethyl-2-furaldehyde (HMF), and formic acid among others [120].

The solid fraction is seen to become more susceptible to enzymatic attack as the cellulose becomes more accessible by the penetration and hydration of cell walls that cause swelling and disruption of the matrix. The primary objective of this pretreatment is, therefore, to lessen the solubilisation of cellulose as much as possible while keeping HDS solubilisation high. After the separation of these two fractions comes a second stage, where the solid product would be subjected to enzymatic hydrolysis forming glucose (cellulose fraction) and xylose (hemicellulose fraction) simple sugars [117, 120]. In order to be an effective pretreatment, the lignin portion of the lignocellulosic material would want to be included in the liquid fraction as to not inhibit the formation of these simple sugars during enzymatic hydrolysis. It has been seen that lower process temperatures during the LHW treatment will increase the solubilisation of the lignin allowing for higher enzymatic hydrolysis of the solid fraction [120].

This process can be seen as advantageous from a cost standpoint in that no additives such as acid catalysts are required; furthermore, expensive reactor systems are not necessary due to the low corrosive nature of this pretreatment technique and the chemicals that are involved. Neutralization of degradation products will not be needed due to their fractionation and utilization in the liquid fraction. In the same sense, inhibitory products have not been seen to form overwhelmingly in the respective fractions allowing for higher yields under specific conditions within this system. There is, however, the necessity for higher energy costs over steam pretreatment due to the higher pressures and the need for a large amount of water supplied to the system. Like many of the other pretreatments, the severity of the process will depend primarily on the type of lignocellulosic material that is being used and will have to be tailored as such.

4.2.3. Ammonia Fiber Explosion (AFEX). The ammonia fiber/freeze explosion (AFEX) process is another physicochemical process, much like steam explosion pretreatment, in which the biomass material is subjected to liquid anhydrous ammonia under high pressures and moderate temperatures and is then rapidly depressurized. The moderate temperatures (60°C to 100°C) are significantly less than that of the steam explosion process, meaning less energy input and overall costs associated with the process. The degree of disruption to the biomass structure will depend on the temperature, as it will affect the rapidness of the ammonia vaporization within the reactor during depressurization. The residence time can be altered from low (5 to 10 min) to moderate (30 min) lengths depending on the degree of saturation needed for the type of biomass. Ammonia loading is another significant variable that needs to be taken into consideration and is typically chosen to be 1 kg ammonia per kg dry biomass for many feedstocks [123–125].

Much like the steam-explosion process, the ammonia and biomass mixture is saturated for a period of time in a pressurized reactor before being released to atmospheric temperature. This rapid expansion of the ammonia gas causes swelling of the biomass feedstock, creating a disruption in the lignin-carbohydrate linkage, hemicellulose hydrolysis and ammonolysis of glucuronic cross-linked bonds, and partial decrystallization of the cellulose structure, all leading to a higher accessible surface area for enzymatic attack [125–127]. Recovery of the ammonia gas is then needed to reduce the operating costs of the pretreatment. This pretreatment does not remove lignin or any other substances from the biomass; however, the lignin-carbohydrate complexes are cleaved, and the lignin is deposited on the surfaces of the material possibly causing blockage of cellulases to cellulose.

There have been extensive literature reviews on this type of pretreatment over the last decade, focusing on the advantages and disadvantages of the AFEX process used for different feedstocks [12, 19, 73, 74]. An overview of some of the advantages include lower moisture content, lower formation of sugar degradation products due to moderate conditions, 100 percent recovery of solid material, and the ability for ammonia to lessen lignin's effect on enzymatic hydrolysis. A smaller number of disadvantages can be seen in the form of costs due to recycle and treatment of chemicals that are being used.

4.2.4. Ammonia Recycle Percolation (ARP). Ammonia recycle percolation (ARP) has been paired with the AFEX pretreatment process by many authors, but it can have some different characteristics that need to be taken into consideration when looking at different pretreatment options. In this process, aqueous ammonia of concentration between 5–15% (wt%), is sent through a packed bed reactor containing the biomass feed stock at a rate of about 5 mL/min. Moderately high temperatures (140°C to 210°C) and longer reactions times are seen in comparison to the AFEX process, creating higher energy costs [128]. A low-liquid ARP (LLARP) process has also been used in which 3.3 mL/g-biomass and 10 to 12 minutes for the liquid throughput and residence times, respectively, have been achieved without any reduction in effectiveness [129].

The advantage with this process over AFEX is its ability to remove a majority of the lignin (75–85%) and solubilise more than half of the hemicellulose (50–60%) while maintaining high cellulose content [129]. This is due to the selectivity of ammonia and its ability to break down lignin by ammonolysis while also solubilising hemicellulose over the longer retention times. What is left is a treated solid material consisting of short-chained cellulosic material containing

Table 3: Effect of various pretreatment methods on the chemical composition and chemical/physical structure of lignocellulosic biomass (adapted from [12]). H: high effect, L: low effect, ND: not determined, *Depends on the chemical nature of the solvent.

Pretreatment	Increases accessible surface area	Decrystallizes cellulose	Removes hemicellulose	Removes lignin	Alters lignin structure
Steam explosion	H		H		L
Liquid hot water	H	ND	H		L
Dilute acid	H		H		H
AFEX	H	H	L	H	H
ARP	H	H	L	H	H
Lime	H	ND	L	H	H
Green solvents (NMMO and ionic liquids)	H	H	L	H or L*	L
Supercritical fluid	H	H	H		L

a high amount of glucan (removal of 59–70% lignin, solubilisation of 48–57% xylan, and >86% enzymatic digestibility's achieved) [9, 129]. Primarily, herbaceous biomass have been most treated with this process—60–80% delignification has been achieved for corn stover and 65–85% delignification for switchgrass [130]. This process will also limit the production of inhibitors to a point where a washout is not needed for processes downstream [131]. High energy costs and liquid loadings, along with many disadvantages associated with the AFEX process, are still some major concerns that need to be addressed before this process is proven to be economical. The ability to retain high cellulose content after pretreatment will help make this process more attractive, however work still needs to be done for overall improvement.

4.2.5. Supercritical Fluid (SCF) Pretreatment. A supercritical fluid is a material which can be either liquid or gas, used in a state above the critical temperature and critical pressure where gases and liquids can coexist. It shows unique properties that are different from those of either gases or liquids under standard conditions—it possesses a liquid like density and exhibits gas-like transport properties of diffusivity and viscosity. Thus, SCF has the ability to penetrate the crystalline structure of lignocellulosic biomass overcoming the mass transfer limitations encountered in other pretreatments [132]. Additionally, supercritical fluids show tunable properties such as partition coefficients and solubility. Small changes in temperature or pressure close to critical point can result in up to 100-fold changes in solubility, which simplifies separation [133].

Supercritical carbon dioxide (CO_2) with a critical temperature (T_c) of 31°C and a critical pressure (P_c) of 7.4 MPa, has excellent potential for biomass pretreatment. Typically used as an extraction solvent [134] in a number of applications, it has gained importance as a solvent for pretreatment of different varieties of biomass. When used in combination with water, it forms carbonic acid which favors polymer hydrolysis. Once the biomass is pretreated, explosive release of CO_2 disrupts the cellulose and hemicellulose structure, thus increasing the accessible surface area for enzyme hydrolysis. The lower temperatures used in the process aids in the stability of the sugars and prevents degradation observed in other pretreatments. Kim and Hong [135] investigated supercritical CO_2 pretreatment of hardwood (Aspen) and southern yellow pine with varying moisture contents followed by enzymatic hydrolysis. SCF pretreatment showed significant enhancements in sugar yields when compared to thermal pretreatments without supercritical CO_2. Alinia and coworkers [136] investigated the effect of pretreatment of dry and wet wheat straw by supercritical CO_2 alone and by a combination of CO_2 and steam under different operating conditions (temperature and residence time in the reactors). It was found that a combination of supercritical CO_2 and steam gave the best overall yield of sugars. Recent work by Luterbacher and coworkers [137] dealt with high pressure (200 bar) CO_2-H_2O pretreatment of high loadings (40%) of a wide variety of biomass (switchgrass, corn stover, big bluestem, and mixed perennial grasses). The pretreatments were investigated over a wide range of temperatures (150°C to 250°C) and residence times of 20 seconds to 60 minutes. It was found that under these operating conditions, a biphasic mixture of H_2O-rich liquid phase and CO_2-rich supercritical phase coexists and this greatly aids in pretreatment. Such biphasic pretreatment produced glucose yields of 73% for wood, 81% for switchgrass and 85% for corn stover. Even though SCF is being investigated by a number of researchers for pretreatment, the whole process has not proven to be economically viable with the high pressures involved being a deterrent. Improvements need to done to implement the process on a large scale.

5. Summary of Lignocellulosic Biomass Pretreatments

The main aim of pretreatment is to increase accessible surface area, to decrystallize cellulose, and to remove hemicellulose and lignin. The advantages and disadvantages of different methods are listed in Table 1 and the effects of different pretreatments are listed in Table 3 (adapted from Mosier and coworkers [12]). The vast array of biomass types precludes the use of just one type of pretreatment for different feedstocks. What is efficient and economical for one feedstock might not translate to an efficient process for another biomass type. Eggeman and Elander [138] carried out an

Table 4: Capital costs of different pretreatment technologies (adapted from [138]).

Pretreatment method	Pretreatment direct fixed capital, $MM	Pretreatment breakdown, % Reactor/% other	Total fixed capital, $MM	Ethanol production, MM gal/yr	Total fixed capital, $/gal annual capacity
Dilute acid	25.0	64/36	208.6	56.1	3.72
Hot water	4.5	100/0	200.9	44.0	4.57
AFEX	25.7	26/74	211.5	56.8	3.72
ARP	28.3	25/75	210.9	46.3	4.56
Lime	22.3	19/81	163.6	48.9	3.35
No pretreatment	0	—	200.3	9.0	22.26
Ideal pretreatment	0	—	162.5	64.7	2.51

economic analysis of different pretreatment technologies for the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) as part of an initiative of the United States Department of Agriculture. Each pretreatment process was embedded in a full bioethanol facility model and an economic analysis done. The same feedstock for the different pretreatment strategies was used in the analysis, and the resulting solid and fluid streams after pretreatment were characterized, and gathered data were used to close material and energy balances for all the processes. The results of their analysis in terms of the impact of the pretreatment approaches on capital and operating cost investment and glucose and xylose sugar yields are given in Table 4. Their conclusion was that the low-cost pretreatment options are often counterbalanced by the higher costs to recover catalysts/solvents and the higher costs of ethanol product recovery. Thus, there is little difference in the projected economic performance of the different pretreatment options.

However, it was also clearly stated that further process improvements such as identification of optimum enzyme blends for each pretreatment approach and conditioning requirements of the hydrolyzates may lead to greater differentiation of projected process economics. Sendich and coworkers [139, 140] used updated parameters and ammonia recovery configurations in the model of Eggeman and Elander and calculated the cost of ethanol production using AFEX. They found out that the minimum ethanol selling price reduced from $1.41/gal to $0.81/gal. A new research tool—the Biorefinery and Farm Integration Tool (BFIT) was also developed in which the production of fuel ethanol from cellulosic biomass was integrated with crop and animal production models. Such tools will be beneficial in evaluating the economic viability of biorefinery technologies in different landscapes and also show the effect of the biorefinery in improving farm economics and reducing emissions. A comprehensive economic and life-cycle analysis on the use of green solvents (NMMO and ionic liquids) has not yet been carried out. The advantage in using these solvents is that they can be recovered for reuse and in addition the chemistry of the ILs can be tuned to treat a wide variety of biomass in a single process. High loadings of cellulose can be dissolved, thus leading to high sugar yields when enzymatic hydrolysis is carried out. Current efforts are focused on combining the pretreatment using green solvents with subsequent hydrolysis and fermentation steps (SSF) to maximize the volumetric productivity of the desired product. Such efforts require a greater fundamental understanding of the chemical and physical mechanisms that occur during pretreatment and the effect of the chemical structure of the lignocellulosic biomass on subsequent enzymatic hydrolysis and fermentation. Emphasis is also being placed on genetically modifying organisms to ferment at higher temperature and with a capacity to ferment the xylose so that the yield of ethanol is increased.

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Research Article

Study of Soybean Oil Hydrolysis Catalyzed by *Thermomyces lanuginosus* Lipase and Its Application to Biodiesel Production *via* Hydroesterification

Elisa d'Avila Cavalcanti-Oliveira,[1] Priscila Rufino da Silva,[1,2] Alessandra Peçanha Ramos,[1,2] Donato Alexandre Gomes Aranda,[2] and Denise Maria Guimarães Freire[1]

[1] *Laboratório de Biotecnologia Microbiana (LaBiM), Centro de Tecnologia, Instituto de Química, lab. 549-1, Universidade Federal do Rio de Janeiro, CEP 21945-970, Rio de Janeiro, RJ, Brazil*
[2] *Laboratório de Tecnologia Verde (GreenTec), Centro de Tecnologia, Escola de Química, lab. 211, Universidade Federal do Rio de Janeiro, CP. 68542, CEP 21945-970 Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Denise Maria Guimarães Freire, freire@iq.ufrj.br

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The process of biodiesel production by the hydroesterification route that is proposed here involves a first step consisting of triacylglyceride hydrolysis catalyzed by lipase from *Thermomyces lanuginosus* (TL 100L) to generate free fatty acids (FFAs). This step is followed by esterification of the FFAs with alcohol, catalyzed by niobic acid in pellets or without a catalyst. The best result for the enzyme-catalyzed hydrolysis was obtained under reaction conditions of 50% (v/v) soybean oil and 2.3% (v/v) lipase (25 U/mL of reaction medium) in distilled water and at 60°C; an 89% conversion rate to FFAs was obtained after 48 hours of reaction. For the esterification reaction, the best result was with an FFA/methanol molar ratio of 1:3, niobic acid catalyst at a concentration of 20% (w/w FFA), and 200°C, which yielded 92% conversion of FFAs to soy methyl esters after 1 hour of reaction. This study is exceptional because both the hydrolysis and the esterification use a simple reaction medium with high substrate concentrations.

1. Introduction

Biodiesel is composed of esters of fatty acids with a short-chain alcohol (methanol or ethanol). It is an alternative fuel obtained from renewable sources such as vegetable oils and animal fats and emits fewer atmospheric pollutants than petroleum diesel. Because its properties are similar to those of petroleum diesel, biodiesel can be used directly in diesel engines without extensive mechanical modifications or maintenance expenses [1].

Biodiesel production by the hydroesterification route occurs in two stages. First, all the material is hydrolyzed to free fatty acids (FFAs) and glycerol. Next, the FFAs are separated and esterified with a short-chain alcohol, generating biodiesel and water. The hydroesterification process has some advantages over the alkaline transesterification process, which is the method that is most often used industrially. In the latter, a high-quality oil or fat is transesterified with methanol in the presence of NaOH, KOH, or sodium methoxide to produce biodiesel and glycerol [2]. However, the disadvantage of the alkaline transesterification route is the high final cost of the biodiesel, mainly because of the price of high-quality oil/fat (which comprises around 80% of the biodiesel cost) and the low selling price of glycerol (by-product). For the alkaline transesterification process, the raw material must be free of FFAs and water; otherwise, soap is formed, resulting in neutralization of the catalyst and difficulty of purification. Also, the glycerol by-product is contaminated with the salt resulting from the catalyst neutralization and methanol and brings a low price [3–7]. One advantage of hydroesterification for alkaline transesterification is the generation of a glycerol without

contact with methanol (which is highly toxic), and this product can be a higher-value food-grade glycerol. Another major advantage is the ability to employ as raw material oils and fats with high concentrations of FFAs and water, which are considered low quality and therefore have a much lower market price [2]. Economic analysis has shown that the hydroesterification process can be as attractive as or more so than the alkaline transesterification process for biodiesel production [5].

Minami and Saka [8] studied a catalyst-free hydroesterification process to obtain biodiesel, involving a subcritical hydrolysis and a supercritical esterification with methanol, and obtained conversion rates above 90% in the two steps (both conducted at a temperature of 270°C and a pressure of 20 MPa). ("Supercritical" refers to a condition above the critical temperature and pressure of the reagent.) Investigations of biodiesel production by hydroesterification using niobic acid in pellets as a catalyst in hydrolysis [9] as well as in the esterification reaction [9–11] have reported high conversion rates (over 90%) in both stages.

Enzymatic hydrolysis (catalyzed by lipases, E.C. 3.1.1.3) has some advantages over chemical or thermal hydrolysis, because enzyme reactions require lower temperatures, which prevents the degradation of products and reduces energy costs. Furthermore, enzymes are biodegradable and consequently are less polluting than chemical catalysts. The main disadvantages of enzyme catalysis are the longer reaction time and the higher cost of the biocatalysts [12]. Enzymatic esterification is more complicated, because often the enzyme is deactivated by the alcohol used as a substrate [13]. The use of a heterogeneous acid catalyst in this step is very interesting, since it is not deactivated by short-chain alcohol, it can be easily separated from the product and reused, and acid catalysts are quite effective in converting FFAs to esters [6, 14].

Talukder et al. [15] studied the process of enzyme-chemical hydroesterification with lipase from *Candida rugosa* and the chemical catalyst Amberlyst 15 (acidic sulfonated styrene-divinylbenzene ion-exchange resin) and obtained complete conversions in the enzymatic hydrolysis and chemical esterification. Our group also studied enzyme-chemical hydroesterification, but employed as catalysts the lipase of germinated seeds of *Jatropha curcas* and niobic acid in pellets, and also obtained high conversion rates [11].

The majority of enzymatic hydrolysis procedures reported in the literature are impractical because they use a low oil content in the reaction, a high concentration of lipase, or a buffer, organic solvent, and emulsifier, all of which increase costs and complicate the purification step. Therefore, we believe that it is important to search for reaction media that are as simple as possible and use higher substrate concentrations.

The present study evaluated biodiesel production using hydroesterification, with the hydrolysis reaction catalyzed by the lipase from *Thermomyces lanuginosus* (TL 100L) and the esterification reaction catalyzed by niobic acid in pellets or without a catalyst, using simple reaction media with a high substrate concentration. The TL 100L lipase is sold by Novozymes and is recommended by this company for the hydrolysis of triacylglycerides [16].

Table 1: Coded levels and real values of tested variables in the CCRD.

Variables	Levels				
	−1.68	−1	0	1	1.68
Temperature (°C)	47	50	55	60	63
Lipase concentration (%v/v*)	0.5	2.3	5	7.7	9.5
Oil concentration (%v/v*)	55	62	73	84	91

*volume of oil + buffer.

2. Material and Methods

2.1. Catalysts. The liquid lipase from *Thermomyces lanuginosus* (Lipozyme TL 100L) was purchased from Novozymes. This lipase is recommended by Novozymes for hydrolysis and is a "food-grade" enzyme [16]. The lipase TL 100L showed activity of 1064 ± 31 U/mL determined on soybean oil (5% w/v) emulsified with Triton X-100 (25% w/v) in sodium phosphate buffer 0.05 M, pH 7.0 (50% v/v), and distilled water, at 37°C, 200 rpm; the FFAs produced were titrated with 0.04 N NaOH until pH 11. One lipase activity unit (U) was defined as the amount of enzymes that produced 1 μmol FFA per min under the assay conditions.

The chemical catalyst used was niobic acid in pellets (HY-340, surface area 80 m^2/g, and pore volume 0.6 cm^3/g) purchased from CBMM (Companhia Brasileira de Metalurgia e Mineração).

2.2. Enzymatic Hydrolysis of Soybean Oil. The reactions were carried out with a volume of 10 mL, in thermostated reactors, under magnetic stirring. The reaction medium was composed of soybean oil, 10 mM sodium phosphate buffer (pH 8.0), and lipase TL 100L. The concentrations of soybean oil and lipase, as well as the temperature and reaction time were alternated during the study, and the conditions used in each experiment are described below. At the end of the reaction, the oil was extracted with hexane and concentrated in a rotary evaporator, and then the concentration of FFAs was determined from the final acidity of the oil.

A Central Composite Rotatable Design (CCRD) was used to investigate the conversion of the hydrolysis reaction. The levels of the variables studied are shown in Table 1. The results were analyzed using Statistica 7.0.

The hydrolysis reactions to produce the FFAs to be esterified (results of Section 3.2) were conducted with 1 L volume, in a thermostated reactor, under mechanical stirring, because a larger volume was necessary for the esterification reactor. All other conditions were as described above.

Table 2: CCRD matrix and the results of soybean oil hydrolysis by lipase TL 100L. The reactions were carried out in 10 mM sodium phosphate buffer (pH 8.0) for 19 hours.

Assay	Temperature (°C)	Concentration of lipase (% v/v)	Concentration of oil (% v/v)	Final concentration of FFAs (% w/w)
1	50	2.3	62	80.4
2	60	2.3	62	**85.4**
3	50	7.7	62	85.6
4	60	7.7	62	85.6
5	50	2.3	84	66.9
6	60	2.3	84	72.5
7	50	7.7	84	74.3
8	60	7.7	84	71.7
9	47	5.0	73	78.6
10	63	5.0	73	81.0
11	55	0.5	73	70.1
12	55	9.5	73	81.7
13	55	5.0	55	86.8
14	55	5.0	91	66.1
15	55	5.0	73	82.0
16	55	5.0	73	82.1
17	55	5.0	73	84.0
18	55	5.0	73	83.1

2.3. Esterification of Free Fatty Acids. The esterification reactions were carried out using the optimum conditions described by Lima [9]: molar ratio FFAs/alcohol of 1:3 for methanol and 1:4 for ethanol; niobic acid catalyst in pellets at a concentration of 20% (w/w of FFAs), or without catalyst; temperature 200°C; pressure 350 psi; and reaction time 1 hour. At the end of the reaction, the catalyst was removed by filtration and the product was held at 110°C for 5 minutes to eliminate the residual alcohol and water formed. The conversion of FFAs to alkyl esters was determined by titration of the remaining FFAs (acidity).

2.4. Determination of Acidity. The acidity was determined by titration of FFAs with 0.25 N NaOH, and the result was expressed in grams of FFAs per 100 g of the sample [9].

3. Results and Discussion

3.1. Enzymatic Hydrolysis of Soybean Oil. Initially, the reaction conditions for the hydrolysis of soybean oil catalyzed by lipase TL 100L were investigated. A previous experimental design (data not shown) indicated that higher conversion rates could be obtained by fixing the sodium phosphate buffer in 10 mM/pH 8.0 and optimizing the temperature and the concentrations of lipase and soybean oil. This optimization was carried out using a Central Composite Rotatable Design (CCRD). The design matrix and the results obtained are shown in Table 2.

The best conversion of FFAs was obtained in assay 13, in the lowest concentration of soybean oil (55%), at 55°C and with 5% of the biocatalyst (corresponding to 53 U/mL of reaction medium). Based on the statistical analysis of the results, an empirical model was constructed (1) to describe the variation in the FFAs concentration as a function of temperature (T), lipase concentration (L), and oil concentration (O). For the model construction, variables with P-values lower than .05 were considered statistically significant (Figure 1). The interactions between temperature and oil concentration and between lipase concentration and oil concentration were not statistically significant and were not included in the model (1).

$$\text{FFA (\% w/w)} = 82.76 + 1.76T - 1.8T^2 + 4.62L - 4.56L^2 - 12.66O - 4.17O^2 - 3.3T^*L. \quad (1)$$

The percentage of variance explained by the model was satisfactory ($R^2 = 0.98$), indicating that the model had a good adjustment to the experimental data, as observed in Figure 2

Figures 3, 4, and 5 show the contour plots of the concentrations of FFAs obtained by the model of (1).

As apparent from Figure 3, the best conversion rates should be obtained at temperatures between 50°C and 60°C, and higher temperatures require lower lipase concentrations. In the literature, there is no consensus about the optimum reaction temperature for *T. lanuginosus* lipase. While some studies have found values of 37°C [17] and 40°C [18], other authors have obtained values of 50°C [19] and 55°C [20, 21]. The behavior observed in Figure 3, that higher temperatures require lower lipase concentrations to obtain the same conversion rate, may be related to a higher enzyme activity at 60°C in these reaction conditions; consequently, less lipase is needed at higher temperatures because the enzyme is more active. Another possibility is related to

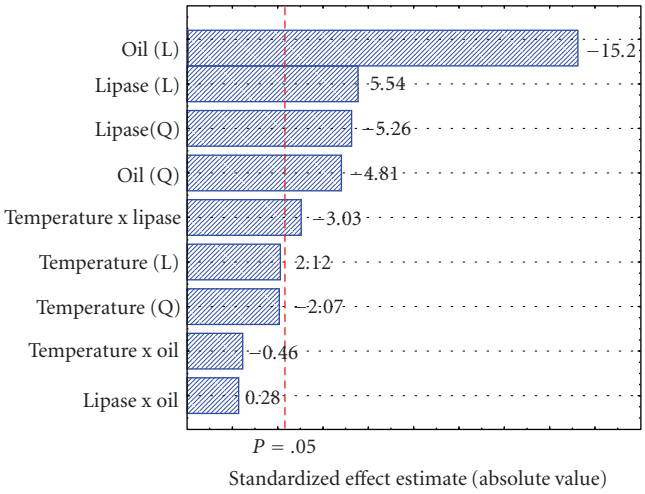

Figure 1: Pareto graph showing the effects of different variables on the concentration of FFAs, at the CCRD.

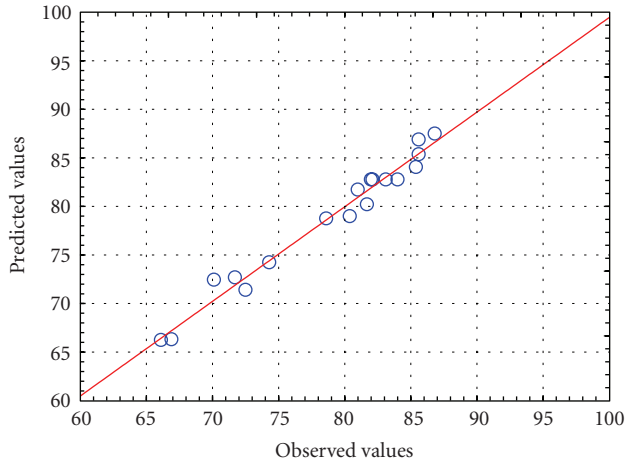

Figure 2: Graph of the values of FFA concentrations (%) predicted by the model, versus values observed experimentally.

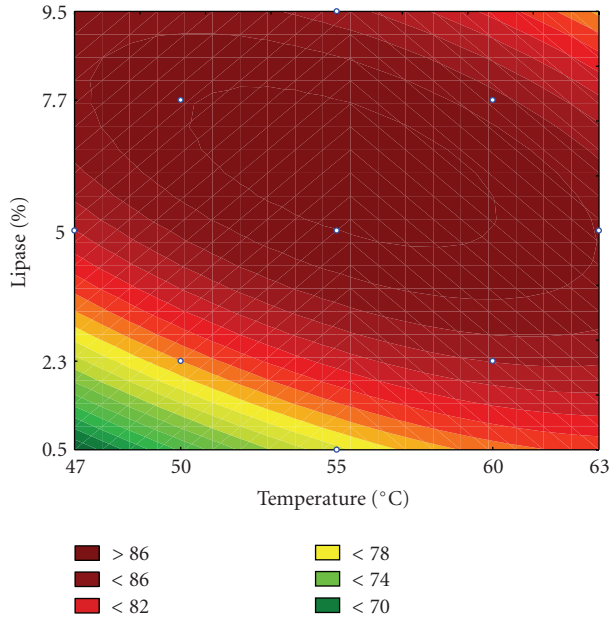

Figure 3: Contour plot for FFAs concentration as a function of lipase content and temperature, at an oil concentration of 62%.

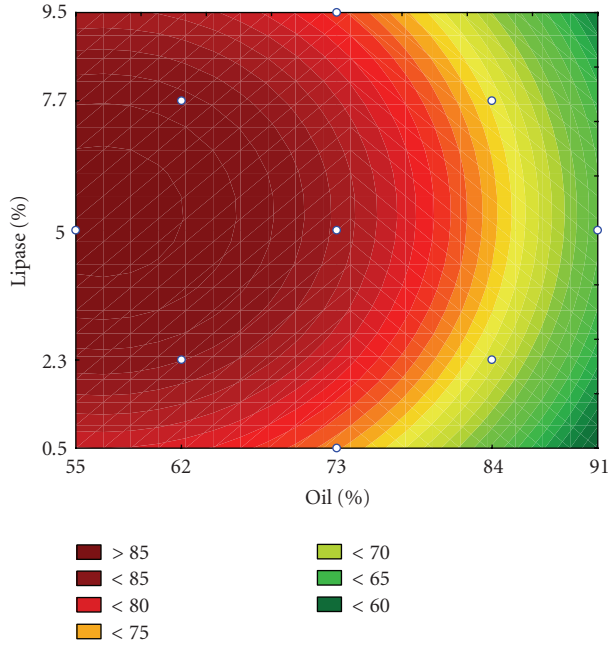

Figure 4: Contour plot for FFAs concentration as a function of lipase content and oil concentration, at 60°C.

increased acyl migration, since this lipase is 1,3-specific and therefore complete hydrolysis of triglyceride depends on the migration of the acyl radical in position 2 to positions 1,3 [22]. Li et al. [23] observed that the increase in temperature increased the acyl migration in their experiments without an enzyme: when they changed the incubation temperature of 30°C to 55°C, the half-life of 1,2-diacylglycerol decreased 10-fold and the half-life of 2-monoacylglycerol decreased 6-fold. For the next experiments, we selected the temperature of 60°C since it gave the highest conversion rate with a lower enzyme concentration (Figure 3).

As can be seen in Figure 4, the model indicated that the best conversions at 60°C should be obtained with concentrations of lipase between 4% and 8%. The existence of an optimum enzyme concentration range may be explained by two factors: at concentrations lower than 4%, more enzyme may favor the accessibility of a large amount of reactants to the active sites, and at concentrations above 8%, dimers with lower activity may be forming. Fernandez-Lafuente [22] reported a strong tendency of *Thermomyces lanuginosus* lipase to dimer formation through the union of two molecules of lipase in the regions of their active centers. This dimer is more stable than the monomer; however, it has lower activity [22]. We opted for a lipase concentration of 2.3%, since this concentration also gives good results

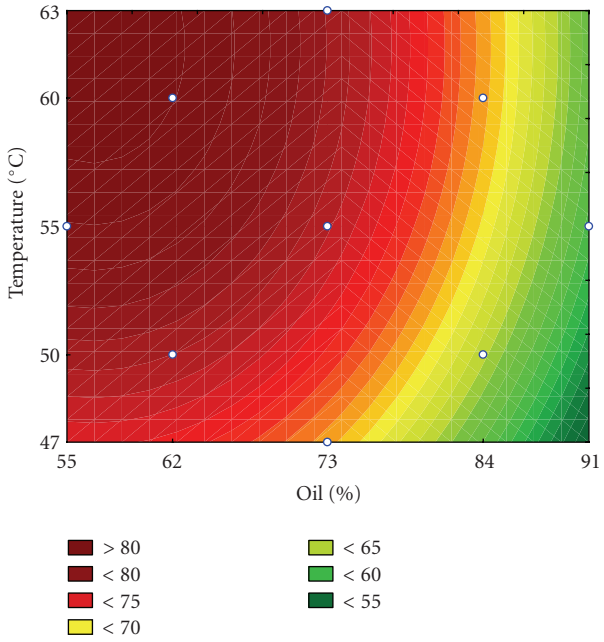

Figure 5: Contour plot for FFAs concentration as a function of temperature and oil concentration, for a lipase content of 2.3%.

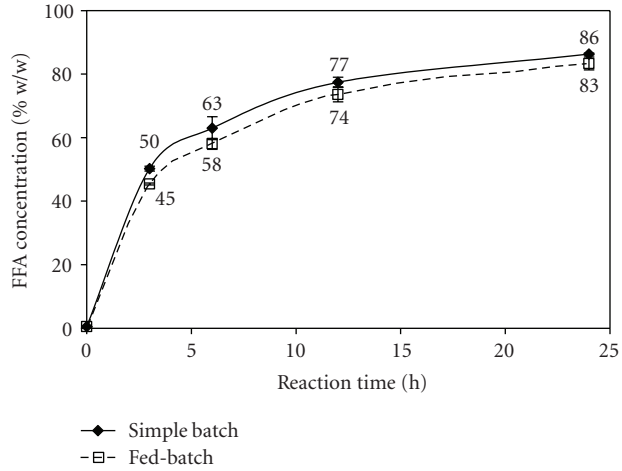

Figure 6: Reaction kinetics of simple batch and fed-batch hydrolysis of soybean oil by TL 100L lipase. The reaction conditions were 2.3% (v/v) lipase (for the fed-batch this volume of enzyme was divided into 3 equal parts that were added at times zero, 6, and 12 hours), 62% (v/v) soybean oil, 10 mM buffer (pH 8.0), at 60°C, for 24 hours.

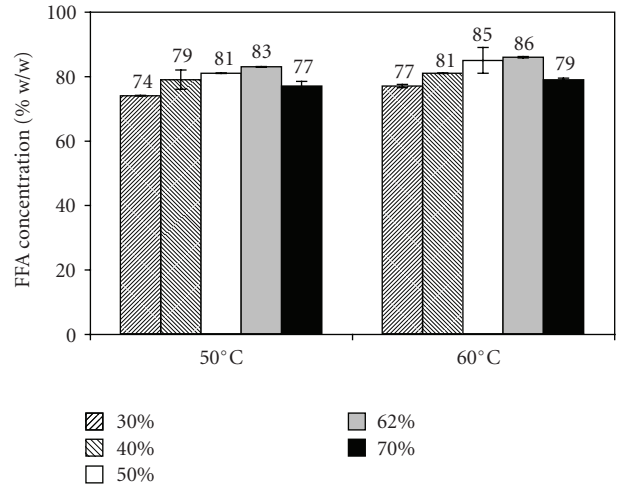

Figure 7: Effect of oil concentration on the soybean oil hydrolysis reaction catalyzed by lipase TL 100L. The reactions were conducted with 2.3% (v/v) lipase, with different concentrations of soybean oil, in 10 mM phosphate buffer, pH 8.0, 50°C, or 60°C, for 24 hours.

(Figure 4) and reduces expenses with the biocatalyst in the process.

For a lipase concentration of 2.3%, the model showed the highest conversion rates with oil concentrations lower than 62% and temperatures higher than 58°C, as illustrated in Figure 5. The higher conversion rates for lower substrate concentrations were expected, because there are more substrate molecules present to access the active sites of enzymes.

In the conditions selected based on the CCRD (60°C; 2.3% lipase; 62% oil), reaction kinetics were carried out in a simple batch and in a fed-batch of enzyme, as shown in Figure 6. The fed-batch of enzyme was chosen for evaluation based on the observation of Jurado et al. [19] that after only 30 minutes at 60°C, the *T. lanuginosus* lipase lost 13% of its activity.

Our results in Figure 6 showed very similar reaction kinetics for the simple batch and fed-batch of enzymes (reaching 86 ± 0.3% and 83 ± 2.0% FFAs after 24 hours, resp.); lipase hydrolytic activity could be observed after 24 hours in the simple batch, showing that the enzyme was not sufficiently deactivated to compromise the reaction. Other investigators have also observed that the lipase from *T. lanuginosus* is capable of maintaining its activity at elevated temperatures such as 50°C–60°C, with maximum activity near pH 9 [22]. This thermostability was expected, since *T. lanuginosus* is a thermophilic fungus that produces other thermostable enzymes [24–28].

We also investigated the utilization of lower concentrations of soybean oil, in an attempt to attain higher concentrations of FFAs, as indicated in Figure 5. The results of the reactions with lower oil concentrations are shown in Figure 7.

One can observe in Figure 7 that the best conversions were obtained for reactions conducted with 50% and 62% (v/v) of oil at 60°C. Although the lower rate of conversion at oil concentrations below 50% was unexpected, it was previously observed by Talukder et al. [7] in the hydrolysis reaction of crude palm oil catalyzed by *Candida rugosa* lipase.

The best concentrations of soybean oil (50% and 62% oil at 60°C) (Figure 7) were evaluated over a longer reaction time. For the reaction carried out with 50% (v/v) soybean oil, the results were 91 ± 0.9% FFAs at 48 hours and 93 ± 0.8% at 72 hours. The reaction with 62% (v/v) soybean oil yielded 87 ± 0.5% FFAs at 48 hours and 86 ± 0.6%

at 72 hours. Although both substrate concentrations gave similar results at 24 hours reaction time (Figure 7), the 50% concentration led to higher concentrations over longer times.

Subsequently, the use of distilled water instead of buffer was evaluated, in order to reduce the cost of the process. The reaction was carried out with 50% soybean oil in the same reaction conditions as described above, and we obtained 84 ± 0.6% FFAs at 24 hours, 89 ± 0.3% at 48 hours, and 90 ± 0.1% at 72 hours. The reaction with distilled water gave practically the same result as the reaction carried out in the buffer, so it was selected as the best option to be added to the reaction medium.

Thus, the selected conditions for soybean oil hydrolysis by lipase TL 100L were 50% (v/v) soybean oil, distilled water, 2.3% (v/v) lipase (corresponding to 25 U/mL of reaction medium), 60°C, and 48 hours. Under these conditions, we obtained 89% hydrolysis.

This study is exceptional among enzymatic hydrolysis studies in the literature, in combining the high oil content with the low concentration of lipase, the use of aqueous media with no buffer, no organic solvent, and no emulsifier, which makes the reaction and purification steps easier and less costly (very important for a low value product as it is the biodiesel).

Freitas et al. [29] obtained 70 and 53% soybean oil hydrolysis using lipases from *Candida rugosa* and *Thermomyces lanuginosus*, respectively. In addition to obtaining lower conversion rates, these authors used an oil content of 25%, half that used in the present study, and 2.5% gum Arabic (emulsifier). In the study of Park et al. [30], combinations of lipase from *Penicillium* sp. + *R. niveus* and *Penicillium* sp. + *R. delemar* hydrolyzed up to 95%–98% of the soybean oil; the reaction was conducted with 3 g oil and 4 mL buffer for 10 hours. Park and colleagues used two lipases, whereas we used only one and did not use a buffer.

Talukder et al. [7] obtained complete hydrolysis of crude palm oil, but they used 33% oil, 33% organic solvent (isooctane), and a buffer in the reaction medium. Edwinoliver et al. [31] obtained a palm oil hydrolytic rate of 89% with a reaction medium containing 1 g oil in 20 mL buffer; in this case, the oil content was very low and they also used a buffer. In the study by Noor et al. [32], nearly all the palm oil was hydrolyzed in only 1.5 hours, but they employed an oil concentration of approximately 2.5% and an emulsifier.

In the study by Goswami et al. [33], castor oil was 60% hydrolyzed by *Candida rugosa* lipase, employing a 3:1 buffer phase volume to oil weight ratio. This is a lower oil content than that used in the present study, and also made use of a buffer, although they achieved a good reaction time of 6 hours.

Our research group [11] obtained complete hydrolysis of physic nut (*Jatropha curcas*) oil catalyzed by the lipase from its own seed. The reaction was conducted with 50% oil and 2.5% crude extract containing lipase, without adding organic solvent or emulsifier, although a buffer was used. Another promising study, by Talukder et al. [15], on the hydrolysis of waste cooking oil by *Candida rugosa* lipase, achieved complete conversion after 10 hours at an oil concentration of 50% and lipase concentration of 0.025%, without additives to the reaction medium (water).

Some studies have employed even more complicated techniques in enzymatic hydrolysis, such as ultrasound [34, 35] and supercritical CO_2 [36] to increase the oil-water interface area where the lipase acts.

3.2. Esterification of FFAs to Biodiesel. After the hydrolysis of soybean oil with lipase TL 100L (under the optimum conditions described above), the esterification of FFAs with methanol and ethanol was carried out, catalyzed by niobic acid in pellets or without a catalyst. The results are shown in Table 3.

Based on the data presented in Table 3, the best esterification rate was 92%, using methanol as the alcohol and niobic acid as the catalyst.

The higher reactivity of methanol compared to ethanol can be observed with and without the catalyst (Table 3). Similar behavior was observed by Aranda et al. [6] in the esterification of FFAs from palm oil using heterogeneous acid catalysts; they obtained up to 70% lower conversion rates using ethanol instead of methanol. Steric hindrance inherent to both the carboxylic acid and the alcohol species seems to be important, making reaction with methanol (a smaller molecule) faster, especially with heterogeneous catalysts [6]. Furthermore, the anhydrous ethanol has much more water than anhydrous methanol, and the water acts to shift the reaction equilibrium toward hydrolysis rather than esterification [14]. Although ethanol has some advantages, such as being a renewable resource and having low toxicity, as opposed to methanol, the latter is still widely used in biodiesel production due to its higher reactivity and lower cost [5, 37].

The niobic acid catalyst had a positive effect on the conversion of the esterification reaction of FFAs, giving 10.4% and 6.0% higher conversions with methanol and ethanol, respectively (Table 3). Because it is a heterogeneous catalyst, niobic acid in pellets can be easily separated from the product and reused.

Aranda et al. [6] evaluated the esterification of palm-oil FFAs with methanol, using different heterogeneous catalysts and obtained conversion rates similar to those in this study. Talukder et al. [15] used Amberlyst 15 as a catalyst for biodiesel production by esterification of FFAs from waste cooking oil with methanol, and obtained 99% esterification; however, they added an organic solvent to the reaction medium, which increases the cost of reagents and the purification process. Lima [9] studied biodiesel production from soybean and castor bean oils, using the process of hydroesterification catalyzed by niobic acid in pellets, and obtained conversion rates of 84% for the reaction of hydrolysis of soybean oil and 82% for castor bean oil; for the esterification reactions, the highest conversion rates were 92% for FFAs from soybean oil and 87% for those of castor bean oil. In the present study, we obtained higher conversion rates (89%) for the hydrolysis reaction of soybean oil catalyzed by lipase TL 100L, and a similar conversion rate (92%) for the esterification reaction of FFAs.

Table 3: Esterification reactions of FFAs of soybean oil. The reactions were carried out using a molar ratio of FFAs/alcohol of 1:3 for methanol and 1:4 for ethanol, 20% (w/w of FFAs) of the catalyst niobic acid in pellets, at 200°C and 1 hour reaction time.

Alcohol	Catalyst	FFAs % (w/w)		Conversion*
		Initial	Final	
Methanol	No	90.6%	15.1%	83.3%
	Yes	91.3%	7.3%	92.0%
Ethanol	No	90.6%	19.9%	78.0%
	Yes	91.3%	15.8%	82.7%

*Conversion was calculated based on the initial concentration of FFAs.

4. Conclusion

The process of biodiesel production through the enzyme-chemical hydroesterification route catalyzed by *Thermomyces lanuginosus* lipase and niobic acid in pellets yielded 89% FFAs in the hydrolysis reaction of soybean oil, and 92% of these FFAs were esterified to biodiesel.

The hydrolysis reaction was carried out with a low lipase concentration (2.3%) and high oil concentration (50%), in simple aqueous medium without a buffer, emulsifier, or solvent, to lower the cost of the process. In the esterification reaction, we also used a solvent-free medium with high substrate concentration (75% FFA). The use of a heterogeneous catalyst, which can be easily separated and reused, also reduced the cost of the process.

The enzymatic-chemical hydroesterification appears to be a promising alternative to the traditional process of biodiesel production by alkaline transesterification.

Acknowledgments

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Research Article

Enzymatic Biodiesel Synthesis Using a Byproduct Obtained from Palm Oil Refining

Igor Nascentes dos Santos Corrêa,[1] Susana Lorena de Souza,[1] Marly Catran,[1] Otávio Luiz Bernardes,[1] Márcio Figueiredo Portilho,[2] and Marta Antunes Pereira Langone[1]

[1] *Institute of Chemistry, Rio de Janeiro State University, Rua São Francisco Xavier 524, PHLC/IQ sala 310, Rio de Janeiro, RJ 20550-013, Brazil*
[2] *Cenpes, Petrobras, Rio de Janeiro, RJ 21949-900, Brazil*

Correspondence should be addressed to Marta Antunes Pereira Langone, langone@uerj.br

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An alternative route to produce biodiesel is based on esterification of free fatty acids present in byproducts obtained from vegetable oil refining, such as palm oil fatty acid distillate (PFAD). PFAD is a byproduct of the production of edible palm oil, which contains 96 wt.% of free fatty acids. The purpose of this work was to study biodiesel synthesis via esterification of PFAD with methanol and ethanol, catalyzed by commercial immobilized lipases (Novozym 435, Lipozyme RM-IM, and Lipozyme TL-IM), in a solvent-free system. The effects of reaction parameters such as type of lipase, enzyme amount, type of alcohol, alcohol amount, and enzyme reuse were studied. Fatty acid conversion of 93% was obtained after 2.5 h of esterification reaction between PFAD and ethanol using 1.0 wt.% of Novozym 435 at 60°C.

1. Introduction

Biodiesel is defined as monoalkyl esters of long chain fatty acids, preferentially methyl and ethyl esters, derived from renewable feedstock, such as vegetable oils or animal fats [1]. Biodiesel is a biodegradable and nonpolluting fuel that has received increasing attention in the recent past. However, the major barrier for the commercialization of biodiesel is its high manufacturing cost [2]. An alternative route to produce biodiesel is based on esterification of free fatty acids present in high concentrations in byproducts obtained from vegetable oil refining [3]. Deodorizer distillates and soapstock are the major byproducts from vegetable oil refining. They have little commercial value and are sold at a fraction of the oil cost [4].

Considering the increase of edible oils production caused by the enhancing of biodiesel production and the human population increase [4], it is necessary to investigate uses for these byproducts in order to avoid their discharge on the environment and produce potential high-value products as well.

In these way, the majority of the published research focuses on the recuperation of high-value chemicals such as tocopherols and sterols from vegetable oil deodorizer distillates (VODD) [5–9]. Notwithstanding, there are also publications about the use of this feedstock to biodiesel synthesis [3, 10–14].

The composition of deodorizer distillates varies widely as it depends on several factors including the raw material and the conditions of the oil refining process [6, 9]. Usually, the free fatty acids content in VODD varies between 25 and 75% [9, 11]. For example, the palm oil fatty acid distillates (PFAD) is a byproduct of physical refining of crude palm oil and is composed mainly of free fatty acids (~82%) with smaller amounts of glycerol esters and nonglyceride components (vitamin E, squalene, and phytosterols) [15]. It is estimated that the total production of PFAD represents 4% of crude palm oil production and the average monthly

export price of PFAD in 2008 was 501 (range 302–730) (FOB USD/Tonne) [15].

Conversion of free fatty acids (FFA) present in VODD to fatty acid alkyl esters can be performed by esterification reactions using lipases as biocatalysts:

$$\text{FFA} + \text{alcohol} \longrightarrow \text{fatty acid alkyl esters (biodiesel)} + \text{water}. \quad (1)$$

Enzyme-based processes are carried out under mild reaction conditions that reduce energy cost as well as tend to have lower waste treatment costs [16]. Furthermore, the reuse of immobilized enzymes is possible, minimizing costs and turning these processes economically available [17].

Following the alternative current trend to develop environmentally acceptable use for byproducts from vegetable oil refining, the aim of this work is to produce biodiesel by the esterification reaction of fatty acids from PFAD with short-chain alcohols using immobilized commercial lipases. The effects of reaction parameters such as type of enzyme, enzyme amount, type of alcohol, alcohol amount, and enzyme reuse were investigated.

2. Materials and Methods

2.1. Materials. PFAD from palm oil refining process was provided by Piraquê S.A. (Brazil). Commercial immobilized lipases used were Lipozyme RM-IM (lipase from *Rhizomucor miehei*), Lipozyme TL-IM (lipase from *Thermomyces lanuginosus*), and Novozym 435 (lipase from *Candida antarctica*), all kindly donated by Novozymes Latin America Ltda. (Araucária, Brazil). Methanol (P.A.), ethanol (P.A.), butanol (P.A.), acetone (P.A.), and n-hexane (99%) were obtained from Vetec Química Fina Ltda (Rio de Janeiro, Brazil). Oleic acid (extra pure) was purchased from Merck (Darmstadt, Germany).

2.2. PFAD Characterization. Acidity and acid value of PFAD were determined according to AOCS Te 1a-64 [18]. Iodine index and moisture content were determined according to AOCS Cd 1d-64 and Ca 2e-84 [18], respectively. Fatty acids present in PFAD were determined according to AOCS Ce 1f-96 [18], using an HP 6890N GC equipped with a flame ionization detector and capillary column SP 2340.

2.3. Measurement of Lipases Activities. The esterification activity of commercial lipases, Lipozyme RM-IM, Lipozyme TL-IM, and Novozym 435, was measured by the consumption of oleic acid at 45°C in the esterification reaction with butanol (oleic acid/butanol molar ratio of 1) with the enzyme amount of 3 wt.%. One esterification unit was defined as the amount of enzyme that consumed 1 μmole of oleic acid per minute (U) per g of enzymatic preparation under the experimental conditions described herein. The esterification activities of commercial lipases, Lipozyme RM-IM, Lipozyme TL-IM and Novozym 435, were 1,510, 454, and 2,960 (μmoles of acid$\cdot$min$^{-1}\cdot$g^{-1}), respectively.

2.4. Esterification Reaction. Esterification reactions took place in a closed 15 mL batch reactor magnetically stirred, coupled to a condenser in order to avoid alcohol loss. Water circulating in the condenser was cooled by a thermostatic bath. Reacting medium temperature (60°C) was kept constant by circulating hot ethylene glycol through the reactor jacket. A thermostatic bath (Haake DC30) allowed a close control over the process temperature. The mass of PFAD was always 8 g while the alcohol mass ranged between 1 and 4 g. The alkyl esters (biodiesel) synthesis was evaluated as a function of the type of lipase (Novozym 435, Lipozyme RM-IM, and Lipozyme TL-IM), enzyme amount (0.1, 0.5, 1, 1.5, 3, 4.5, 6, 7.5, and 9 wt.% based on PFAD mass), and type of alcohol used (methanol or ethanol) and in respect to stepwise addition of alcohol (single addition, at time 0, or two consecutive alcohol additions, i.e., 1/2 of alcohol added at time 0 and 1/2 after 30 minutes).

2.5. Quantification of Free Fatty Acids (FFAs). Reaction progress was monitored by taking duplicate samples (100 μL) each 30 min until 2.5 h of reaction. The amount of FFAs in reaction medium samples was analyzed by titration with NaOH 0.02 mol$\cdot$L^{-1} using a Mettler DL 25 autotitrator. Conversion was defined as the number of moles of fatty acids reacted per mole of fatty acids fed to the system.

2.6. Chromatography Analysis (Biodiesel Yield). The fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE) content in the reaction medium was analyzed on Varian gas chromatograph (CP-3380 model), equipped with a flame ionization detector (FID), a CP WAX 52 CB capillary column 30 m $\times$ 0.25 mm $\times$ 0.25 mm, and a split injection system with a 1 : 20 ratio. Injector and detector temperatures were kept at 280 and 300°C, respectively. The oven was initially maintained at 200°C for 4.5 min, then heated up to 250°C at a 20°C min^{-1} rate, and kept constant at the final temperature for 5 min. Hydrogen was used as the carrier gas at a 2 mL min^{-1} flow rate; column pressure was set at 20 psi. A computer loaded with the Star Workstation 6.2 software was connected to the GC by a Star 800 Module Interface to automatically integrate the peaks obtained. Methyl heptadecanoate was used as internal standard. The yield of biodiesel was calculated as grams of biodiesel produced per grams of PFAD.

2.7. Enzyme Reuse. The enzyme reuse was evaluated in the esterification reaction of PFAD with alcohol (methanol, ethanol) at 60°C, with the enzyme concentration of 3 wt.%. The alcohol was added in a stepwise manner (T_0 = 0.5 g, $T_{30\,\text{min}}$ = 0.5 g). The enzyme was separated from the reaction medium by decantation, washed with n-hexane, vacuum filtered, and placed in a desiccator for 24 h. After this treatment, the enzyme was reused in a new batch.

3. Results and Discussion

3.1. PFAD Characterization. The byproducts from vegetable oil refining process are complex mixtures, and there are

Table 1: Physicochemical properties of PFAD.

Acidity (wt.%) (as oleic acid)	96.3
Acid value (mg KOH/g sample)	191.6
Iodine index (I_2/100 g)	63.8
Moisture content (%)	0.70

Table 2: Fatty acid composition of PFAD (wt.%).

Fatty acid	Composition (%)
Caprilic acid C8	0.0
Capric acid C10	0.1
Lauric acid C12	0.3
Myristic acid C14	0.9
Palmitic acid C16	**46.1**
Palmitoleic acid C16:1	0.3
Stearic acid C18	5.5
Oleic acid C18:1	**36.6**
Linoleic acid C18:2	9.2
Linolenic acid C18:3	0.3
Arachidic acid C20	0.3
Gadoleic acid C20:1	0.1
Behenic acid C22	0.0
Lignoceric acid C24	0.0
Other fatty acids	0.1
Total trans isomer	0.2

few publications concerning their full analysis and characterization [4]. PFAD was characterized according to its physicochemical properties (Table 1). Fatty acids are the major components of PFAD. They represent approximately 96% (as oleic acid) of the PFAD composition on a wet basis (Table 1). This value agrees with the free fatty acid content in the PFAD used by Chongkhong et al. [13], 96 wt.%, and by Top [15], 70–90 wt.%.

Acid value and iodine index of PFAD are also shown in Table 1. Some chemical properties of raw materials influence biodiesel quality. The iodine index is related to the grade of oil unsaturation, and, in general, its value has to be lower than 115% for biodiesel production [19]. An iodine index of 63.8% was determined for the PFAD used in this work (Table 1).

The fatty acid profile of the raw material plays an important role on biodiesel properties. The chemical composition of PFAD regarding fatty acids is shown in Table 2. Palmitic (46.1%) and oleic (36.6%) acids are predominant in the PFAD studied in this work. The PFAD composition showed in Table 2 is similar to the fatty acid profile of the two samples of PFAD reported by Top [15].

As reported in the literature, fatty acid content of byproducts obtained from vegetable oil refining is a reflection of the parent oil composition [4]. Usually, palm oils contain 44% of palmitic acid and 39% of oleic acid [20]. So, the composition showed in Table 2 agrees with the expected values.

3.2. Effects of the Type of Lipase. Methanol is the most used alcohol for biodiesel production, and to a lesser extension, ethanol. As methanol is cheaper and more reactive and the fatty acid methyl esters (FAME) are more volatile than those obtained from ethanol (FAEE, fatty acid ethyl esters), it is chosen [21]. However, considering that Brazil is the largest world producer of ethanol and that ethanol can be considered more renewable because it can be produced from renewable sources by fermentation, the reactions for biodiesel synthesis from PFAD were carried out with methanol and ethanol.

The most widely used commercial immobilized lipases (Novozym 435, Lipozyme RM-IM, and Lipozyme TL-IM) were compared regarding conversion of fatty acids from a PFAD. Reactions were carried out at 60°C, considering the melting point of PFAD (~50°C), using 3 wt.% (based on PFAD weight) of enzyme and 2 g of alcohol (methanol or ethanol). The alcohol was added in a stepwise manner (T_0 = 1.0 g, $T_{30\ \mathrm{min}}$ = 1.0 g) to avoid high amounts of alcohol in the reaction medium at the beginning of the reaction. According to the results shown in Figure 1, Novozym 435 presented the highest conversion in the presence of methanol (95%) and ethanol (91%). These results are expected considering the esterification activity values obtained in this work for the three commercial lipases. The esterification activities of Lipozyme RM-IM, Lipozyme TL-IM, and Novozym 435 were 1,510, 454, and 2,960 (μmoles of acid$\cdot$min$^{-1}\cdot$g^{-1}), respectively. Novozym 435 showed the highest esterification activity. It has been reported as one of the most active and versatile enzyme [3, 22–24]. This enzyme shows high stability even in the presence of low-molecular-weight alcohols, like methanol and ethanol.

Besides commercial lipases presented different activities for esterification, conversions of fatty acids showed in Figure 1 may be explained by several reasons. For instance, whereas the reaction was carried out in a batch reactor, one cannot disregard the possible denaturation of enzyme in presence of reagents or products during the reaction time. Moreover, considering the variety of fatty acids present in PFAD, lipases may exhibit different selectivities depending on the number of carbon atoms as well as on the presence of unsaturations in the acid molecule.

Lipozyme RM-IM is also a quite active and stable lipase in anhydrous media and thus has been widely used in esterification reactions [22]. Using this enzyme, a conversion of 52.3% was obtained with methanol and 53.4% with ethanol.

Lipozyme TL-IM was less active for the esterification reaction than the other studied lipases. The PFAD conversion values were 28.8% and 33.1% in the reactions with methanol and ethanol, respectively. This enzyme shows better performance in transesterification reactions, as reported in literature [22].

Similar results were obtained by Souza et al. [3] in soybean oil deodorizer distillate (SODD) esterification with ethanol using immobilized commercial lipases. Reactions were carried out at 50°C using 3 wt% of enzyme (Novozym 435, Lipozyme TL-IM, and Lipozyme RM-IM) and 2 g of ethanol (T_0 = 1 g; $T_{30\ \mathrm{min}}$ = 1 g). Novozym 435 presented the

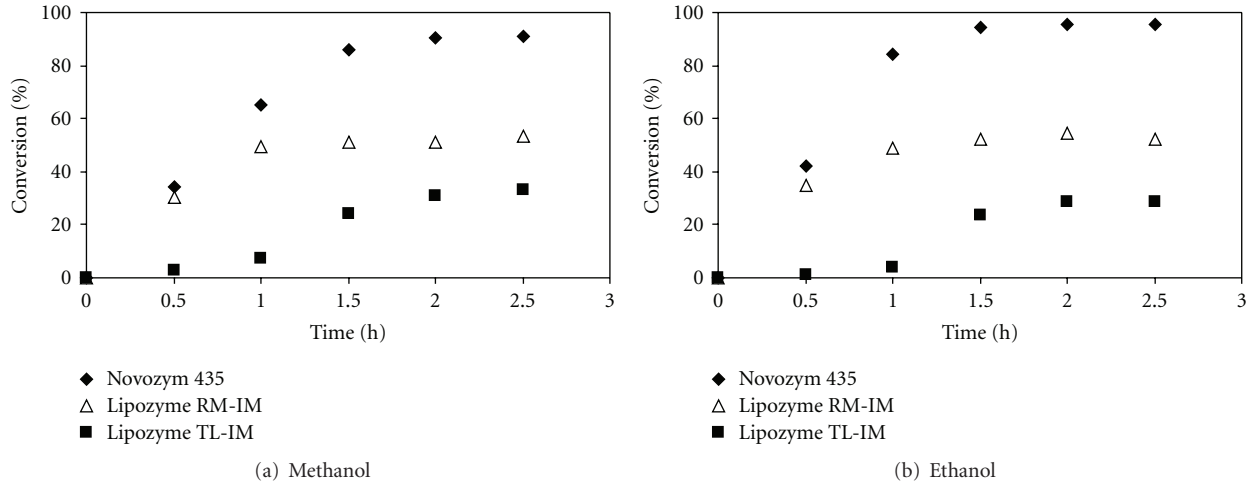


Figure 1: Effects of type of lipase on fatty acids conversion during 2.5 h, using 3 wt.% of commercial lipase, 8 g of PFAD, and 2 g of alcohol added in two steps (T_0 = 1 g, $T_{30\ \mathrm{min}}$ = 1 g) at 60°C. (a) Methanol; (b) ethanol.

highest SODD conversion (83.5%), while Lipozyme RM and Lipozyme TL-IM showed conversion values equal to 59.1 and 16.4, respectively. The chemical composition of PFAD and SODD (byproducts from vegetable oil refining) regarding fatty acids and lipase selectivity may explain the differences in conversion values observed for the three commercial lipases. Palmitic (46.1%) and oleic (36.6%) acids are predominant in the PFAD while in the SODD the predominance of palmitic acid (30.9%), followed by oleic acid (23.8%) and stearic acid (23.7%), was observed.

As Lipozyme TL-IM was the less active enzyme tested in this work conditions, the following experiments were carried out using just Lipozyme RM-IM and Novozym 435.

3.3. Effects of the Type and Amount of Alcohol. The effects of initial amount of alcohol and its feeding technique to the reactor (stepwise or single addition) on the fatty acid conversion were investigated. The amount of PFAD was always 8 g while methanol and ethanol mass ranged between 1 and 4 g. Based on the PFAD composition shown in Table 2, we can admit that the proportion 8 g of distillate : 1 g of alcohol corresponds nearly to a molar ratio 1 : 1 of fatty acids to alcohol. So, the PFAD fatty acids would be the limiting reagent.

Figure 2(a) shows that an increase in methanol amount when it was completely added at the beginning of the reaction leads to a decrease in fatty acid conversion for both enzymes. Talakuder et al. [25] also observed that the biodiesel yield in the PFAD esterification reactions drops at a methanol content of more than 13 wt.% of PFAD. These results are in agreement with those obtained in our work in the reactions using 1 g of methanol and 8 g of PFAD (methanol content = 12.5 wt.% of PFAD).

When ethanol was employed, lower conversion values were obtained with increasing concentration of alcohol only for the reactions catalyzed by Lipozyme RM-IM (Figure 2(b)). So, these results show that the destabilizing effect of alcohol on lipases decreased with increasing alcohol molecular weight as previously reported by other authors [3, 22, 24]. Similar results were observed in esterification reactions of ethanol and soybean oil deodorizer distillate [3]. Moreover, loss of lipase activity caused by the polar short-chain alcohols was greater for Lipozyme RM-IM than for Novozym 435 as has already been reported [23, 24].

The stepwise addition of alcohol has been the main strategy to avoid lipase denaturation caused by high initial concentration of alcohol. In this system, alcohol concentration in the reaction medium was always kept low and thus reducing enzyme deactivation. As shown in Figure 3(a), higher fatty acid conversion was obtained with stepwise addition of methanol (two consecutive alcohol additions, i.e. 1/2 of alcohol added at time 0, and 1/2 after 30 minutes). Using ethanol, this effect was not as pronounced for the reactions catalyzed by Novozym 435 (Figure 3(b)). Novozym 435 has a greater resistance to the presence of short-chain alcohols than the immobilized lipase Lipozyme RM-IM. This explains that stepwise addition has a minor impact on the conversion in the case of Novozym 435.

3.4. Effects of Lipase Amount. Effects of enzyme amount on fatty acids conversion were studied using 0.1, 0.5, 1, 1.5, 3, 4.5, 6, 7.5, and 9 wt.% of enzyme (Lipozyme RM-IM and Novozym 435) at 60°C, with stepwise addition of 2 g of alcohol. Results are shown in Figure 4.

When Lipozyme RM-IM was used in esterification reactions of PFAD with methanol, an increase in conversion as enzyme concentration increased up to 9 wt.% has been observed, achieving a conversion of 73.8%. For the reactions conducted with Novozym 435, conversions above 93% were obtained with only 1 wt.% of enzyme. However, under the same conditions (1 wt.%), Lipozyme RM-IM enabled a lower conversion (45%).

The effect of the concentration of lipase (0.1 to 9 wt.%) was also evaluated in reactions carried out with ethanol

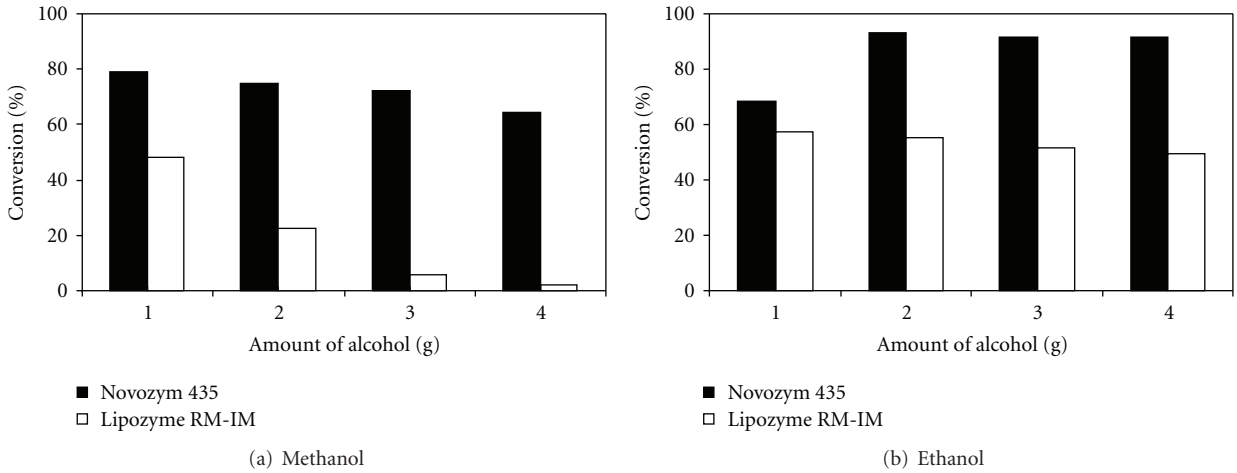


Figure 2: Effects of alcohol amount on fatty acids conversion after 2.5 h of reaction, using a single addition of the alcohol at the beginning of reaction, 3 wt.% of commercial lipase, and 8 g of PFAD at 60°C. (a) Methanol; (b) ethanol.

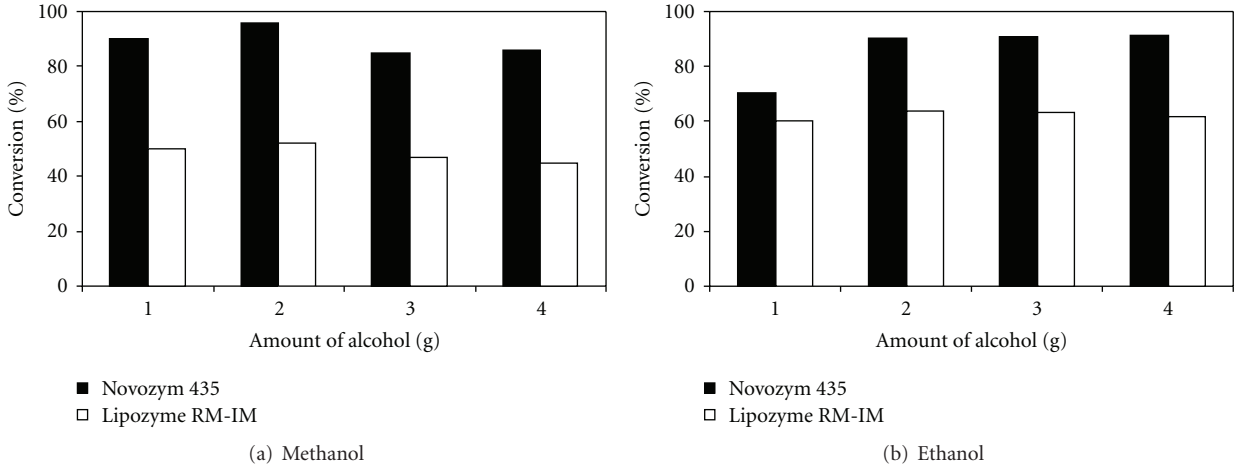


Figure 3: Effects of alcohol amount on fatty acids conversion after 2.5 h of reaction, using a stepwise addition of alcohol (two consecutive alcohol additions, i.e. 1/2 of alcohol added at time 0 and 1/2 after 30 minutes), 3 wt.% of commercial lipase, and 8 g of PFAD at 60°C. (a) Methanol; (b) ethanol.

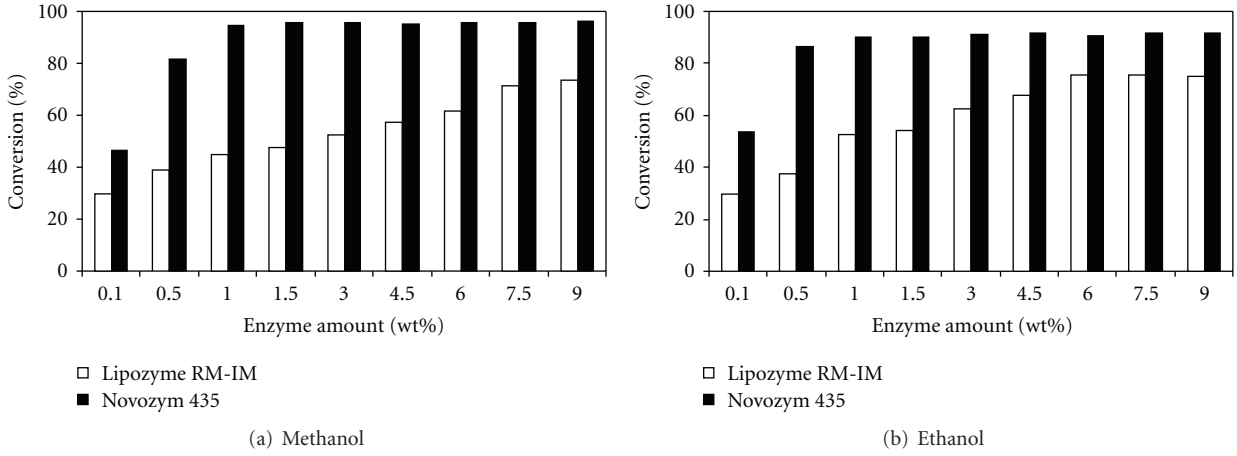
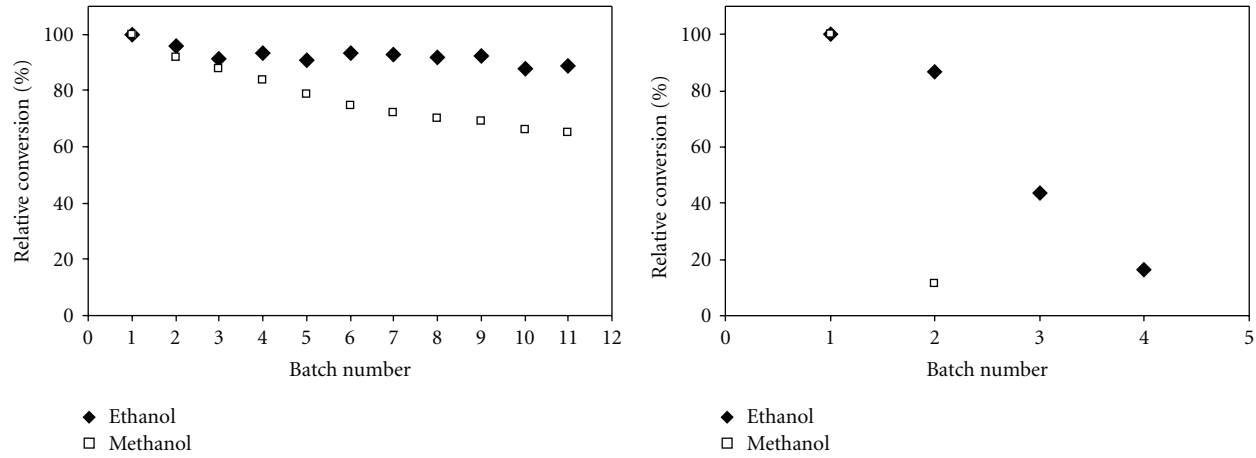


Figure 4: Effects of enzyme amount on fatty acids conversion after 2.5 h, using 8 g of PFAD and 2 g of ethanol added in two steps (T_0 = 1.0 g, $T_{30\ \mathrm{min}}$ = 1.0 g) at 60°C. (a) Methanol; (b) ethanol.



(a) Novozym 435 (b) Lipozyme RM-IM

FIGURE 5: Effects of enzyme reuse on relative fatty acids conversion. All of reactions were carried out for 2.5 h, using 8 g of PFAD and 2 g of alcohol added in two steps (T_0 = 1.0 g, $T_{30\ \mathrm{min}}$ = 1.0 g) at 60°C. (a) Novozym 435; (b) Lipozyme RM-IM.

(Figure 4(b)). With Lipozyme RM-IM, an increase in conversion with increasing enzyme amount up to 6 wt.% was observed, achieving a conversion of 75.5%. Higher amounts of enzyme did not result in a significant increase in conversion. For the reactions conducted with Novozym 435, conversions of 86.7% were obtained with only 0.5 wt.% of enzyme. The initial rate values for the reactions using 0.5 (2.59 μmol·min^{-1}) and 9 wt.% (2.79 μmol·min^{-1}) of Novozym 435 were similar. Thus, no significant increase in initial rate was observed when the loading of Novozym 435 increased from 0.5 to 9%, which is interesting from an economic point of view. Despite Novozym 435 is more expensive than Lipozyme RM-IM, it presents a higher activity and can be used in smaller quantities than Lipozyme RM-IM to obtain higher conversions.

Talukder et al. [25] studied the production of biodiesel from PFAD using Novozym 435 in the range of 0.5–6.0 wt.% of PFAD. The minimum amount of Novozym 435 required for obtaining the maximum biodiesel yield (>80%) was 1.0 wt.% in relation to PFAD amount.

3.5. Lipase Reuse. One of the main drawbacks of the use of enzymes for biodiesel production is the cost of the biocatalyst. Thus, several studies are reported in the literature investigating the reuse of immobilized lipases or continuous processes employing a system of packed-bed columns with immobilized lipases [26]. Therefore, the reuse of Lipozyme RM-IM and Novozym 435 was investigated in this work in the reactions carried out with 8 g of PFAD and 1 g of alcohol (methanol or ethanol), added in a stepwise manner (T_0 = 0.5 g, $T_{30\ \mathrm{min}}$ = 0.5 g), at 60°C. Figure 5 shows the relative fatty acid conversion in each cycle.

It can be seen in Figure 5 that Novozym 435 remains active after more cycles than Lipozyme RM-IM and that methanol has a much more deleterious effect on lipase activity than ethanol, mainly for Lipozyme RM-IM, confirming the results previously observed in Section 3.3. Novozym 435 was reused 10 times with conversion reaching 88% and 65% after the eleventh reaction with ethanol and methanol, respectively. However, the fatty acid conversion was decreasing with successive use of Lipozyme RM-IM. Only 16% and 11% of fatty acid conversion was obtained after the third and the first reuse of Lipozyme RM-IM in the esterification reaction of ethanol and methanol, respectively. These results could be attributed to lipase denaturation caused by short-chain alcohols [27].

3.6. Biodiesel Production. A comparison between Novozym 435 and Lipozyme RM-IM was also evaluated using the same esterification activity units (2,000 U). The use of the same activity units values resulted in employing different amounts of Novozym 435 (0.68 g) and Lipozyme RM-IM (1.32 g) in esterification reactions of PFAD. These reactions were performed at 60°C with stepwise addition of alcohol (T_0 = 1.0 g, $T_{30\ \mathrm{min}}$ = 1.0 g). Under these conditions, biodiesel yield and fatty acids conversion were similar for both enzymes tested, as can be seen in Figure 6, confirming the higher esterification activity of Novozym 435, which results in the use of smaller amount of this enzyme preparation.

Both biodiesel yield and fatty acids conversion have the same profile during 2.5 h of reaction indicating that consumption of fatty acids was really for the production of FAME or FAEE. Biodiesel yield was higher than 80% after 2 h of reaction in these conditions. These results are similar to those reported by Talakuder et al. [25] who obtained 90% of biodiesel yield after 2 h, at 60°C, using Novozym. Wang et al. [11] have used a mixture of commercial lipases (3% of Lipozyme TL-IM and 2% of Novozym 435) in SODD esterification reaction with methanol, employing t-butanol as solvent, and they have obtained a yield of 84% in methyl esters after 12 h.

The PFAD fatty acids esterification has been also studied using homogeneous acid catalyst. Chongkhong et al. [12] obtained more than 90% of FAME content (wt.%) after 4 h

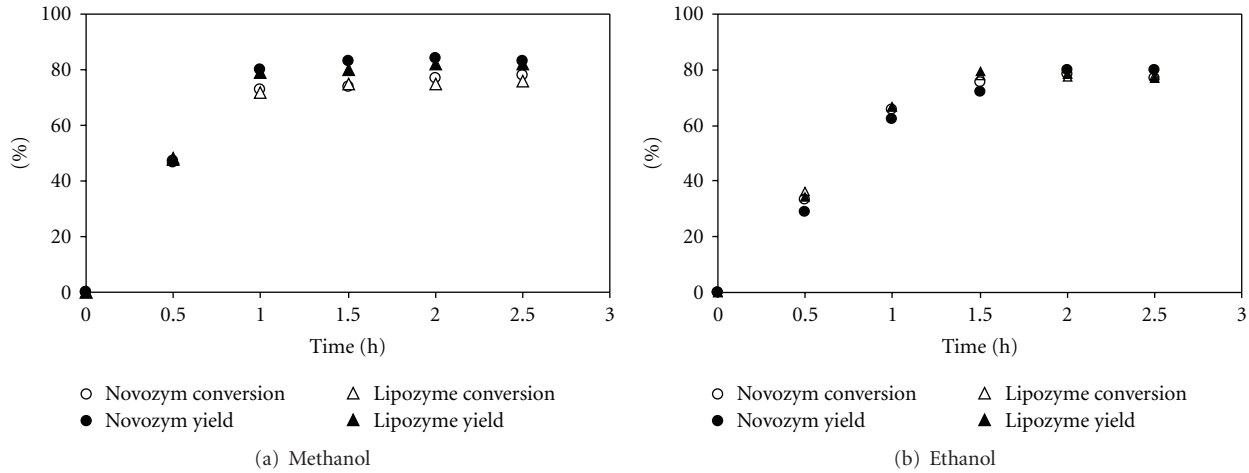


Figure 6: Biodiesel yield and fatty acids conversion in enzymatic esterification of PFAD. All of reactions were carried out for 2.5 h, using 2000 U (esterification activity of lipase), 8 g of PFAD, and 2 g of alcohol added in two steps (T_0 = 1.0 g, $T_{30\ min}$ = 1.0 g) at 60°C. (a) Methanol; (b) ethanol.

of PFAD esterification with methanol, at 90°C, with a molar ratio of methanol to PFAD higher than 4, in the presence of 1.834 wt.% of H_2SO_4. When a molar ratio of methanol to PFAD equal to 1 was used, less than 70 wt.% of FAME was obtained.

Thus, the use of lipase for the biodiesel production from a byproduct of palm oil industry offers some advantages in relation to chemical catalysis. The immobilized lipase could be recovered and reused for several cycles by a simple procedure. Moreover, in the chemical process with H_2SO_4 [12], an increase of dark color of product occurred when the temperature increased. This dark color was not observed in enzymatic reactions. The homogeneous acid catalyzed esterification also presents difficulties in biodiesel purification, catalyst recovery, and wastewater treatment [25]. Finally, high yields were achieved in enzymatic process by using smaller amounts of alcohol.

4. Conclusions

The biodiesel synthesis using a byproduct from palm oil refining and commercial immobilized lipases was demonstrated in this work. High fatty acids conversions were obtained in mild reaction conditions. Biodiesel yields higher than 80% were obtained in the reactions conducted with methanol or ethanol employing Novozym 435. The use of such a cheap feedstock and a biocatalyst to produce biodiesel, in addition to economic gains, represents an environment friendly process.

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Research Article

Cellulolytic Enzymes Production *via* Solid-State Fermentation: Effect of Pretreatment Methods on Physicochemical Characteristics of Substrate

Khushal Brijwani and Praveen V. Vadlani

Bioprocessing Laboratory, 201 Shellenberger Hall, Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA

Correspondence should be addressed to Praveen V. Vadlani, vadlani@ksu.edu

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We investigated the effect of pretreatment on the physicochemical characteristics—crystallinity, bed porosity, and volumetric specific surface of soybean hulls and production of cellulolytic enzymes in solid-state fermentation of *Trichoderma reesei* and *Aspergillus oryzae* cultures. Mild acid and alkali and steam pretreatments significantly increased crystallinity and bed porosity without significant change inholocellulosic composition of substrate. Crystalline and porous steam-pretreated soybean hulls inoculated with *T. reesei* culture had 4 filter paper units (FPU)/g-ds, 0.6 IU/g-ds β-glucosidase, and 45 IU/g-ds endocellulase, whereas untreated hulls had 0.75 FPU/g-ds, 0.06 IU/g-ds β-glucosidase, and 7.29 IU/g-ds endocellulase enzyme activities. In *A. oryzae* steam-pretreated soybean hulls had 47.10 IU/g-ds endocellulase compared to 30.82 IU/g-ds in untreated soybean hulls. Generalized linear statistical model fitted to enzyme activity data showed that effects of physicochemical characteristics on enzymes production were both culture and enzyme specific. The paper shows a correlation between substrate physicochemical properties and enzyme production.

1. Introduction

With increasing emphasis on bio-based fuels and chemicals, the cellulase market is expected to increase dramatically [1]. To create a sustainable bioeconomy, cellulases need to be produced cost-effectively and possess excellent biocatalytic properties [2]. Solid-state fermentation (SSF) offers a low-cost alternative for producing cellulases using natural polymers derived from agroindustrial residues [3, 4].

SSF is defined as a discrete solid phase in which microorganisms grow on the surface of moist particles as well as inside and between them. The space between particles is occupied by a continuous gas phase [5]. Gas phase in SSF is strongly affected by the size, shape, and tortuosity of a network of gas-filled pores. The air- or gas-filled pores are referred to as bed porosity, which is defined as the volume of gas contained in the system at any given time (void fraction) [6]. Availability of spaces between particles ensures availability of oxygen that improves enzyme production in aerobic fungal cultures [7–9]. Chutmanop et al. [10] showed that by blending rice bran with wheat bran resulted in substantial improvement in the morphology of rice bran which improved protease production during solid-state culturing of *A. oryzae*. The increase in bed porosity of the substrate could be the reason behind improved production; however, no attempts were made to measure bed porosity to show its relationship to enzyme production. Several authors in the past have suggested the merits of open porous solid beds but no explicit investigation has been conducted yet that relates bed porosity with enzyme production in SSF. In industrial scale SSF processes, bed porosity is essential but not sufficient for complete process control. Other parameters, such as microbial cell physiology, composition of the solid substrate, and substrate reactivity also could influence the productivity of the process [11, 12].

Substrate reactivity, especially in case of cellulosic substrates, is influenced by physicochemical characteristics of

the substrate at different levels. At microfibril level it is crystallinity of cellulose, and at fiber level it is specific surface area (characterizing pore size or degree of swelling) [13–15]. The increase in cellulase reactivity due to increase in specific surface area is attributed to the creation of surface openings or internal slits, voids, or spaces, by the removal of cell wall components, that enhances the direct physical contact between the enzymes and the substrate [16]. During growth on complex substrates, propagation of fungal mycelium occurs *via* production of enzymes that drive hydrolytic reactions. The hydrolytic reactions are responsible for generation of soluble sugars that facilitate fungal growth. It has been proposed that the hydrolysis occurs efficiently when the pores within the substrate are large enough to accommodate both large and small enzyme components to maintain the synergistic action of the enzyme system [14, 17, 18]. On the other hand, reduced surface area impedes this synergistic action.

Crystalline cellulose digestion requires concerted action of exo- and endoglucanases. The crystalline nature of the carbon source used to induce cellulolytic expression in many species of fungi significantly influences the hydrolytic potential of the enzyme preparation [19]. Evans et al. [20] showed that crystalline-cotton-induced cellulolytic complex derived from submerged *T. reesei* cultures exhibited higher potential in hydrolyzing crystalline cellulose than Solka-Floc-induced cellulases. Fungi growing on complex cellulosic substrates are prone to catabolite repression by glucose [21]. The extent of catabolite repression depends on the rate of glucose formation, which in turn depends on the secretion of enzymes that degrade cellulose. Fan et al. [22, 23], and, more recently, Ciolacu et al. [24] and Hall et al. [25] have shown that the rate of cellulose degradation is dependent on crystallinity of the cellulosic substrate. In other words, crystallinity of cellulosic sample could alter not only the quality of enzymes (the proportion of various activities with cellulolytic enzyme complex) but also the quantity of enzymes produced. Thus, studies delineating the effects of crystallinity on enzyme production in SSF are of significant interest.

The growth of fungi in natural substrates is usually slow and this limitation must be overcome by suitable mechanical and chemical pretreatment of the raw substrate [26]. However, pretreatments are known to induce structural changes in cellulosic substrates, which could alter the physicochemical properties of the substrate [2]. The effect of pretreatment methods on physicochemical characteristics of substrate and its repercussions on cellulolytic enzyme productivity in fungal solid-state fermentation has not been investigated so far, which is evident from the recent reviews on SSF [3, 27]. An in-depth understanding of the role of physicochemical characteristics of substrate on cellulase production in SSF would provide a framework for comprehensive analysis of critical design issues that should facilitate cellulase production with enhanced biocatalysis.

The present study aimed to determine the role of pretreatment techniques in altering the physicochemical characteristics—bed porosity, volumetric specific surface, and crystallinity of solid-state substrate. In addition, the effect of change in physicochemical attributes on enzyme production in fungal solid-state fermentation was studied with respect to type of fungal species and different cellulolytic enzyme activities. The pretreatments were carefully chosen to limit the effect on the chemical compositional changes of solid substrate, which would otherwise diminish the role of physicochemical attributes. Since crystallinity is critical to this study, a new method of measuring crystallinity of complex cellulosic substrate was also discussed.

2. Materials and Methods

2.1. Sample Preparation. Untreated ground soybean hulls (purchased from Archer Daniels Midland, Salina, KS, USA), herein referred to as native soybean hulls, had a geometric mean diameter, d_{gw}, of 0.61 ± 0.002 mm. Native soybean hulls were subjected to four different treatments before being used for production of the cellulolytic enzyme system: (1) steam pretreatment, in which a 5% (w/v) slurry of soybean hulls in distilled water was pressure cooked at 121°C for 60 min; (2) hydrochloric acid pretreatment, in which a 5% (w/v) slurry of soybean hulls in 1 N HCl was kept on a gyratory shaker (150 rpm) for 24 h at ambient temperature; (3) sulfuric acid pretreatment, in which a 5% (w/v) slurry of soybean hulls in 1 N H_2SO_4 was kept on a gyratory shaker (150 rpm) for 24 h at ambient temperature (4) sodium hydroxide pretreatment, in which a 5% (w/v) slurry of soybean hulls in 1 N NaOH was kept on a gyratory shaker (150 rpm) for 24 h at ambient temperature. After acid and alkali pretreatments, treated soybean hulls were collected by filtration and extensively washed with distilled water. The pH was adjusted to approximately 5.5. Steam-pretreated soybean hulls were washed once. All treated substrates were dried overnight at 45°C in a forced-draft oven (Fisher Scientific, USA). Dried substrates were used for compositional analysis, analysis of physicochemical characteristics, and production of enzymes. Treatments were performed in quadruplets.

2.2. SSF for Cellulolytic Enzyme System Production in Native and Pretreated Soybean Hulls. Two fungal cultures *T. reesei* (ATCC 26921) and *A. oryzae* (ATCC 12892) were used for SSF of native and pretreated soybean hulls. Cultures were used as both mono and mixed (1 : 1). Native and pretreated dried soybean hulls (5 g) were adjusted to 70% (wet basis) moisture content (mc) by using Mandels media [28] of pH 5 and were sterilized in a vertical sterilizer (121°C/15 psi gauge) for 30 minutes. Cultures were added as spore suspensions (10^8 spores/mL-suspension) at the loading of 0.1 mL per gram dry substrate. The propagation, maintenance, and generation of spore suspensions are described in [29]. Flasks containing two cultures in the ratio of 1 : 1 were labeled as mixed. Flasks were incubated for 5 days at 30°C. The conditions of temperature, pH, moisture (70%), and incubation days of the SSF process used in this study were optimized previously [29]. Following incubation, enzymes were extracted and analyzed per section analytical methods.

2.3. Analysis of Physical Parameters: Bed Porosity. Porosity (ε) of the samples was computed from the values of true density and bulk density by using the relationship described in [30] as follows:

$$\varepsilon = \left(1 - \frac{\rho_b}{\rho_t}\right) \times 100. \tag{1}$$

True density (ρ_t) was determined using a standard liquid pycnometer by determining the volume of the sample at various moisture contents. Volume (V, cm^3) was calculated from the following relationship [31]:

$$V = \frac{\left(M_{ps} - M_p\right) - \left(M_{pts} - M_t\right)}{\rho_{\text{tol}}}, \tag{2}$$

where M_t is mass of the pycnometer filled with toluene, M_{ps} is the mass of pycnometer and sample, M_p is mass of the pycnometer, M_{pts} is mass of the pycnometer filled with toluene and sample, and ρ_{tol} is the density of toluene. Knowing V, the true density (g/cc) then can be calculated from the following expression:

$$\rho_t = \frac{\left(M_{ps} - M_p\right)}{V}. \tag{3}$$

Bulk density (ρ_b) is estimated by weighing the samples (70% mc) after pouring in a vessel of known volume (10 mL) [30].

2.4. Analysis of Physical Parameters: Volumetric Specific Surface (cm^{-1}). Volumetric specific surface is defined as external surface area per unit volume of the samples [32]. Volumetric specific surface of samples was determined from particle size analysis [33]. Samples were sieved using USA standard testing sieves stacked in order of decreasing aperture size above the collection pan placed in Ro-Tap sieve sifter (Laval Lab Inc., Canada). Weight of oversize generated during sieving was used to compute geometric mean diameter (d_{gw}) and geometric standard deviation (S_{gw}) according to the following equations:

$$d_{\text{gw}} = \log^{-1}\left(\frac{\sum\left(W_i \log d_i\right)}{\sum W_i}\right)$$

$$S_{\text{gw}} = \log^{-1}\sqrt{\left(\frac{\sum\left[W_i\left(\log d_i - \log d_{\text{gw}}\right)^2\right]}{\sum W_i}\right)}, \tag{4}$$

where d_i is the diameter of the ith sieve in the stack and W_i is the weight fraction on the ith sieve. Using d_{gw} and S_{gw}, surface area per gram was calculated as follows [33]:

$$S\ (\text{cm}^2/\text{g}) = \frac{\beta_s}{\rho\beta_v}\exp\left(0.5\ln^2 S_{\text{gw}} - \ln d_{\text{gw}}\right). \tag{5a}$$

Volumetric specific surface (SA, cm^{-1}) can then be obtained from (5a) by multiplying it with specific weight (ρ) (g/cm^3), that is,

$$SA\ (\text{cm}^{-1}) = \frac{\beta_s}{\beta_v}\exp\left(0.5\ln^2 S_{\text{gw}} - \ln d_{\text{gw}}\right), \tag{5b}$$

where β_s is the shape coefficient for calculating surface area of particles (fixed at 6) and β_v is the shape coefficient for calculating volume of particles (fixed at 1) [33].

2.5. Analysis of Physical Parameters: Wide-Angle X-Ray Diffraction. Wide-angle X-ray diffraction (XRG 3100 X-ray generator, Phillips Electronics Instrument Inc., TX, USA) was used to estimate the crystallinity of native and pretreated soybean hulls. The X-rays from a Cu tube operating at 35 KV and 20 mA were collected by an energy dispersive detector that is able to resolve CuK_α line. Counts were collected at a step size of 0.02° at a series of angles between 5° and 40°. Speed of count collection was 0.6°/min.

2.6. Analysis of Physical Parameters: Crystallinity Calculations Using Deconvolution Method. The raw diffractograms were subjected to a fitting procedure using a nonlinear least squares numerical procedure. The deconvolution method separates amorphous and crystalline contributions to the diffraction spectrum under curve-fitting process by selecting a shape function [34]. In this method it is very important to understand the major sources that contribute to the shape function of the observed X-ray profile $h(2\theta)$, which is a convolution (Θ) of the intrinsic specimen profile $f(2\theta)$ with the spectral distribution (W) and the instrumental function (G) superimposed over the background b [35], as given below:

$$h(2\theta) = \left[(W\Theta G)\Theta f\right](2\theta) + b. \tag{6}$$

The Voigt function, which is a convolution of Gaussian and Lorentzian peak functions, would include both Gaussian intrinsic broadening of the specimen along with the Lorentzian instrumental profile that considers the background from amorphous scattering. The Voigt function, therefore, appropriately takes into account the peak broadening due to diffusive scattering [35, 36].

Using the Voigt function intensity of the reflection is represented by following equation [35]:

$$f(2\theta) = \frac{a_o \int_{-\infty}^{\infty}\left(\exp\left(-(2\theta)^2\right)/\left(a_l^2 + \left((x - a_c)/a_g - 2\theta\right)^2\right)\right)d(2\theta)}{\int_{-\infty}^{\infty}\left(\exp\left(-\left(-(2\theta)^2\right)\right)/\left(a_l^2 + (2\theta)\right)\right)d(2\theta)}, \tag{7}$$

where a_o is the amplitude of the peak, a_c is the center of the peak, a_l is the width of the Lorentzian component, and a_g is the width of the Gaussian component of the peak. The major reflective planes in cellulosic material from plant sources correspond to the following Miller indices (hkl): 101, $10\overline{1}$, 002, 021, and 040, with 002 as the prominent reflection representing crystalline cellulose (sometimes resolved into 021 plane as well) [37]. X-ray peaks were fitted using Voigt function as profile shape function using Peakfit (SeaSolve Software Inc., MA, USA) program. The program was rerun locking these planes; consequently, five Voigt functions were fitted. The fitted peaks were used to evaluate degree of

crystallinity (X_{cr}) of the sample per (8) described by Wada et al. [36],

$$X_{cr}\ (\%) = \frac{I_{002} + I_{021}}{I_{101} + I_{10\bar{1}} + I_{002} + I_{021} + I_{040}} \times 100, \quad (8)$$

where I followed by a subscript represents the integrated intensity of the particular Bragg plane. Crystallinity, therefore, represents the fraction of α-cellulose represented by planes 002 and 021 present in a particular sample.

2.7. Analytical Methods: Compositional Analysis. The lignocellulosic composition of soybean hulls was determined with an ANKOM 200 Fiber Analyzer (ANKOM Technology, USA). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were analyzed per procedure specified by the manufacturer (http://www.ankom.com). Protein content ($N \times 6.25$) was determined by the Kjeldahl method after digestion and distillation with an autoanalyser (Leco FP-2000, Leco Corporation, MI, USA). All moisture measurements were carried out using Denver Infrared Moisture Analyzer (Model IR35; Fisher Scientific, USA). Ash content of soybean hulls was measured using muffle furnace from Fisher Scientific.

2.8. Enzyme Assay. Crude cellulases were extracted from various production steps described in Section 2.4 by adding 30 mL of citrate buffer (50 mM, pH 5) to each flask and shaking the contents at 150 rpm for 30 minutes. Contents were filtered using coarse filter paper (Fisher Scientific, P-8 coarse grade), and the filtrate obtained was centrifuged at 10,000 ×g for 15 minutes at 4°C (Sorvall RC-6, Thermo Scientific, USA). The supernatant was analyzed for filter paper activity (FPU/g-ds), endocellulase (IU/g-ds), β-glucosidase (IU/g-ds), and xylanase (IU/g-ds) activities. Enzymatic assays were carried out using standard protocols described in Brijwani et al. [29]. Enzyme activities were reported as units per gram of dry substrate (g-ds).

2.9. Statistical Analysis. Statistical analysis was carried out using the GLM procedure in SAS software version 9.1 (SAS Institute, Cary, NC, USA). Multiple comparisons were conducted using Tukey Kramer HSD at $P < .05$.

3. Results and Discussion

3.1. Effect of Pretreatments on Compositional Changes in Soybean Hulls. Effects of various pretreatments on compositional changes in soybean hulls are shown in Table 1. Data is represented only to outline holocellulose (cellulose + hemicellulose), lignin, protein, and ash content of soybean hulls, and not necessarily embody composition fully. Soybean hulls are known to contain appreciable amount of pectin (~15%) and lipids (<4%) as well [38, 39]. Both acid and alkali pretreatments enriched the cellulosic fraction and extracted a small part of the hemicellulosic fraction. Steam-pretreated soybean hulls, on the other hand, had a composition similar to that of native soybean hulls. An interesting finding was that holocellulosic content was fairly constant (no significant difference, $P < .05$) across the spectrum of treatments used in this study (Table 1). Total cellulosic content may be useful to consider because both cellulose and hemicellulose are implicated in induction of cellulolytic enzyme complex [40]. Henceforth, subjecting soybean hulls to mild pretreatments preserved the holocellulosic composition of native soybean hulls.

3.2. Effect of Pretreatments on Changes in Physical Attributes of Soybean Hulls: Bed Porosity and Volumetric Specific Surface of Pretreated Soybean Hulls. There was a substantial increase in the bed porosity (Table 2), estimated at 70% mc, for pretreated soybean hulls compared with native soybean hulls. The increase in bed porosity is likely due to modification of the internal structure of soybean hulls that led to redistribution and partial solubilization of hemicellulose and swelling of the substrate [42]. Volumetric specific surface (cm^{-1}), on the other hand, was similar for pretreated and native soybean hulls. Notably, volumetric specific surface measurements were the outcome of particle size analysis that accounted only for external surface area; however, fibers have lumen characterized by hollow space. It is the interfibrillar space sometimes referred to as "internal porosity" that has capability of accommodating large enzyme molecules and leads to thorough digestibility. Chemical pretreatment tends to enlarge intermicrofibrillar spaces by dissolution of cell wall capillaries [18]. Unfortunately, finding a simple technique to determine lumen internal surfaces is difficult, and volumetric specific surface incorporating external particle diameter is unable to capture the internal specific area, which characterizes microfibrillar spaces [32]. This was evident in the current study when the volumetric specific surface of pretreated and untreated soybean hulls were not significantly different ($P < .05$). Apparently, it seems essential to identify or modify current techniques that can easily implement rapid and routine analysis of internal surface area, and therefore warrants future investigations.

3.3. X-Ray Crystallinity of Native and Pretreated Soybean Hulls. Wide-angle X-ray diffraction has been used extensively to measure the crystallinity of cellulosic substrates. Crystallinity in the polymeric sample may be measured in several ways from an X-ray diffractogram; the most common is the peak intensity method [43]. The method requires amorphous material to diffract with the same intensity at 18° (~$10\bar{1}$ plane) and 22° (002 plane), and does not account for peak shifting or overlap. Moreover, the crystallinity values predicted by this method are usually overestimated [25]. Further, this method assumes highest peak (002) as the only determinant of the cellulose crystallinity [34], which is certainly not the case as five planes have been identified responsible for the characteristic reflection. Finally, lignocellulosic substrates contain appreciable amounts of hemicellulose and lignin that lead to diffusive X-ray scattering (reflection), a hallmark of paracrystalline substances [44, 45]. Given these drawbacks of the peak intensity method, a sophisticated technique using deconvolution was successfully applied in our studies to X-ray spectra of both native and pretreated

Table 1: Composition of various substrates (dry basis).

Sample	Cellulose (ADF-ADL)	Hemicellulose (NDF-ADF)	Holocellulose*	Lignin (ADL)	Protein	Ash
Native soybean hulls	45.90±0.60	19.59 ± 0.57	65.48 ± 1.14^{A}	0.75 ± 0.09	11.96 ± 0.06	5.21 ± 0.01
Steam-treated soybean hulls	49.99 ± 2.67	19.32 ± 0.83	69.31 ± 3.38^{A}	1.19 ± 0.15	10.43 ± 0.07	2.67 ± 0.05
HCl-treated soybean hulls	57.19 ± 0.40	15.33 ± 0.96	72.52 ± 1.08^{A}	1.33 ± 0.07	9.60 ± 0.03	2.55 ± 0.05
H_2SO_4-treated soybean hulls	54.74 ± 0.47	17.39 ± 0.77	72.14 ± 1.23^{A}	1.47 ± 0.19	10.11 ± 0.11	2.78 ± 0.08
NaOH-treated soybean hulls	60.45 ± 1.61	15.66 ± 1.58	76.11 ± 3.15^{A}	1.23 ± 0.03	3.45 ± 0.06	3.26 ± 0.06

*Represents sum of cellulose and hemicellulose; data are expressed as mean ± SE; $n = 4$; means with same letters do not differ significantly. Pairwise comparisons between total cellulosics were tested using Tukey Kramer HSD at $P < .05$.

Table 2: Physical attributes of various substrates.

Sample	Degree of crystallinity (%)	Adj. R^2 for X-ray fitting	RMSE for X-ray fitting	Bed porosity (%)	Volumetric specific surface (cm^{-1})
Native soybean hulls	42.56 ± 3.34	0.91	15.06	40.41 ± 1.91	122.28 ± 1.91
Steam-treated soybean hulls	57.16 ± 2.39	0.94	12.48	57.45 ± 0.50	120.41 ± 2.34
HCl-treated soybean hulls	56.29 ± 0.12	0.94	13.40	53.65 ± 0.12	120.28 ± 2.47
H_2SO_4-treated soybean hulls	56.53 ± 0.12	0.95	13.35	50.02 ± 0.68	120.77 ± 2.16
NaOH-treated soybean hulls	59.72 ± 0.43	0.96	11.70	56.77 ± 0.57	128.09 ± 1.84

Data are expressed as mean ± SE; $n = 4$. It should be noted that RMSE values are scaled on y-axis that represents X-ray intensities of various peaks corresponding to Bragg planes. Peak values are usually in the range of 100–500 counts.

soybean hulls for crystallinity measurements. This method is relatively new in the arena of lignocellulosic biofuels research, although it is routinely used in polymer science research [41].

The fitted X-ray diffractograms using Voigt function are shown in Figures 1(a)–1(e) for both native and pretreated soybean hulls. Fit was assessed using R^2. Almost all diffractograms using this scheme had $R^2 > 0.95$. Also, featured in the Table 2 are adjusted R^2 (Adj. R^2) and root mean square error (RMSE) of the fit. The higher value of adjusted R^2 and lower RMSE further confirmed the goodness of fit. Notice the five peaks corresponding to identified lattice planes and gradual evolution of peaks in pretreated soybean hulls compared to native soybean hulls indicating increase in degree of crystallinity due to pretreatments. Degree of crystallinity was calculated from (8), and the values are listed in Table 2. The steam, acid, and alkali pretreatments all resulted in a significant increase in degree of crystallinity compared to native soybean hulls. The pretreated soybean hulls had crystallinity from 57 to 59% (Table 2). The enhancement in crystallinity is due to enrichment in the α-cellulose fraction in the pretreated samples due to reduction in the interlocking amorphous cellulosic chains and plausible correction in lattice defects of cellulose during pretreatments [46, 47]. The α-cellulose fraction is the crystalline cellulose of plant polymers and is responsible for the characteristic X-ray diffraction. Additionally, due to the mild nature of pretreatments, enrichment in α-cellulose fraction was possible by selective reduction of the amorphous phase. The outcome could have been different if harsh chemical pretreatments (using high temperature and pressure) were employed.

3.4. Effect of Pretreatment Methods on Production of Cellulolytic Enzyme System. Production of a cellulolytic enzyme system was assessed through measurement of four leading activities: filter paper units (FPU/g-ds (dry substrate)), β-glucosidase (IU/g-ds), endocellulase (IU/g-ds), and xylanase (IU/g-ds). Inspection of Figure 2 reveals that enzyme production in both mono and mixed cultures of *T. reesei* and *A. oryzae* was significantly reduced in alkali-pretreated soybean hulls compared to native, steam-, and acid-pretreated substrates. Gossett et al. [48] stated that an important aspect of alkali pretreatment is that biomass itself consumes some of the alkali. As a result, changes brought about by alkali pretreatment can cause solubilization, distribution, and condensation of lignin and hemicellulose and modification of cellulosic structure. These effects can counter the positive effects rendered by alkali pretreatment. Aiello et al. [49] showed that alkali-pretreated sugarcane bagasse in liquid fermentation of *T. reesei* (QM 9414) significantly decreased cellulase yield over untreated bagasse. Cellulolytic enzyme production in HCl- and H_2SO_4-pretreated soybean hulls was significantly ($P < .05$) lower as well for both cultures compared to production in both native and steam-pretreated substrates. Acid pretreatment of lignocellulosics is known

Table 3: Effect of interaction between crystallinity and bed porosity of substrates on cellulolytic enzyme production in both mono and mixed SSF of *T. reesei* and *A. oryzae*.

Interaction	Culture	Cellulolytic enzyme system				Treatments considered
		Filter paper units (FPU/g-ds)	β-glucosidase (IU/g-ds)	Endoglucanase (IU/g-ds)	Xylanase (IU/g-ds)	
Crystallinity × porosity	*Trichoderma reesei*	<0.0001*	0.0388*	<0.0001*	0.0472	Native, steam
Crystallinity × porosity	*Aspergillus oryzae*	0.4629	0.9218	0.0005*	0.9912	Native, steam
Crystallinity × porosity	Mixed	0.0044*	0.0449	0.0257**	0.9061	Native, steam

*Indicates Tukey probability for a particular interaction is significant at 95% confidence. **Indicates significance at $P < .05$ but not significance at $P < .01$. Model (9) ran in SAS 9.1.
Abbreviations: native, untreated soybean hulls; steam, steam-pretreated soybean hulls.

to generate inhibitory compounds as result of sugar and lignin degradation during the treatments [50, 51]. Though the acid pretreatment may result in increased digestibility of lignocellulosic substrate, the inhibitory compounds have deleterious effects on enzyme and microbial activity.

Steam pretreatment resulted in significant ($P < .05$) and substantial enhancement in production of all cellulolytic activities in *T. reesei* culture compared to production in untreated soybean hulls. The production of xylanase, though, was not significantly ($P < .05$) different. Steam-pretreated soybean hulls had about 4 FPU/g-ds compared with 0.75 FPU/g-ds in native and endocellulase of 45 IU/g-ds compared with 7.29 IU/g-ds in native. β-glucosidase activity also improved significantly ($P < .05$) in steam-pretreated compared with native soybean hulls. The preponderance of these results is apparent from the fact that both native and steam pretreated soybean hulls had compositional similarity (Table 1) but significantly different enzyme production (Figure 2). This is a key indication that in SSF, in which fungal mycelium is in direct contact with the substrate particles, the physicochemical nature of the substrate is important in addition to its composition.

In *A. oryzae* no significant differences ($P < .05$) occurred in enzyme production between steam-pretreated and native soybean hulls except in endoglucanase levels. In steam-pretreated soybean hulls, *A. oryzae* produced a significantly higher amount of endoglucanase (47 IU/g-ds) compared to that in native substrate (31 IU/g-ds). Mixed culture had similar results as in *A. oryzae*, where production in steam-pretreated soybean hulls was not significantly different ($P < .05$) compared to native soybean hulls (Figure 2).

The foregoing indicated steam pretreatment had disparities in enzyme production, which were both enzyme and culture specific. To relate the trends in enzyme production with physicochemical characteristics of the substrate in the two fungal cultures, *T. reesei* and *A. oryzae*, additional statistical analysis was performed.

3.5. Effect of Interaction between Crystallinity and Porosity in Cellulolytic Enzyme System Production in Pretreated Substrates. The interaction of crystallinity and porosity was modeled using the general linear model of SAS with the following expression:

$$y_{ijk} = \mu + ab_{ij} + \epsilon_{ijk}, \quad (9)$$

where is one of the enzyme activities as the dependent variable, μ is the grand mean ($n = 4$), ab_{ij} is the interaction effect of crystallinity and porosity, and ϵ_{ijk} is random error with mean 0 and experimental error variance as its variance. Both composition (holocellulose) and volumetric specific surface were excluded as they were nearly constant across pretreatments (Tables 1 and 2). In addition, only native and steam pretreated substrates were considered in our analysis because enzyme production in acid- and alkali-pretreated substrates was lower due to their inhibitory effects on microbial propagation. Crystallinity and porosity were considered together because both of them were simultaneously altered when substrates were subjected to pretreatments. It was not possible to keep one constant and make other variable during pretreatments. Broadly speaking, the model represented by (9) is more reflective of one-way variance analysis than factorial variance analysis.

Examination of data (Table 3) shows that for *T. reesei*, with an increase in crystallinity and porosity due to steam pretreatment, all cellulolytic enzyme activities increased significantly except xylanase. In *A. oryzae* fermentation, significant improvement was noticed only in endoglucanase production whereas, in mixed culture fermentation, significant decrease occurred in filter paper units at $P < .01$ and endoglucanase at $P < .05$ as a result of increased crystallinity and porosity.

Bed porosity ensures oxygen availability between the moist substrate particles. It is plausibly implicated in the propagation of fungal cultures and, therefore, affects enzyme production. Rahardjo et al. [8, 9] explained this phenomenon by using various model substrates that differed in the amount of open spaces for production of α-amylase in solid-state cultures of *A. oryzae* and explicitly showed that model substrates with more porous structure had better enzyme production compared to less porous substrates. Therefore, decrease in filter paper and endoglucanases activities in mixed culture compared to *T. reesei* could be attributed

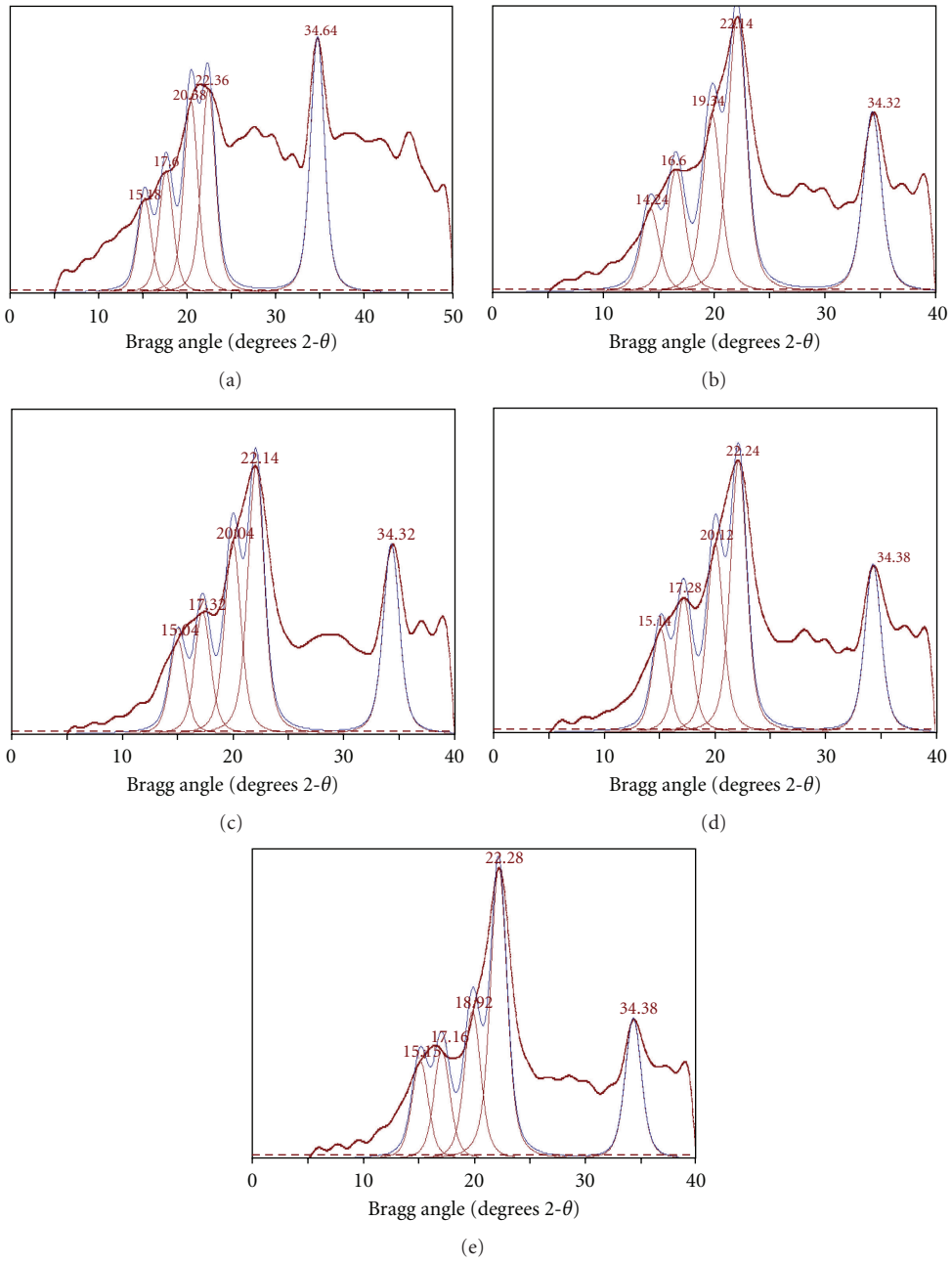

FIGURE 1: X-ray diffractograms. Gaussian smoothing followed by Voigt function was used to fit the diffractogram output of the instrument. (a) Native soybean hulls. (b) Steam-pretreated soybean hulls. (c) HCl-pretreated soybean hulls. (d) H_2SO_4-pretreated soybean hulls. (e) NaOH-pretreated soybean hulls. Planes corresponding to 2θ are 101 plane (~15°), $10\bar{1}$ (~17°), 021 plane (~20°), 002 plane (~22°), and 040 plane (~34°). (Adapted from [41]).

to another factor that is, increase in crystallinity. It is apparent from the literature that *T. reesei* cellulases are particularly active towards crystalline cellulose [20, 52, 53]; however, enzymes from *Aspergillus* spp. lack ability to degrade crystalline cellulose [54, 55]. In mixed culture fermentation wherein *A. oryzae* was dominant, filter paper and endocellulase activities were reduced due to the inability of *A. oryzae* to digest crystalline substrate. This is further confirmed by observing the data of *A. oryzae* fermentation, where no improvement in cellulolytic activities in steam-pretreated soybean hulls over native substrate was observed except in endoglucanase activity.

Evidently, results highlighted that effect of crystallinity was specific for type of culture as it brought enhancement in cellulolytic activities of *T. reesei*, and this enhancement was not particularly observed in *A. oryzae*. The analysis also showed that within the spectrum of cellulolytic activities studied not all activities got altered on exposure to crystalline substrate. The results are interesting in view of the fact that pretreatments due to their ability to induce changes in

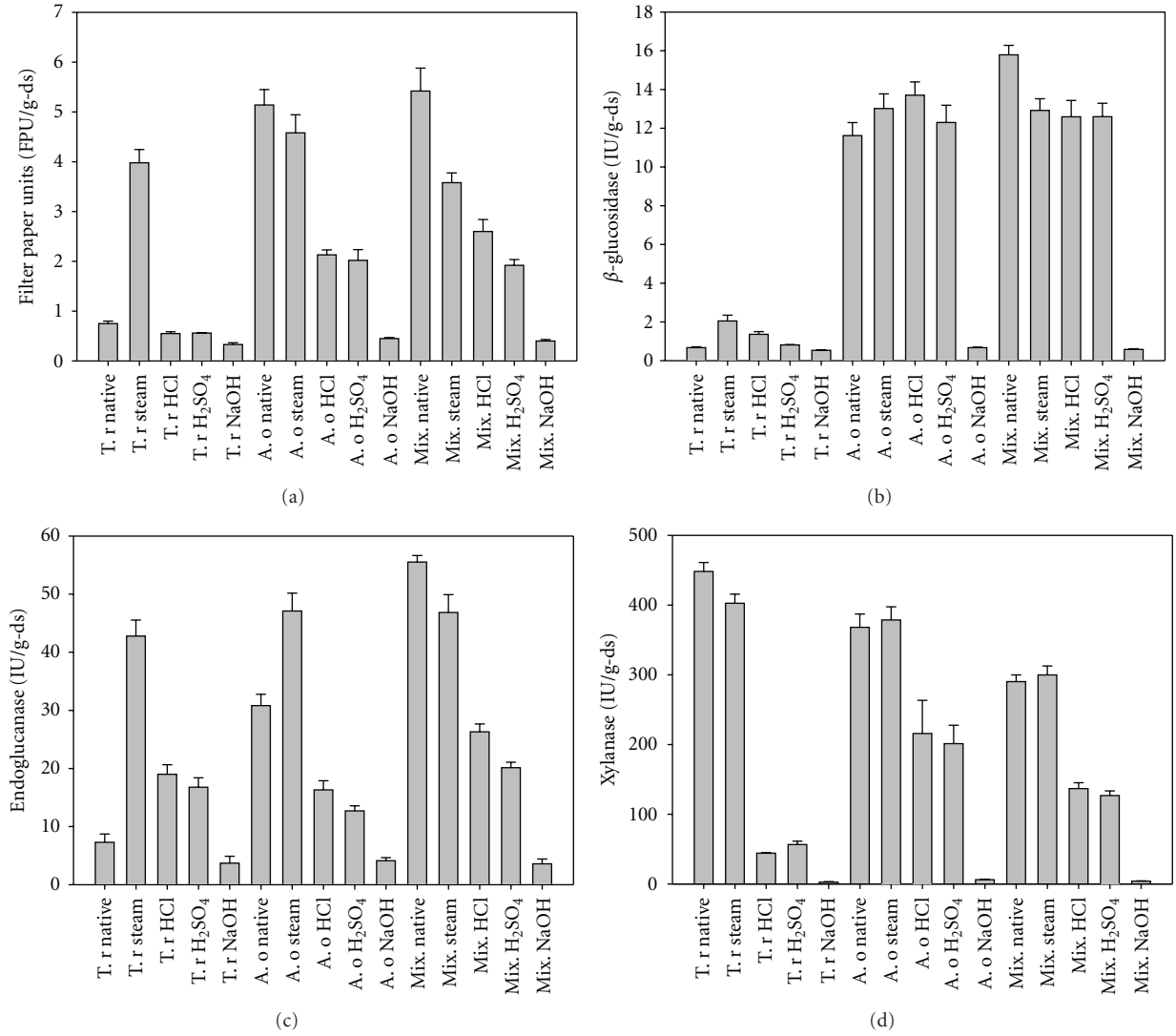

Figure 2: Effect of different pretreatments on cellulolytic enzyme production in 5 days grown mono and mixed cultures of *Trichoderma reesei* and *Aspergillus oryzae*. (a) Filter paper activity. (b) β-glucosidase activity. (c) Endocellulase activity. (d) Xylanase activity. Abbreviations: T. r, *T. reesei*; A. o, *Aspergillus oryzae*; mix, 1 : 1 mixture of *T. reesei* and *A. oryzae* cultures; native, untreated soybean hulls; steam, steam-pretreated soybean hulls; HCl, hydrochloricacid-pretreated soybean hulls; H_2SO_4, sulfuric-acid-pretreated soybean hulls; NaOH, sodiumhydroxide-pretreated soybean hulls. Refer to text for more details on conditions of pretreatments. Data are expressed as mean ± SE, $n = 4$.

physicochemical attributes resulted in altered enzyme production in fungal SSF of soybean hulls.

4. Conclusions

For the first time, current work demonstrated that mild pretreatment methods could significantly alter the physicochemical attributes of the substrate (soybean hulls) without significant changes in holocellulosic composition. The altered physicochemical attributes due to pretreatment had significant effects on the production of cellulolytic enzyme activities, and these effects were both culture and enzyme specific. A sophisticated deconvolution method was used to determine X-ray crystallinity from raw diffractograms of both treated and untreated substrates. This method takes into account diffusive scattering due to paracrystalline nature of celluloses found in plant material, and therefore provides consistent and reliable measurements. Steam pretreatment significantly increased both porosity and crystallinity of soybean hulls, and production of all the three cellulase activities in *T. reesei* culture (i.e., filter paper, β-glucosidase, and endocellulase) compared to untreated substrate. Xylanase production, however, remained unaltered. While using *A. oryzae* culture, significant improvement was observed only in endocellulase whereas in the mixed culture fermentation, filter paper, and endocellulase activities decreased in steam-pretreated soybean hulls.

Further study of porosity and crystallinity and their effects on enzyme production is necessary if we are to understand fully the effects of physiochemical attributes. Our studies highlighted the effects of pretreatment methods,

changes in the physiochemical characteristics of substrates, and choice of fungal culture in SSF on enzyme production. Experimental methods to enhance enzyme production are imperative for the success of the biofuels industry, which uses enzymatic and microbial fermentation platform.

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Research Article

Evaluation of *Chlorella* (Chlorophyta) as Source of Fermentable Sugars via Cell Wall Enzymatic Hydrolysis

Marcoaurélio Almenara Rodrigues[1] and Elba Pinto da Silva Bon[2]

[1] *Laboratory of Studies Applicable to Photosynthesis (LEAF), Biochemistry Department, Center of Technology, Chemistry Institute, Federal University of Rio de Janeiro (UFRJ), Bloco A, Avenida Athos da Silveira Ramos 149, Ilha do Fundão, 21 941-909 Rio de Janeiro, RJ, Brazil*
[2] *Enzyme Technology Laboratory (ENZITEC), Biochemistry Department, Center of Technology, Chemistry Institute, Federal University of Rio de Janeiro (UFRJ), Bloco A, Avenida Athos da Silveira Ramos 149, Ilha do Fundão, 21 941-909 Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Marcoaurélio Almenara Rodrigues, almenara@iq.ufrj.br

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The cell wall of *Chlorella* is composed of up to 80% carbohydrates including cellulose. In this study, *Chlorella homosphaera* and *Chlorella zofingiensis* were evaluated as source of fermentable sugars via their cell wall enzymatic degradation. The algae were cultivated in inorganic medium, collected at the stationary growth phase and centrifuged. The cell pellet was suspended in citrate buffer, pH 4.8 and subjected to 24 hours hydrolysis at 50°C using a cellulases, xylanases, and amylases blend. The measurement of glucose and reducing sugars concentration in the reaction mixture supernatant, on a dry biomass base, showed hydrolysis yields of 2.9% and 5.03% glucose and 4.8% and 8.6% reducing sugars, for *C. homosphaera* and *C. zofingiensis*, respectively. However if cells were washed with chilled ethanol, cold dried, and grounded the biomass hydrolysis yields increased to 23.3% and 18.4% glucose and 24.5% and 19.3% reducing sugars for *C. homosphaera* and *C. zofingiensis*, respectively.

1. Introduction

The commitment of several nations to reduce the carbon dioxide emissions has given a great impulse to the development of alternative energy sources not based on fossil sources but rather on renewable resources. Plants and algae are good candidates, as alternative energy sources, as they obtain their energy from the sunlight and build up their biomass by removing carbon dioxide from atmosphere through photosynthesis. In this way, any time a fuel originated from plants or algae is burnt, the carbon dioxide emitted is the very same that was previously removed by those organisms. However, differently from plants, algae cultivation does not compete for land crop occupation. Microalgae are particularly attractive as they are photoautotrophic organisms that grow in simple inorganic medium and contrary to higher plants, each cell is photosynthetically competent so that the amount of carbon dioxide fixed is much higher in a biomass base [1]. Moreover, its cell wall structure does not present lignin, which can be advantageous to the enzymatic hydrolysis of its polysaccharide components [2].

Considering the structure of the cell wall, some *Chlorella* species possess an outer cell wall layer and an inner cell wall layer, while other species present only the inner cell wall layer [3]. The outer cell wall layer consists of two types of ultrastructure. In the first one it is observed a trilaminar structure and the presence of algaenan which is a highly resistant, nonhydrolyzable aliphatic biopolymer composed of long-chain even-carbon-numbered ω9-unsaturated ω-hydroxy fatty acid monomers varying in chain length from 30 to 34 carbon atoms. These monomers are intermolecularly ester linked to form linear chains in which the unsaturations act as the starting position of ether cross-linking [4–6]. The second type of outer cell wall layer shows a homogeneous ultrastructure and as such, the trilaminar layer and algaenan are absent [6, 7]. *C. zofingiensis* and *C. homosphaera* are

amongst those species that possess trilaminar layer and possibly algaenan in their cell wall [6, 8]. The inner cell wall layer shows high cellulose content [9, 10] and is prone to chemical and enzymatic degradation. It is composed of a matrix and a rigid fibrillar structure, which was called by Takeda and Hirokawa "rigid cell wall" [11]. The matrix is soluble in alkalis and trifluoroacetic acid while the so-called "rigid cell wall" is hydrolyzed upon HCl or H_2SO_4 treatment.

The sugar composition of the algae cell wall has been studied. The cell wall of microalgae belonging to the Chlorophyte division may reach up to 80% in carbohydrate content, as is the case for *Chlorella fusca* [12]. The composition and the cell wall structure is species dependent and may be used as taxonomical markers [13–15]. As such, the genus *Chlorella* comprises of species in which the "rigid cell wall" is composed of polysaccharides formed mainly by glucose and mannose and those in which this structure is composed of polysaccharides formed mainly by glucosamine. The sugar composition of the cell wall matrix of the first group is dominated by mannose and fucose whereas in the second group galactose, fucose, and sometimes xylose are the main sugars found [11, 13, 14]. The cell wall sugar composition of *C. zofingiensis* is 70% glucose and 30% mannose in its "rigid cell wall" and 65% mannose, 30% glucose, plus minor amounts of rhamnose and galactose in its matrix cell wall [14]. Concerning cell wall sugar composition of *C. homosphaera*, whose synonymy with *Chlorella minutissima* has been recently established [8], it presents 85% glucose 15% mannose in its "rigid cell wall" and 70% mannose, 20% glucose, and 10% galactose in its matrix cell wall [14].

Accordingly, *Chlorella* cell wall is a good source of fermentable sugars, mostly cellulose derived, not to mention its starch content [16], provided these polysaccharides are properly hydrolyzed.

This work aims to evaluate the use of *Chlorella*, a green microalgae, as source of fermentable sugars for second generation ethanol production, via the enzymatic hydrolysis of its cell wall polysaccharides and intracellular starch content.

2. Materials and Methods

2.1. Cells and Growth. Cells of *Chlorella zofingiensis and C. homosphaera* were maintained in inorganic WC medium [17] at 21°C under 60 μmol photons$\cdot$m$^{-2}\cdot$s^{-1} irradiance of daylight white fluorescent light (Osram, Osasco, SP, Brazil) with 12 hours of photoperiod. Algae were kept in 250 mL erlenmeyers with 50 mL of medium and shaken occasionally. In order to obtain larger quantities of biomass, cells were grown in 4000 mL erlenmeyers with 3500 mL of medium under continuous aeration (10 mL$\cdot$min^{-1}).

Cells were collected at the middle of stationary phase of growth given that at this stage of growth, the maximum batch biomass yield is obtained. Moreover, it is at the stationary phase that algae stop dividing and begin to accumulate photosynthates, among them structural polysaccharides, besides showing less night biomass loss [18].

Cells were centrifuged at 10, 400 × g for 10 min in a Sorval centrifuge (Sorval Instruments, Wilmington, Del, USA), washed twice with distilled water, resuspended in 50 mM citrate buffer pH 4.8 to form a concentrated suspension and frozen until use. Before the freezing step, an aliquot of 1 mL of this suspension was used to determine the dry cell mass content whereby 64 mg cell dry weight were recovered from 1 mL of the concentrated cell suspension. Alternatively, instead of being resuspended in citrate buffer and frozen, cells were washed twice with chilled 95% ethanol, cold dried and ground with a pestle and mortar until a fine powder was obtained. The dried, cell powder was kept frozen until use.

2.2. Enzymatic Hydrolysis. Cells were hydrolyzed by a mixture of cellulases, xylanases, and amylases enzymes produced by *Tricoderma reesei*—RUT C30 and *Aspergillus awamori* [19, 20] in a proportion of 10 FPU/g dry mass and 1 g cell (dry mass)/10 mL of the reaction medium. The algae frozen suspensions were thawed, its volume measured, and its dry mass concentration was used to calculate the amount of enzyme preparation necessary to provide an enzyme load of 10 FPU/g dry mass. The reaction volume was afterwards corrected with 50 mM citrate buffer pH 4.8 to give a final cell (dry mass) concentration of 1 g/10 mL of the reaction medium which was incubated at 50°C in a rotatory shaker (Innova, New Brunswick Scientific, Edison, NJ, USA) using a glass capped erlenmeyer. Aliquots were withdrawn after 0, 2, 4, 6, and 24 h of hydrolysis, incubated for 5 min in a boiling water bath to quench the enzymatic reaction, centrifuged to sediment solid particles and the supernatants were used for glucose and reducing sugars determination. The conditions for the hydrolysis experiments, which were carried out using chilled 95% ethanol, cold dried, and ground cells were the same, however sampling was only carried out after 24 h of hydrolysis.

2.3. Sugar Determination. YSI 2730 glucose analyzer (Yellow Springs Incorporated, Ohio, USA) was used for glucose concentration measurement. Reductant sugars were determined by the 3,4-dinitrosalicylic acid method (DNS) [21] using a solution of 10 mM glucose as standard.

2.4. Biomass Hydrolysis Yield Calculation. Hydrolysis yields were expressed on a dry biomass base. Data for glucose concentration (g$\cdot$L^{-1}) and reductant sugars concentration (μmol$\cdot$mL^{-1}) were normalized so that hydrolysis yields were expressed in gram of glucose/reducing sugar per 100 g of dry biomass.

2.4.1. Calculation for Glucose Yield. The glucose analyser gives the results in g$\cdot$L^{-1}. Let C be the glucose concentration in the reaction mixture supernatant and V (mL) the total volume of the hydrolysis reaction. For the determination of the total glucose amount in grams (m), resulting from a known amount of dry biomass, the concentration C must be divided by 1000 and multiplied by V, as shown in

$$m = \frac{V}{1000} \cdot C. \tag{1}$$

The hydrolysis yield (Y) was expressed in gram of glucose per 100 g of dry biomass according to (2), where the mass

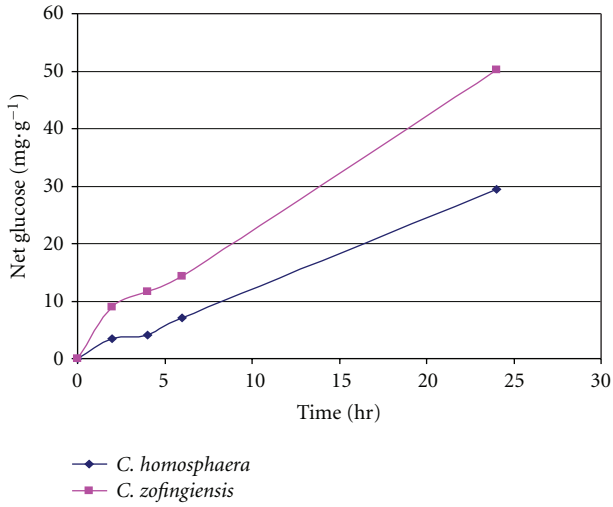

Figure 1: Time course for glucose accumulation upon the enzymatic hydrolysis of *C. homosphaera* and *C. zofingiensis polysaccharides*. Cells suspensions (100 mg dry mass·mL^{-1}) in 50 mM citrate buffer pH 4.8 were hydrolyzed by a mixture of cellulases, xylanases, and amylases enzymes produced by *Tricoderma reesei*—RUT C30 and *Aspergillus awamori*—for 24 h at 50°C. Aliquots were withdrawn at 0, 2, 4, 6, and 24 h hydrolysis.

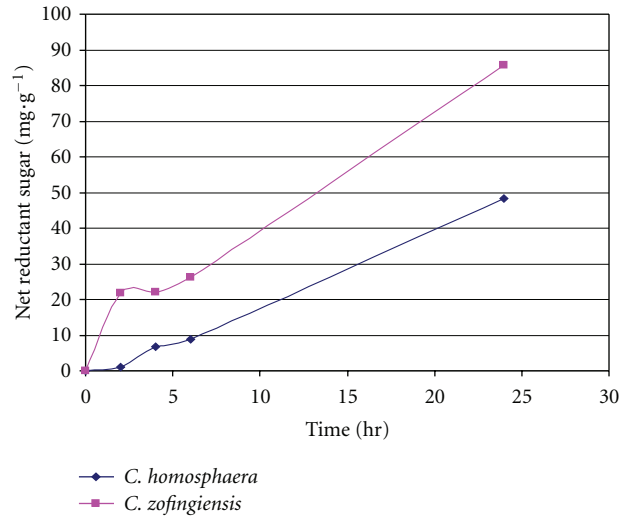

Figure 2: Time course for reductant sugars accumulation upon the enzymatic hydrolysis of *C. homosphaera* and *C. zofingiensis polysaccharides*. Conditions as described in Figure 1.

in gram of biomass used in the experiments of hydrolyses is represented by m_b.

$$Y \equiv \frac{100}{m_b} \cdot m = \frac{100}{m_b} \cdot \frac{V}{1000} \cdot C$$
$$\therefore Y = \frac{V \cdot C}{10 \cdot m_b}. \quad (2)$$

2.4.2. Calculation for Reducing Sugar Yield (Hexose Equivalents). The results obtained from DNS method gives the results in μmol reducing sugar·mL^{-1}. Let c be the concentration of reducing sugar in the reaction mixture supernatant. In order to express this concentration in mol·mL^{-1} (c'), c must be divided by 10^6 or multiplied by 10^{-6}

$$c' = 10^{-6} \cdot c. \quad (3)$$

Multiplying (3) by the molar mass it is obtained the concentration in gram per milliliter (cg). All hexoses have the same molecular mass; its value is 180 mol/g. Equation (3) then becomes

$$cg = 180 \cdot c' \quad \therefore cg = 180 \cdot 10^{-6} c$$
$$\therefore cg = 0.18 \cdot 10^{-3} \cdot c. \quad (4)$$

The total amount of reducing sugar in grams (m) within the reactor is obtained multiplying cg by the volume of the hydrolysis reaction mixture (V) as defined previously. Equation (4) becomes:

$$m = V \cdot cg \quad \therefore m = 0.18 \cdot 10^{-3} \cdot c \cdot V. \quad (5)$$

The hydrolysis yield (Y) is calculated as shown previously:

$$Y \equiv \frac{100}{m_b} \cdot m = \frac{100}{m_b} \cdot 0.18 \cdot 10^{-3} \cdot c \cdot V$$
$$\therefore Y = 0.018 \cdot \frac{c \cdot V}{m_b}. \quad (6)$$

If results were to be expressed in milligram of glucose/reducing sugar per gram of dry biomass (Y'), Y had to be multiplied by 1000 and divided by 100: ($Y' = 10 \cdot Y$).

3. Results and Discussion

Figures 1 and 2 show the time course, up to 24 hours, for glucose and reducing carbohydrates (hexose equivalents) accumulation, in the enzymatic hydrolysis experiments using the biomass of *C. homosphaera* and *C. zofingiensis*, respectively. Results were expressed as milligram of sugar per gram of dry biomass. A continuous increase in the amount of hydrolyzed glucose and total reducing carbohydrates was observed within the 24 hours of experimental assay. As a sugar concentration plateau was not reached it is likely that higher concentrations would be reached upon a longer incubation period. The amount of hydrolyzed sugars, either glucose or total reducing sugars, were consistently higher for *C. zofingiensis*. After 24 h hydrolysis, *C. homosphaera* yielded a 2.9% of hydrolyzed glucose and 4.8% of total reducing sugars whereas *C. zofingiensis* yielded 5.0 and 8.6%, respectively, on a dry biomass base, indicating this material to be more prone to enzymatic hydrolysis. When the amount of glucose relative to total reducing sugar, was calculated, it was found that 60.4% of all reducing sugars was glucose for *C. homosphaera* and 58.1% for *C. zofingiensis*. It is also interesting to note that, although the amount of hydrolysed sugars was higher *for C. zofingiensis* the proportion of

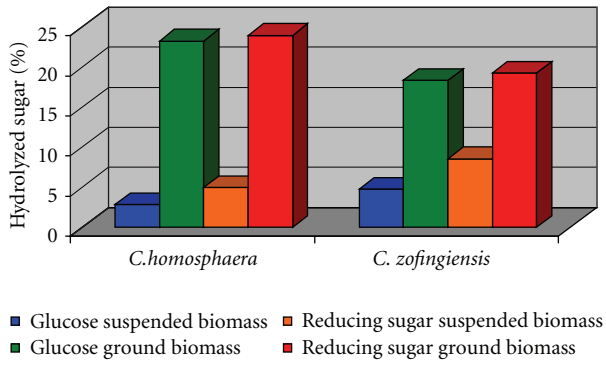

Figure 3: Effect of biomass pretreatment on hydrolyzed sugar yields for *C. homosphaera* and *C. zofingiensis*. Cells pellet were resuspended in citrate buffer pH 4.8 and subjected to enzymatic hydrolysis (blue and orange bars) or washed twice with chilled ethanol 95% (v/v), cold dried and ground, and then subjected to enzymatic hydrolysis in citrate pH 4.8 buffer (green and red bars). Hydrolyzed glucose (blue and green bars) and total reductant sugars (orange and red bars) were determined.

glucose to reducing sugars was the same, suggesting that both species may have similar cell wall composition and structure, allowing a similar enzymatic attack. These results are consistent to the cell wall structure and composition previously reported [14], since their cell walls show similar sugar composition and their polysaccharides are composed predominantly by glucose and mannose.

The set of enzymatic hydrolysis experiments using cells which were washed with chilled 95% (v/v) ethanol, cold dried and ground showed a dramatic increase in the hydrolysis yield after 24 h as shown in Figure 3. *C. homosphaera* yielded 23.3% of hydrolyzed glucose and 24.5% of reductant carbohydrates, whereas *C. zofingiensis* yielded 18.4 and 19.3%, respectively, on a dry biomass base. These data indicated an 8 fold increase in the amount of hydrolyzed glucose and 4.8-fold in that of reductant sugars for *C. homosphaera* whereas for *C. zofingiensis* the increase, nevertheless lower, of 3.8- and 2.2-folds, respectively, was also important. Interestingly, the algae biomass pretreatment procedure leveled the yield of hydrolyzed sugars for both species (Figure 3). It could be argued that these results might not be comparable with those obtained from the suspended biomass (Figure 2) because the hydrolysis time course was not done for the pretreated biomass. That would indeed be true if the subject of comparison were the time course for enzymatic progression. However the purpose of this procedure was solely to compare the hydrolysis sugar yield, for both materials, within 24 h reaction.

Besides the significant improvement in the algae biomass hydrolysis yield, it was found that glucose represented 95.1% of the total hydrolyzed sugars for *C. homosphaera* and 95.3% for *C. zofingiensis* which is quite remarkable considering the diversified sugars composition of the algae cell wall. This might suggest that the dehydration and powdering pretreatment procedures have destabilized the algae cell wall, specially the outer cell wall, allowing the selective action of the enzymes blend in the cellulose ultrastructures of the cell wall that were not available in the native cell wall structure.

Although the algae biomass starch content was not determined, it is not possible to rule out starch hydrolysis into glucose due to the high amylases content of the enzyme blend which was used in this study [19].

In a similar study, Fu et al. [22] investigated the hydrolysis of cell wall of *Chlorella* sp. using immobilized cellulase. The authors reported a yield of 58% of hydrolyzed glucose relative to total sugar content after 24 h of continuous enzyme treatment. Considering that cellulose represents 33% of the algal dry biomass in *C. pyrenoidosa* [2], and assuming that this is true for all species of the genus *Chlorella*, the aforementioned hydrolysis yield corresponds to a 19.4% yield on a dry biomass base. These results are similar to those found in this study.

It is also important to emphasize the concentration of glucose syrup, of 23.3 $g \cdot L^{-1}$ for *C. homosphaera* and 18.4 $g \cdot L^{-1}$ for *C. zofingiensis* showing that higher concentrations may be achieved upon the optimization of these initial working conditions. As such, *Chlorella* sugar syrups are promising as renewable resources for a bioethanol or biorefinery platform.

The continuation of this study will include analysis of the algae biomass starch content, optimization of the pretreatment conditions for both the dehydration and the grinding steps, and improvement of the enzymatic hydrolysis conditions, considering temperature, pH, and incubation time, as well as the enzyme load, aiming to increase yields and to decrease the hydrolysis time. Studies will also determine and compare with the literature the cell wall composition of both species, analyze the hydrolysates reducing sugars composition and the carbohydrate composition of the residual nonhydrolyzed algae polysaccharides, as well as the adequacy of the algae sugar syrups for ethanol fermentation by *Saccharomyces cerevisiae*.

4. Conclusions

The use of *C. homosphaera* and *C. zofingiensis* as a source of biomass for second generation ethanol production showed to be quite promising. High yields of the fermentable sugar glucose were obtained from the algae biomass, by the use of a simple pre-treatment procedure, which involved dehydration and grinding, followed by hydrolysis by an enzyme blend, produced by *Tricoderma reesei*—RUT C30 and *Aspergillus awamori*. This enzyme poll presented a collection of activities which proved to be adequate for the cell wall degradation of the algae species which were evaluated in this study.

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Review Article

Formate Formation and Formate Conversion in Biological Fuels Production

Bryan R. Crable,[1,2] Caroline M. Plugge,[2] Michael J. McInerney,[1] and Alfons J. M. Stams[2]

[1] *Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019, USA*
[2] *Laboratorium voor Microbiologie, Wageningen Universiteit, Dreijenplein 10, 6703 HB Wageningen, The Netherlands*

Correspondence should be addressed to Alfons J. M. Stams, fons.stams@wur.nl

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Biomethanation is a mature technology for fuel production. Fourth generation biofuels research will focus on sequestering CO_2 and providing carbon-neutral or carbon-negative strategies to cope with dwindling fossil fuel supplies and environmental impact. Formate is an important intermediate in the methanogenic breakdown of complex organic material and serves as an important precursor for biological fuels production in the form of methane, hydrogen, and potentially methanol. Formate is produced by either CoA-dependent cleavage of pyruvate or enzymatic reduction of CO_2 in an NADH- or ferredoxin-dependent manner. Formate is consumed through oxidation to CO_2 and H_2 or can be further reduced via the Wood-Ljungdahl pathway for carbon fixation or industrially for the production of methanol. Here, we review the enzymes involved in the interconversion of formate and discuss potential applications for biofuels production.

1. Introduction

Methane has been recognized as an important fuel source since at least 1778 when Alessandro Volta first identified methane as the primary component of swamp gas. In 1884 Louis Pasteur proposed using methane produced from the anaerobic decay of horse dung to light the streets of Paris. Naturally occurring methane, otherwise known as natural gas, currently provides approximately 20–25% of the US energy demand. The United States' 2010 methane consumption was estimated to be approximately 2.43×10^{16} kJ and demand is anticipated to grow at an annualized rate of 0.4% annually through 2035 to 2.74×10^{16} kJ [1]. Current government support and investment underscores the important role of biomethanation. Several countries, including Japan, the United States, and Sweden, have taken leadership roles in designing the next generation of methane-fueled vehicles [2]. As of 2006, Germany had invested in nearly 3500 biogas production facilities that provided approximately 900 MW of electricity [3]. Denmark and Sweden have made similar investments into biogas production facilities and biomethanation will be one of many tools the Dutch dairy industry relies on to achieve energy self-sufficiency by 2020 [4].

Biomethanation is a complex biological process. It is well established that methanogenic ecosystems require multipart cooperation between at least three trophic guilds. This includes fermentative bacteria, acetogenic bacteria, and methanogens. In short, complex organic molecules are fermented to acetate, hydrogen, formate, and a variety of organic acids (lactate, propionate, and butyrate) and ethanol. Acetogenic bacteria convert these compounds to the methanogenic substrates hydrogen, formate, and acetate. In this cascade, interspecies electron transfer (IET) plays an important role. It results in a syntrophic relationship between acetogenic bacteria and methanogens.

This syntrophic relationship is the rate-limiting step for biological methane production [5] and is essential to proper functioning and maintenance of the overall thermodynamic viability of these systems. The role of interspecies hydrogen transfer is rather well studied, but the role of formate in the methanogenic cascade has received less attention. In anaerobic systems, formate is produced through either formate dehydrogenase (FDH) catalyzed reduction of carbon dioxide

or through CoA-dependent cleavage of pyruvate to formate and acetyl-CoA. Conversely, archaeal FDH initially oxidizes formate to H_2 and CO_2. CO_2 can then be reduced by methanogens to form methane. Here, we review the state of our knowledge regarding the production, transfer, and consumption of formate in methanogenic ecosystems by focusing on the properties of the enzymes involved.

2. Formate Production by Pyruvate-Formate Lyase

Pyruvate is the end product of the three major glycolytic pathways: Embden-Meyerhof-Parnas (EMP), Entner-Doudoroff (ED), and Pentose Phosphate Pathways (PPP). Aerobically grown organisms further metabolize pyruvate through NAD-dependent pyruvate dehydrogenase catalyzed oxidation to acetyl-coenzyme A (CoA) and carbon dioxide. In the absence of oxygen, pyruvate is oxidized through either a ferredoxin-dependent oxidoreductase, which catalyzes reduction of ferredoxin coupled to oxidation of pyruvate to acetate and carbon dioxide or through the action of pyruvate formate lyase (PFL). PFL catalyzes CoA-dependent cleavage of pyruvate to form acetyl-CoA without the associated production of NADH or reduced ferredoxin according to the following reaction [6]:

$$\mathrm{CH_3COCOOH + CoASH \longrightarrow CH_3COO\text{–}CoA + CHOOH}$$

$$\Delta G^{\circ'} = -12.9\,\mathrm{kJ\ mol^{-1}}\ [7]. \tag{1}$$

This reaction notably does not result in the production of NADH or reduced ferredoxin and allows for ATP synthesis from acetyl-CoA through the combined action of phosphotransacetylase and acetate kinase [8].

Escherichia coli PFL activity was first demonstrated in 1943 by Kalnitsky and Werkman [9] and was the first glycyl radical enzyme discovered [10, 11]. The forward reaction (1) is energetically favorable and is catalyzed through a "ping-pong" mechanism according to the following reaction scheme [6]:

$$\text{Active PFL + pyruvate} \longleftrightarrow \text{acetyl-PFL + formate}, \tag{2}$$

$$\text{Acetyl-PFL + CoA} \longleftrightarrow \text{active PFL + acetyl-CoA}. \tag{3}$$

Mutagenesis experiments showed that the PFL active site consists of three amino acid moieties; the glycyl radical Gly734, Cys418, and Cys419 [12]. Crystal structures of inactive PFL with and without substrate have been solved [13, 14]. These structures reveal that PFL is a α_2 homodimer of approximately 170 kDa. The active site residues are contained on opposing hairpin loops with a distance of 4.8 Å between the alpha carbon of Gly734 and Cys419 though this orientation may differ in the activated form of the enzyme [13, 14]. PFL is activated by PFL-activating enzyme (PFL-AE), which mediates hydrogen abstraction from the Gly734 residue to form the glycyl radical [15]. The glycyl radical is thought to be relayed to Cys419 and, possibly, Cys418 forming a thiyl radical, which is responsible for the attack on pyruvate, forming acetylated PFL (2) with associated release of a formyl radical anion ($\cdot CO_2^-$) [14].

Two mechanisms for formate formation have been postulated. In the conventional model, the formyl radical is quenched by hydrogen abstraction from Cys419, reforming the Cys419 thiyl radical. Abstraction of hydrogen from CoA facilitates acetyl transfer, releasing acetyl-CoA [14]. Guo and Himo (2004) [16] revisit this reaction mechanism proposing, instead, that the formyl radical is quenched by hydrogen abstraction directly from CoASH without intermediate involvement of a Cys418 thiyl radical [16].

PFL reaction creates a pool of formate that can have diverse fates depending on the environmental conditions in the bioreactor. Under oxygenic- and nitrate-reducing conditions, electrons from the oxidation of formate can be utilized to reduce either oxygen or nitrate. In the absence of nitrate or oxygen, formate can be oxidized directly to CO_2 with the reduction of protons to form H_2 (discussed later).

3. Interconversion of Formate and H_2/CO_2

Formate oxidation and CO_2 reduction are interconvertible processes that are carried out by two main families of enzymes found in Eubacteria. The first group of enzymes is the iron-sulfur formate dehydrogenase (FDH) enzymes. These enzymes catalyze NAD-independent formate oxidation, have complex quaternary structure, and contain redox active molybdenum (Mo) or tungsten (W) prosthetic groups. The second class of enzymes are the NAD^+-dependent FDH enzymes which catalyze the concomitant reduction of NAD^+ to NADH and formate oxidation to CO_2.

3.1. Structure and Function of Iron-Sulfur-Containing FDH. In enteric bacteria such as *E. coli*, the fermentative formate dehydrogenase FDH-H together with the hydrogenase Hyd-3 form the energy conserving formate-hydrogen lyase (FHL) complex that oxidizes formate produced via the pyruvate-formate lyase (PFL) system. In this system, FDH-H reduces the Hyd-3 enzyme with electrons extracted from the two-electron oxidation of formate. Reduced Hyd-3 then produces hydrogen gas through the reduction of two protons [17].

Formate dehydrogenase is a member of the dimethylsulfoxide (DMSO) reductase family of enzymes. The first crystal structure obtained was for FDH-H from *Escherichia coli*; this was found to be a single subunit enzyme with four distinct domains. There is great diversity in subunit composition for these enzymes but the overall topology for all the known crystal structures is very similar; four highly conserved domains are distributed across 1, 2, or 3 subunits. A redox active molybdenum (Mo) or tungsten (W) atom is coordinated in a square-pyramidal manner at the structure's center. Four sulfur atoms distributed on two molybdopterin guanine dinucleotide (MGD) prosthetic groups provide four base ligands. The selenium atom of a selenocysteine residue provides the apical ligand [18, 19].

The active site is approximately 25 Å from the enzyme surface. Formate enters the enzyme through a positively lined funnel-shaped entrance known as the formate cleft. This

funnel leads directly to the active site, which is composed of single arginine, histidine, and selenocysteine residues. For the two electron oxidation of formate to carbon dioxide, formate presumably binds directly to Mo[VI] and displaces the SeCys residue. The free selenol is stabilized by an arginine residue. The molybdenum atom likely accepts two electrons from formate with concomitant production of H^+ and CO_2. A protonable histidine residue accepts the proton from formate. Raaijmakers and colleagues (2002) have identified a putative proton channel in FDH-H and the tungsten-containing formate dehydrogenase of *Desulfovibrio gigas*. This tunnel, which is oriented perpendicular to the formate cleft, is coated with protonatable glutamic and aspartic acid side chains. Also identified in this study was a hydrophobic channel that may allow the release of CO_2 [18, 19].

In addition to the FHL-associated FDH-H enzyme, *E. coli* expresses two additional FDH enzymes with similar functions, but used under different growth conditions. FDH-N was the first FDH isolated and characterized from *E. coli* and is expressed when the organism is grown in the presence of nitrate. FDH-N is a heterotrimer composed of an ~110 kDa α-subunit, an ~32 kDa β subunit, and an ~20 kDa γ-subunit. The catalytic α-subunit catalyzes the periplasmic oxidation of formate. Electrons are transferred via the β-subunit and ultimately reduce the cytochrome *b*-containing γ-subunit; an integral membrane protein which reduces menaquinone to menaquinol. Oxidation of menaquinol by respiratory nitrate reductase Nar results in the translocation of two protons from the cell interior into the periplasm which helps contribute to maintaining proton motive force [17].

FDH-O is topologically similar to FDH-N. FDH-O is expressed when *E. coli* is grown aerobically and is slightly upregulated in the presence of nitrate. Expression studies led to the postulation that this constitutively expressed enzyme complex acts as a minor formate-to-nitrate respiratory chain ensuring rapid adaptation to nitrate-reducing conditions in the absence of physiologically viable amounts of FDH-N. However conclusive evidence for a physiological role of FDH-O remains enigmatic [20].

3.2. FDH-Catalyzed CO_2 Reduction. Carbon dioxide reductase activity has been successfully demonstrated *in vitro* with FDH enzymes containing a W-MGD cofactor instead of the Mo-MGD cofactor. CO_2 reduction is favored because W has a lower redox potential than Mo. It is very likely that CO_2 reductase activity is restricted to W-MGD containing FDH enzymes, however it is not yet clear if the inverse relationship is also true: that formate dehydrogenase activity is restricted to Mo-MGD containing enzymes [21].

This activity is essential for carbon fixation by acetogens that reduce CO_2 to acetate via the Wood-Ljungdahl pathway. Pyruvate generated during glycolysis is metabolized exclusively via the pyruvate:ferredoxin oxidoreductase system in acetogens. This results in the production of acetate, reduced ferredoxin, and CO_2. The first step in acetate synthesis from CO_2 involves FDH-catalyzed reduction of CO_2 to formate with molecular hydrogen as the physiological electron donor [22–24]. There is high sequence similarity between the formate-producing and formate-consuming FDH enzymes. For example, the acetogen *Acetobacterium woodii* contains an FDH isoenzyme whose catalytic subunit has 80% identity [25] with the same subunit of *E. coli*. Given this similarity, it is likely that the catalytic mechanism of carbon dioxide reduction in these organisms is essentially the reverse of the mechanism previously discussed for *E. coli* FDH-H.

3.3. NAD^+-Dependent FDH. The second family of formate oxidizing enzymes is the NAD^+-dependent FDH enzymes which are devoid of any metallic prosthetic groups. These enzymes exist as α_2 homodimers and catalyze reduction of NAD^+ to NADH with concomitant oxidation of formate. This class of enzymes has been studied extensively due to their practical application as a regenerative agent for NADH used in chiral compound synthesis by the pharmaceutical industry. A crystal structure is available [26] and the catalytic mechanisms and structure have been reviewed extensively [27–29]. Recent reports have demonstrated reversibility of these enzymes with NADH-dependent CO_2 reduction to form methanol [30–33] (discussed later).

4. Formate to Methane

4.1. Formate Utilization by Methanogens. Biological methane production from formate or hydrogen and carbon dioxide proceeds through a multistep pathway. This pathway has been reviewed at length [34–36] and we wish only to present a basic introduction to the methanogenic pathway. Readers are directed to *Methanogenesis* edited by Ferry for a more thorough understanding of the methanogens [36].

Carbon dioxide is used to form N-formylmethanofuran from CO_2 and free methanofuran (MFR) (Figure 1, reaction 2). The formyl group is transferred from formylmethanofuran to tetrahydromethanopterin (H_4MPT) by formyl-MFR: H_4MPT formyl transferase to form N^5-formyl-H_4MPT (Figure 1, reaction 3). This undergoes a cyclization reaction catalyzed by N^5, N^{10}-methenyl-H_4MPT cyclohydrolase to form N^5, N^{10}-methenyl-H_4MPT (Figure 1, reaction 4). F_{420}-reducing (NiFe) hydrogenase enzymes catalyze two sequential reductions of N^5, N^{10}-methenyl-H_4MPT; first to N^5, N^{10}-methylene-H_4MPT (Figure 1, reaction 5) and then to N^5, N^{10}-methyl-H_4MPT (Figure 1, reaction 6). The methyl group is then transferred to coenzyme M (CoM) (Figure 1, reaction 7) which is reduced to form free methane and oxidized CoM (Figure 1, reaction 8) [36].

Free formate is not utilized by methanogenic archaea for methanogenesis although there is evidence that formate is needed nutritionally as a formate auxotroph of *Methanothermobacter autotrophicus* was obtained [37]. Formate is first oxidized to CO_2 by formate dehydrogenase with concomitant production of $F_{420}H_2$. $F_{420}H_2$ can then be oxidized by F_{420} (NiFe) hydrogenase to oxidized F_{420} and molecular hydrogen. Alternatively, $F_{420}H_2$ can serve as reductant in the sequential reduction of N^5, N^{10}-methenyl-H_4MPT: first to N^5, N^{10}-methylene-H_4MPT (Figure 1, reaction 5) and then to N^5, N^{10}-methyl-H_4MPT (Figure 1, reaction 6). Free

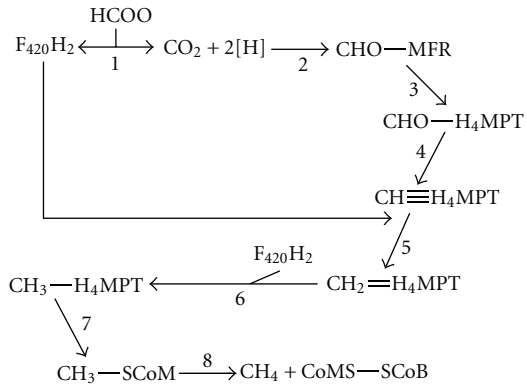

Figure 1: Pathway of hydrogenotrophic methanogenesis from formate. Abbreviations used: $HCOO^-$: formate; $F_{420}H_2$: reduced factor 420; MFR: methanofuran; H_4MPT: tetrahydromethanopterin; CoMS: CoEnzyme S; SCoB: CoEnzyme B. For details regarding enzymes and reactions please see accompanying text.

hydrogen is not a requirement for reduction of CO_2 to methane in organisms growing on formate [36].

There is an important physiological distinction to be made among the hydrogenotrophic methanogens. The presence of cytochromes and the menaquinone analogue methanophenazine seems ubiquitous among members of the *Methanosarcinales* order, however these features appear restricted to only this group of organisms. Interestingly, cytochrome-containing methanogens demonstrate greater than a twofold higher yield when grown with H_2 and CO_2 when compared to noncytochrome-containing methanogens, and growth on formate is restricted to methanogens without cytochromes [38].

Costa and colleagues (2010) have proposed another role for formate dehydrogenase. Reduction of methyl-CoM to CoM-S-S-CoB (Figure 1, reaction 8) is the last step in biological methane formation. Cytochrome-containing organisms catalyze this reaction through the action of a membrane-associated, methanophenazine-reducing (NiFe) hydrogenase (Vho) and a methanophenazine-dependent heterodisulfide reductase (Hdr), which oxidizes molecular hydrogen coupled to concomitant reduction of methyl-CoM to form CoM-S-S-CoB and methane. Organisms without cytochromes lack this energy-conserving mechanism and rely instead, evidence suggests, on a cytosolic complex in which electrons flow from formate to a physically associated Hdr. Bifurcation of the electrons allows a coupling of the exergonic reduction of methyl-CoM to CoM-S-S-CoB to the endergonic reduction of ferredoxin. The reduced ferredoxin is then used to reduce CO_2 to N-formylmethanofuran in step one of methanogenesis [39].

4.2. Formate as an Interspecies Electron Carrier in Methanogenesis. Methanogens are a physiologically specialized group of organisms that use a relatively narrow field of growth substrate: molecular hydrogen and carbon dioxide, formate, acetate, and methanol, and secondary alcohols like 2-propanol or 2-butanol. Primary fermenting organisms (Figure 2, group 1) hydrolyze complex polymers such as polysaccharides, nucleic acids, lipids, and protein to form simple oligomeric and polymeric substrates such as sugars, purines, pyrimidines, fatty acids, glycerol, and amino acids. These monomers are then further fermented to classical fermentation products by the primary fermenting bacteria (Figure 2, group 1): short chain fatty acids such as propionate and butyrate, alcohols such as ethanol, H_2 and CO_2, and acetate [40–43]. The methanogens rely on substrates which are produced from primary and secondary fermenting organisms (Figure 2, groups 1 & 2). Approximately 70% of the methane produced is through the dismutation of acetate (Table 1) in anaerobic bioreactors (Figure 2, group 3) by acetoclastic methanogens. The remaining portion is produced almost exclusively by hydrogenotrophic methanogens (Figure 2, group 4) [44].

Table 1: Free energy changes of some reactions involved in the degradation of fatty acids and alcohols to methane. Calculations based on Thauer et al. 1977 [45].

Reaction	$\Delta G^{\circ\prime}$ (kJ/mol)
ethanol + H_2O → acetate$^-$ + H^+ + 2 H_2	+ 9.6
propionate + 2 H_2O → acetate$^-$ + H^+ + CO_2 + 3 H_2	+ 76
butyrate + 2 H_2O → acetate$^-$ + 2 H_2	+ 48
acetate$^-$ + 2 H_2O → 2 CO_2 + 4 H_2	+ 96
4 H_2 + CO_2 → CH_4 + 2 H_2O	−131
4 formate + 4 H^+ → CH_4 + 3 CO_2 + 2 H_2O	−145
CO_2 + H_2 → formate + H^+	−4.5

In well-balanced ecosystems, carbon flows almost exclusively from the monomeric subunits to the formation of H_2, CO_2, acetate, and formate. If the system becomes unbalanced through the accumulation of short-chain fatty acids, for example, acetate, propionate, and butyrate, the pH will decrease inhibiting methanogenesis. Secondary fermenting organisms are able to metabolize organic acids, but under standard conditions, these fermentation pathways are endergonic. This thermodynamic challenge is solved through syntrophic IET through hydrogenotrophic consumption of hydrogen and/or formate. The classic example of this phenomenon is through hydrogen exchange and was first elucidated with studies of *Methanobacillus omelianskii*; a coculture containing the S-organism, which fermented ethanol to acetate (Table 1), and the methanogen *Methanobacterium bryantii,* which consumed hydrogen with concomitant CO_2 reduction (Table 1) [46].

IET has been documented with both formate and H_2 transfer, and it is unclear whether one mechanism is more physiologically relevant than the other. Metabolic flux calculations [47] coupled with observations that the propionate-oxidizing bacterium *Syntrophobacter fumaroxidans* catalyzed propionate oxidation when grown together with H_2/formate consuming methanogens such as *Methanospirillum hungatei* and *Methanobacterium formicicum,* but not when grown with a H_2-only-utilizing methanogen *Methanobrevibacter arboriphilus* [47] have led to speculation that formate is the more important interspecies electron carrier. Experiments that showed that tungsten (W) and molybdenum (Mo) exclusion inhibited syntrophic growth support that formate

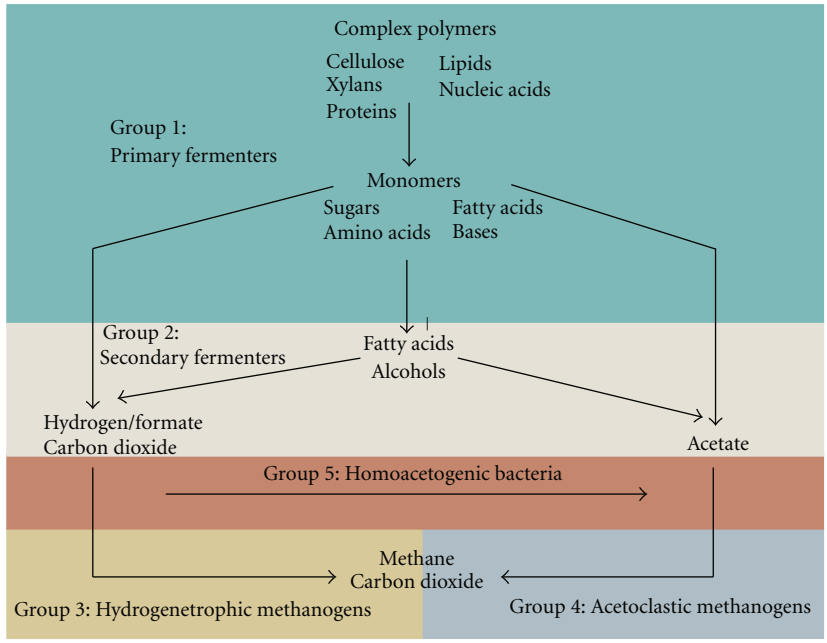

Figure 2: Carbon flux in methanogenic environments. Primary fermenting organisms (Group 1) degrade complex polymers to monomers, fatty acids, alcohols, hydrogen, carbon dioxide, acetate, and formate. Secondary fermenting organisms (Group 2) degrade fatty acids and alcohols to hydrogen, carbon dioxide, acetate, and formate. Hydrogenotrophic methanogens (Group 3) and acetoclastic methanogens (Group 4) convert carbon dioxide and formate or acetate to methane, respectively. For a more detailed discussion see accompanying text.

is important for IET because either element is an essential cofactor for FDH-catalyzed CO_2 reduction. However, these results do not conclusively demonstrate a metabolic preference for H_2 or formate transfer because Mo/W is also essential for formylmethanofuran dehydrogenase (FMDH) catalyzed reduction of CO_2 and formation of N^5-formylmethanofuran [48], an indispensable step in methanogenesis [36]. Recent research indicates that multiple hydrogenases and formate dehydrogenases play a key role in syntrophy [49].

5. Perspectives

Biological methane production is a mature technology for providing renewable alternative fuels from readily available complex organic materials. However, for the fourth generation of (bio)fuels, emphasis is on efficient CO_2 removal from the Earth's atmosphere. The resulting fuels and gases should not only be renewable, but also be carbon-negative. Carbon negative and carbon-neutral energy sources should also be used to drive the production of reduced carbon compounds to be used as fuels in a regenerative energy economy based on CO_2. However, due to its kinetic and thermodynamic stability, strategies to activate and reduce CO_2 are required.

Here, we have reviewed the role of formate production and conversion during methanogenic decay of organic material. It is important to recognize that the conversion of formate to hydrogen and CO_2 is a reversible process with enzymes capable of both CO_2 reduction as well as oxidation identified in the literature. With an oxidation potential close to that of hydrogen, formate is a primary energy source in its own right. Moreover, formate is the first stable intermediate in the biological conversion of CO_2 to methanol according the following overall mechanism:

$$CO_2 + 3NADH + 3H^+ \longrightarrow CH_3OH + 3NAD^+ + H_2O. \quad (4)$$

Cofactor-dependent conversion, however, is not economically viable. According to the above equation, one mole of methanol has a theoretical cofactor requirement of three moles of NADH. At current prices (~\$500/g NADH) this results in a cost per mole greater than \$1,000,000. A recent report demonstrated an efficiency of 127% when *in situ* biocatalytic cofactor regeneration and enzyme immobilization were employed in bench scale experiments. This would effectively lower the cost per mole methanol to \$850,000.

However, as described above, W-MGD-containing iron-sulfur FDH enzymes catalyze CO_2 reduction with reductants other than NADH and are physiologically active in homoacetogenic bacteria. In acetogens, hydrogen serves as the physiological electron donor and metabolic engineering pursuits may provide an industrially relevant way to reduce CO_2 to formate via W-MGD-containing FDH catalysis. This would provide formate as a precursor molecule for biological methanol formation [30]. However, NADH dependency of downstream enzymes such as formaldehyde dehydrogenase and alcohol dehydrogenase would still pose a considerable economic challenge to cost-effective biological production of methanol.

Potentially of more importance would be developing ways of controlling metabolic flux and directing electrons towards increased formate production and, just as importantly, directing electrons from formate consumption directly into

hydrogen or methane production and limiting electron flow down other branches of the metabolic network. Recently, Maeda and colleagues (2008) [50] engineered a strain of *E. coli* with quadruple mutations that produced 141 times more H_2 from formate than the wild-type strain in bench-top experiments. First, hydrogen oxidation by Hyd-1 and Hyd-2 hydrogenase enzymes was inactivated by deleting the corresponding genes (*hyaB* and *hybC*, resp.). Next, the gene for the FHL repressor (*hycA*) was deleted and expression of the FHL inducer encoded by *fhlA* was increased. These alterations alone contributed to an 80-fold increase in hydrogen production rate compared to wild-type strains. When these mutations were combined with inactivation of the aerobic nitrate dependent FDH-O and FDH-N, a further 1.7-fold increase in hydrogen production rate to 113 (±12) μmol mg-protein^{-1} hr^{-1} was observed for a cumulative 141-fold increase in hydrogen production rate compared to the wild-type strain [50]. Thus, streamlining the metabolic flux by channeling electrons from formate directly to the FHL complex rather than down extraneous branches of the metabolic network enhances hydrogen production.

Abbreviations

ΔG:	Change in Gibb's free energy under conditions specified
ΔG°′:	Change in Gibb's free energy under standard conditions at pH = 7.0
CoA:	Coenzyme A
CoM:	Coenzyme M
CoB:	Coenzyme B
DMSO:	Dimethyl sulfoxide
ED:	Entner Doudoroff pathway of glycolysis
EMP:	Embden-Meyerhof-Parnas pathway of glycolysis
FDH:	Formate dehydrogenase
FHL:	Formate-hydrogen lyase
FMDH:	Formylmethanofuran dehydrogenase
H_4MPT:	Tetrahydromethanopterin
IET:	Interspecies electron transfer
MFR:	Methanofuran
MGD:	Molybdopterin guanine dinucleotide
Mo-MGD:	Molybdenum containing MGD
NAD^+:	Nicotinamide adenine dinucleotide
NADH:	Reduced NAD^+
PFL:	Pyruvate formate lyase
PFL-AE:	Pyruvate-formate lyase activating enzyme
PPP:	Pentose phosphate pathway of glycolysis
W-MGD:	Tungsten containing MGD.

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