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Green Chemistry

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Green Chemistry

REVIEW

Advances in plant materials, food by-products, and algae conversion into biofuels: Use of environment-friendly technologies

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Green technologies have emerged as useful tools for the generation of clean fuels with the potential to minimize the effect of human activity on the environment. Currently, these fuels are mainly composed of hydrocarbons obtained from crude oil. Over the last two decades, biomass has gained significant attention as a renewable feedstock for more sustainable biofuel production and has been a great candidate to replace fossil fuels. Principal components of most of the available biomass are cellulose, hemi-cellulose, and lignin. Although available green technologies for biofuel production are progressing rapidly, productivity and chemical yield from these techniques are still below the required values. Therefore, there is a need for interdisciplinary studies to meet the requirements for more global and efficient production by streamlining processes, integrating technologies and achieving techno-economic improvements. In this context, we aim to give an overview of available biomass such as agricultural wastes suitable for the generation of different classes of biofuels including next-generation biofuels. Unfortunately, expensive, wasteful and energy-consuming pretreatment processes are still used.

Therefore, novel technologies that allow a more efficient separation with low resource consumption and the generation of a low number of residues are required. In this regard, the novel technologies such as efficient fractionation techniques, genetic and metabolic engineering including the application of CRISPR/Cas tools, as well as microfluidic platforms to improve the overall yield of biofuel production are discussed.

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Introduction

Sustainability is a key-element for worldwide economic development. Energy is an essential part of people's daily life. There are diverse energy sources such as natural gas, coal, and oil which can be utilized for the production of fuel, heat, electricity etc¹. With increasing world population, the demand for diverse types of fuels has sharply risen mainly due to industrialization and motorization over the world²⁻⁴. This excessive consumption has generated a high concentration of atmospheric pollution and, in particular, a steep increase in the amount of greenhouse gasses including carbon dioxide, sulfur dioxide, and nitrogen oxides from the burning of fossil fuels^{1,3,5}.

This has likely led to many adverse consequences notably changes in climate, loss of glaciers, the rise in global sea level, and loss of biodiversity. On the other hand, fossil fuels have limited sources which are being exhausted due to overconsumption per capita^{4,6}. Therefore, most countries have been revising their policies and shifting their focus towards clean and renewable fuels to meet their future demands¹. In this respect, the Kyoto protocol ratified the target of decreasing carbon dioxide concentration through reduction of dependency on fossil fuels^{1,5}.

The scientific community has made a lot of efforts to employ versatile sources and emerging technologies for developing renewable fuels which are more cost-effective, efficient and sustainable with less emissions^{4,5,7}. Among all energy sources, biomass has gained particular attention due to its numerous advantages over fossil resources^{2,4}. It is a favorable source for production of clean energies like biofuels. Biofuels are being explored as an attractive choice for addressing these crises i.e., reliance on fossil source and greenhouses gas emissions⁴.

They can be solid, liquid or gaseous fuels, which are produced from biomass and can be used either purely or as blended forms with other fuel types^{2,3}.

Biofuels have many benefits which are: (a) availability from existing biomass sources, (b) environmentally friendly potential and lower threat to the ecosystem, (c) biodegradability, renewability and contribution to sustainability, (d) beneficial for the economy including extending the opportunities for agriculture, (e) increasing industrial investment, boosting agricultural income, creating rural manufacturing jobs and (f) achieving energy security^{2,4,8-10}. Due to all these advantages, biofuels are becoming competitive with fossil fuels, and are forecast to grow even faster in the next decade². Therefore, the major goal of this review is to provide a detailed discussion on definition, reaction pathways, agricultural sources, production method (conventional and innovative green techniques), and existing challenges with common forms of liquid and gas biofuels.

Biofuels production

Agricultural wastes as a biomass source for biofuels

The major source of biofuel is biomass and, for this reason, biofuel is also called biomass-based fuel^{3,10}. Biomass is defined as an organic substance, which has stored sunlight in the form of energy by photosynthesis⁹. It refers to any renewable type of plant-based material which can be used for the production of energy, like transport fuel, power or heat^{1,10,11}. It is considered to be a relatively attractive feedstock because of a) renewability, b) positive environmental properties (lower release of carbon dioxide and sulfur content than fossil energy) and c) significant economic potential when compared to fossil resources^{2,6,11}. Biomass can be converted into biofuels using different thermal, physical or biological processes⁵. There are various categories of plant-based biomass,

which fall into two main groups including agricultural biomass (e.g., grasses, straw from rice and wheat, stalks, bran/husk, crop residue, seeds and plant food by-products), and forestry resources (e.g., thinned wood and sawdust, logging residues and leaves). However the non-plant-based biomass are classified into livestock resources (e.g., butchery waste), fishery sources (e.g., industrial fishery processing by-products), industrial biomass (sewage sludge), household biomass (e.g., garbage waste) and plantation sources (e.g., aquatic algae, photosynthetic organisms)^{9,12,13}. **Fig. 1** shows the potential agro-residues, which can be used as biomass for biofuels production^{3,4,7,14}.

Among these categories, agricultural waste conversion represents approximately 64% of the total energy demand and has the most significant contribution to biomass energy⁹. Agricultural waste refers to the residues produced in fields or on farms during harvesting and other activities^{9,13}. Many developing countries have a wide range of agricultural wastes in abundant quantities, which are regularly disposed instead of being used as biomass source⁹. For instance, rice straw is globally produced at around 600-900 million tons per year. Only a small portion of this straw is directly used (as animal feed), and the remainder is mostly burnt from the field¹. Another example is corn straw, where more than 90% is left in the fields in the United States¹. On the other hand, the current disposal methods for these agricultural residues have led to huge environmental issues. For instance, straw burning results in atmospheric pollution and affects human health¹³.

Plant-derived terpenes (or terpenoids) are the other attractive sustainable resource, which can be considered as powerful platform for production of plant-based biofuels. Many plants such as *Eucalyptus polybractea*, mints, eucalypts, pines and citrus produce

wide varieties of terpenes such as α -pinene, β -pinene, camphene, limonene, 3-carene, 1,8-cineole, spathulenol, myrcene etc. It has been reported that global industry recovers 3 million tons of these hydrocarbons per year. However, some of them (such as β -pinene or myrcene) have been identified to meet current chemical and industrial requirements (e.g., viscosities, freezing and flash points and density), and therefore, have potential to be used directly or blended with existing fuel like jet fuel (e.g., JP-5, Jet-A, and JP-8), gasoline, or other types diesels¹⁵.

Therefore, in view of the importance and capability of above-mentioned resources, utilizing these potential plant/agro-resources for fuel production in an appropriate way is highly necessary providing the "double green" benefits of avoiding uncontrolled release of pollutants into the atmosphere and substituting non-renewable fossil fuels.

Biofuel classification

Biofuels can be broadly classified based by the type and sources of biomass, e.g., residues from agriculture, food industry, fishery or municipal wastes⁴. Biofuels can be also categorized according to primary or secondary generation or based on their forms and applications (**Table 1**)⁴. First generation biofuels were produced without processing biomass and used mainly for heat and electricity generation, while second generation biofuels are obtained by highly-processed biomass and can be employed in diverse industrial applications. Second generation biofuels are further divided into three sub-categories based on technologies and materials used for their production⁴. The common forms of liquid or gaseous biofuels are bio-liquids (including bio-alcohols such as bioethanol and biomethanol), biodiesel and biogas^{3,4} (**Fig. 2**).

Table 1. Different generation and categories of biofuels

Category/ generation	Source/substrate	Product
Primary	Firewood, wood chips, pellets, animal waste, forest and crop residues	Used as unprocessed form, mainly for heating, cooking or electricity purposes
	Seeds, grain and sugars	Bioethanol/ butanol (by fermentation of starchy or sugar-rich crops), Biodiesel (by transesterification of plant oils)
Secondary	Lignocellulosic biomass	Bioethanol /butanol (using enzymatic hydrolysis), Methanol, mixed alcohol and green diesel (by thermochemical processes) Biomethane (by anaerobic digestion)
	Algae, seaweeds	Biodiesel and bioethanol from algae and seaweeds, Hydrogen from microbes and green algae

Bio-alcohols

Alcohols are known as oxygenated fuels¹⁶. Each molecule of alcohol possesses a various number of oxygen atoms, and the number of these atoms is inversely associated with its heating value. In other words, the heating rate for the combustion stage decreases as the number of oxygen atoms increases. Practically speaking, any of the organic molecules of the alcohol family can be considered as a biofuel. The prime examples of this family are butanol (C₄H₉OH), propanol (C₃H₇OH), ethanol (C₂H₅OH) and methanol (CH₃OH) which are suitable for commercial purposes^{10,17}. Bioalcohols are defined as alcohols biologically obtained from renewable biomass sources^{3,18}. Among all types of bioalcohols, bioethanol and

biomethanol are the most common due to suitable economic and technical potentials for internal combustion engines^{3,19}.

Bioethanol

Ethanol is a colorless, clear liquid with an agreeable odor and pungent taste. Pure ethanol can be used as a vehicles fuel-like gasoline additive/petrol substitute to increase octane number and improve the emissions released by motor vehicles^{3,19}. Due to the properties of ethanol, bioethanol is highly regarded as a renewable alternative for motor vehicles and transportation system. Consequently, it reduces the consumption of crude oil^{10,16,19} and decreases the adverse environmental impact by reduction of CO₂ build up¹⁰.

Direct use of bioethanol or in the form of a mixture with gasoline has a long history. Its usage was widespread in the United States and Europe until the early 1900s. After the Second World War, the potential of bioethanol was largely ignored until the appearance of the oil crisis in the 1970s. Since the 1980s, there was a growing interest regarding the use of bioethanol as a substitute fuel especially for transportation¹⁰. Brazil and the United States are the world's leading bioethanol producing countries with more than 80% of global production. The United States is the leading producer with an estimated production of more than 15,000 million gallons per year, which accounts for more than half of global production^{19,20}. Brazil is another major producer with an estimated production of more than 7,000 million gallons per year²⁰. **Fig. 3** depicts bioethanol production in different countries around the world²¹.

Bioethanol can be produced from plentiful agricultural residues² (**Fig. 4**). Bioethanol is also known as grain alcohol since it is mostly made from the sugar components of plant materials and starchy crops^{9,19}. It is

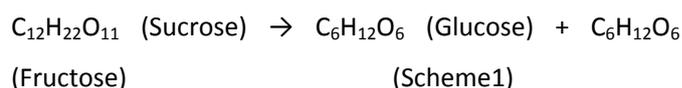
generated by fermentation of the sugar components of these substances¹⁹. Any kind of carbohydrates can be used for bioethanol production^{3,10}. These raw agricultural wastes were generally categorized into two groups, which are a) sucrose-containing substances; and b) starchy crops^{3,10,19}.

Eventually owing to the development of advanced technologies, lignocellulosic waste materials/cellulosic biomass such as wood and straw have also been added as suitable agro-wastes for economical production of bioethanol^{3,10,19}. The primary examples of lignocellulosic agro-wastes are rice straw, wheat straw, corn straw, and bagasse, in which cellulose is the chief component and which are available throughout the year¹. Nevertheless, bioethanol production from lignocellulosic biomass is more expensive than traditional starchy crops¹⁷, as the fermentation process of these cellulosic biomasses is more complex and longer¹⁹.

The raw material used for bioethanol production is a crucial parameter for energy yield. For instance, sugarcane and cellulosic bioethanol yield 9 times as much energy as the fossil energy used to produce them. It is also reported that bioethanol from corn yields 20-30% more energy than fossil fuel energy consumed to make it¹⁰. Among all agro-wastes, sugarcane juice and molasses have been much exploited in recent years, yielding hydrated and anhydrous bioethanol. Brazil is one of the biggest producers of sugarcane with 31% of global production. There are approximately 9 million hectares of sugarcane cultivated in Brazil. Sugar beet is another popular crop which is grown in many European countries and yields a higher amount of bioethanol than grains such as wheat. The United States mainly uses cornstarch to produce bioethanol, whereas Europe utilizes starch obtained from wheat and barley. Canada also reported plans for the significant future development of corn-based

bioethanol, and countries like Argentina are considering the possibility of corn as a source of biofuel in the future^{1,6,10}.

Three processing steps are required for bioethanol production from sugar-rich crops: enzyme hydrolysis, fermentation, distillation/dehydration^{6,17,19}. Hydrolysis of carbohydrate by enzymatic treatment (also called saccharification) is the initial step which releases sugars from stored carbohydrate. This results in a fermentable sugar-containing solution^{19,20}, which can be further hydrolyzed by yeast-derived invertase to release simple sugars, e.g., glucose and fructose (Scheme 1)^{3,6,11}. This step is followed by fermentation, during which simple sugars are converted into ethanol by the action of *Saccharomyces cerevisiae* yeast (Scheme 2)^{3,11,20}. Distillation/dehydration, as the last step, is applied to the fermented broth with the aim of recovery and concentration of ethanol. Distillation is an energy-consuming operation, which accounts for a significant part of bioethanol production cost²⁰. The fermented broth typically contains approximately 12% ethanol. The alcohol can be purified up to 96% by distillation.



The process of bioethanol production from lignocellulosic materials is different. Lignocellulose is a poly-carbohydrate complex which is composed of lignin, cellulose, and hemicellulose. In this type of material, the lignocellulose is first subjected to pre-treatment for delignification to release cellulose and hemicellulose before hydrolysis. The pre-treatment is performed to break the matrix, decrease the degree of cellulose crystallinity and increase the fraction of amorphous cellulose¹. In fact, this step helps making lignocellulose

biomass more susceptible to further treatment such as hydrolysis with improved yield of monomeric sugars. The type of pre-treatment can be physical (e.g., size reduction, pyrolysis, microwave heating and non-thermal irradiation), chemical (e.g., wet oxidation, acid or alkaline treatments), physico-chemical (e.g., steam, ammonia fiber or CO₂ explosion) or biological (e.g., microbial treatment using white, brown and soft rot fungi)¹ (Fig. 5).

After pre-treatment step, enzymatic hydrolysis of cellulose and hemicellulose can be performed to produce fermentable sugars such as glucose, arabinose, mannose, galactose, and xylose. In this stage, hydrolysis breaks down the glycosidic linkages to release pentoses and hexoses. These hydrolyzed sugars can be then fermented into bioethanol^{1,11}.

Although most of the current studies reveals the potential use (by software simulation) of such technologies that could reduce the environmental impact, it is still necessary to evaluate in detail the processing cost, the purity of ethanol obtained from the different plants, as well as the practical implementation of the systems, these being the main relevant obstacles to establish energy saving technologies in the concentration of bioethanol²². Besides, during the process of obtaining bioethanol, many waste and byproducts are generated, which need to be valorized since they can be reused to obtain more bioethanol as well as being a source of other valuable compounds.

Biomass-based Methanol

Methanol (CH₃OH) is a simple organic liquid hydrogen carrier that acts as a hydrogen storage compound A⁹. It is also known as wood alcohol since it was extracted from wood as a co-product of charcoal. It is an alternative for conventional motor fuels or a clean additive to the gasoline^{2,23}. Methanol is mainly manufactured from non-

renewable natural gas, while it can be produced from biomass by gasification process¹⁹. Production of biomethanol from biomass is environmental, economic and consumer benefit process¹⁷. It has been reported that the total cost of methanol production from biomass is remarkably cheaper than its production from CO₂. Furthermore, there is an increasing trend in methanol demand whereas the price of this fuel is expected to rise in the future. Therefore, processing of biomass is the most cost-effective way to produce methanol⁹. For this reason, some countries such as Brazil and the US have paid much attention to the production of biomethanol⁹. Moreover, some other products such as syngas also can be produced from biomass.

Lignocellulosic biomass is a valuable substance for the production of methanol. It contains cellulose, hemicellulose, lignin and small amounts of proteins, lipids, and ash that can be decomposed to produce methanol biofuels⁹. Biomethanol, especially from lignocellulosic materials, has low emissions since the carbon content of alcohol is primarily derived from the carbon that was sequestered in the growing of feedstock and is only being re-released into atmosphere². It has been reported that sugar cane bagasse and corncob with the total biomethanol content of 5.93% and 0.67%, respectively, can be used as a promising source⁹. Nakagawa et al.²⁴ also reported a high yield (55% by weight) for methanol production from rice bran, whereas the yields for rice straw and husks were 36% and 39%, respectively. Apart from the sources above, other agricultural and animal biomass sources such as vegetable residues, wheat straw, butchery waste, fishery waste, and thinned wood have been introduced as potential materials for the biological production of methanol⁹.

Methanol is typically produced from the breakdown of methyl esters or combination of ether with the methoxyl groups⁹. So far, several processes have been introduced for production of biomethanol, such as pyrolysis, bio-synthesis, gasification, electrolysis and photoelectrochemical methods. Each method has its own benefits/limitations and applications. The pyrolysis, as a conventional method, is particularly adapted for methanol production for diesel engines and gas turbine applications on a large scale, whereas electrolysis and photoelectrochemical methods, as new techniques, are still limited to lab scale. Bio-synthesis process is also used as production method for gaseous fuels from a wide range of biomass resources; however gasification is considered as more preferable technique for the same due to its cost-effective benefit⁹.

Pyrolysis is the first synthetic process was introduced by Gulluetal in 1927⁹. This method can produce biofuel with high fuel-to-feed ratios, and as a result, it has been attracting more attention than other production methods⁵. The term “pyrolysis” is taken from the Greek words “pyro” meaning fire and “lysis” meaning decomposition or cleavage into smaller constituent parts using thermal energy^{2,5}.

The pyrolysis process of organic substances is very complex and consists of both simultaneous and successive reactions⁵. It involves a catalyzed reaction of hydrogen and carbon monoxide under high temperature and pressure⁹. In this process, the decomposition of components starts at 350 - 550 °C and rises to 700 - 800 °C in the absence of oxygen. The long chains of hydrogen, oxygen, and carbon compounds break down into smaller parts in the form of gases, condensable vapors and solid -^{5,25}. The products of biomass pyrolysis are bio-oil (or bio-crude), residual char and gases such as CH₄, H₂, CO₂ and

CO^{25,26}. In the later stages, the methanol is derived from the bio-oil through synthesis gas process^{5,25}. -
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The process of pyrolysis can be classified into three different categories named flash, fast and conventional (Table 2)⁵. The categories differ in operating conditions like process temperature, heating rate, solid residence time, biomass particle size. The rate and extent of decomposition during pyrolysis and distribution of intermediate and final products are highly dependent on these effective parameters^{5,9}.

Table 2. Classification of pyrolysis process and its products under different operating conditions

	Type of pyrolysis		
	Conventional/slow pyrolysis	Fast pyrolysis	Flash pyrolysis
Operating conditions			
Heating rate (K/s)	0.1-1	10-200	>1000
Particle size (mm)	5-50	<1	<0.2
Residence time (s)	450-550	0.5-10	<0.5
Temperature (K)	550-950	850-1250	1050-1300
Approx. product yield (%)			
Oil	30	50	75
Char	35	20	12
Gas	35	30	13

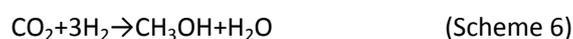
Gasification is another thermochemical processing method for synthesis of methanol from biomass. In this method, the biomass is initially gasified to produce intermediate product i.e., synthesis gas (syngas), which is subsequently transformed into methanol under high pressure and temperature in a MeOH synthesiser^{5,27}. Production of syngas can be done through catalytic and non-catalytic routes. The non-catalytic process requires

high temperature, while the catalytic route can be operated at lower temperature¹⁴. **Fig. 6** is depicted the production steps of biomethanol from carbohydrates biomass by gasification and partial oxidation reaction³. As can be seen, the gasification involves reacting the biomass with oxygen or steam to decompose the complex carbohydrates substance and produce a gaseous mixture consisting of H₂ (22-32%), CO (28-36 %), CO₂ (21-30 %) and other hydrocarbons such as CH₄ and C₂H₄³. The gases are further converted in a conventional steam-reforming/water-gas shift reaction to predominantly produce carbon monoxide and hydrogen (Scheme 3 & 4). This step is then followed by high-pressure catalytic methanol synthesis as shown in Scheme 5 and 6².

Shift reactions



Methanol synthesis reaction



The gasification has advantages over other conversion technologies. Some of these advantages are a) feasibility of use of any type of biomass (e.g., agricultural, forestry, chemical or organic wastes/by-products); b) feasibility of conversion of the entire carbon content of the biomass materials into fuel; c) the product gas can be converted into a wide range of potential biofuels (e.g., methanol, synthetic diesel, gasoline, H₂ and Bio-Synthetic Natural Gas); and d) lower CO₂ emission and high thermal efficiency²⁶.

Gasification process also can be performed in the form of hydrogasification or steam hydrogasification. Hydrogasification uses hydrogen as the gasifying agent, whereas in steam hydrogasification, steam and hydrogen

are used as gasifying agents. These conversion processes are typically suited for the wet biomass/feedstock. These processes may improve the overall process efficiency during conversion of biomass by increasing the contents of products such as CH₄, CO, CO₂, H₂, or other hydrocarbons²⁶.

Biodiesel

Biodiesel is a clear amber-yellow liquid, which is chemically defined as mono-alkyl esters of vegetable oils or animal fats. It is an interesting substitute to petro-fuel, which can be made from both edible and non-edible oils^{11,28}. Biodiesel has been probably received the most attention as a substitute fuel for diesel engines among all biofuels, due to its similar energy content and chemical structure²⁸. It has the remarkable economic potential at industrial scale and has been commercially used in several countries such as the United States, Brazil, Australia, Malaysia as well as over European countries^{28,29}.

Table 3 shows the major benefits of biodiesel over conventional petrodiesel fuel^{8,11,28,30}. Thanks to these advantages, governmental policies are changing towards investment on research and production of biodiesel particularly from crops with higher oil production. Considering the existing trend for biodiesel demand and the potentiality of increasing production, it is possible that the production of biodiesel increases further in the near future.

Table 3. Major advantages and disadvantages of biodiesels as compared to petroleum diesel fuels

Advantages	
Technical benefits	Non-toxic, Non-flammable and non- explosive vapors Perfectly miscible Higher lubricity Lesser flash point than petrodiesel Synthesized from edible and non-

	edible oils Better sulfur and aromatic contents Safer handling and storage
Environmental benefits	Environmentally friendly Reduction of CO ₂ in the atmosphere Reduction of sulfur levels in the atmosphere Biodegradability Renewability
Economic benefits	Job creation Avoidance of urban migration Provision of modern energy carriers to rural communities Availability Energy security
Disadvantages	Lower energy content Lower stability Lower engine speed and power Creation of engine durability problems and corrosion Creation of carbon deposition and polymerization in engine

Since biodiesel is a product for the energy sector, oil for biodiesel production needs to be inexpensively available in large quantities⁷. To increase the availability of oil, various vegetable oils and crops must be taken into consideration. The idea to use vegetable oils for renewable fuel competing with petroleum was proposed since the beginning of the 1980s¹¹. Nowadays, several crops have been put forward as a potential candidate for biodiesel production. Some examples are soybeans, peanut, rapeseed, coconut, babassu, sunflower palm, castor bean, canola, corn, and cotton^{6,7,28}. **Fig. 7** shows the major oils used for biodiesel production in the United States in 2016³¹. Also, there are some other palm species, for instance, *Attalea maripa*, *Syagrus coronata*, *Astrocaryum aculeatum*, *Acrocomia aculeata*, and

Mauritia flexuosa, which are usable for biodiesel production⁷.
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Moreover, some plant-based oils made from seeds have also been introduced to endow great potential for making biodiesel such as assal (*Shorea robusta*), neem (*Azadirachta indica*), mahua (*Mahua indica*), besides karanj (*Pongamia pinnata*) and ratanjyot (*Jatropha curcas*)⁶. Non-edible vegetable oils, such as Karanja (*Millettia pinnata*), *Jatropha curcas*, and *Madhuca longifolia* have also been reported as suitable seed oils to produce biodiesel²⁸.

Different parts of the fruits can be used for oil extraction. Coconut oil is extracted from the endosperm. In oil palm, both mesocarp and seeds of the fruit are used, whereas, in peanut, castor bean, babassu, soybean, rapeseed, sunflower, physic nut, and cotton, the oil is extracted from seeds. The oil content of each part in each crop varies depending on species and anatomical differences. It has been reported that oil palm and physic nut are the most advantageous biodiesel crops as they can produce approximately 8000 and 1500 kg of oil/ha, respectively⁷. It is also obvious that the higher oil yield is corresponding to the lower production costs. Therefore, crops with high oil content are preferable²⁸. Some crops such as soybean have a high value and consequently makes the production of a cost-effective fuel very challenging. However, there are various types of low-cost oils and fats, such as animal-based restaurant waste which can be converted to biodiesel¹¹. The biodiesel can also be made from other sources such as pork lard, beef tallow, and yellow grease. Processing these low-cost oils is usually challenging since the free fatty acids contents are high in these oils and therefore cannot be converted into biodiesel by an alkaline catalysis^{2,11}. Another valuable source of biodiesel is microalgae. The advantages of microalgae as a feedstock for biodiesel production, over

terrestrial plants, are that there is no requirement for soil fertility and, if marine algae are used, there is no need to draw upon valuable and often scarce supplies of freshwater²⁹. The other main advantages as well as disadvantages of using microalgae for biofuel production are shown in **Table 4**³².

Table 4. The major advantages and disadvantages of biofuel produced from microalgae

Advantages	Disadvantages
High growth rate	Low biomass concentration
Less water demand than land crops	Higher capital costs
High-efficiency CO ₂ mitigation	
More cost effective farming	

Although many plants resources and also technologies have been introduced for biodiesel production, only few of them are economically viable and can be implemented in commercial scale. One this handful resource is *Camelina sativa*, which is a fast-growing plant with high oil content (35–38%). Camelina-based fuel has been in use for commercial and military aircrafts and it is a more efficient solution than commercial biodiesel that absorbs water too easily³³. Another potential option for commercial biodiesel production is Pennycress (*Thlaspi arvense L.*). It contents 36% oil with high net energy output. A minimum amount of 907 kg of this plant can be harvested per acre, which allows for approximate production of 115 gallons of biodiesel. This plant is very short growing season and its biodiesel properties is found to be excellent, and

therefore, it is considered as great option for commercially production of biofuel³³.

To commercialize a biodiesel fuel on a large scale, several determining factors such as costs of processing and technology, transportation and storage of feedstock and land use changes are involved³³. In this respect, Singh and Gu, 2010³⁴ stated three requirements, which must be fulfilled for a successful replacement of conventional fuel by biofuel production process. These requirements are a) availability of sufficient sources for production at commercial scale; b) having standard specifications and quality; and c) having a lesser finishing cost as compared to conventional fossil fuel³⁴. In order to fulfil these requirements, more research studies are required to assess commercial viability of plant-oil resources, their economic efficiency, feasibility and modifications of technological process for commercialization of biodiesel production³³.

As previously mentioned, herbal oil is the endless primary source of biodiesel with a similar energetic content to diesel fuel¹¹, which can be used as fuel for combustion engines after applying some modifications. Pure oils generally have a higher viscosity than diesel fuel (approximately 10-20 times) and lower volatility¹¹. Therefore, the complete burning does not occur and consequently results in the formation of deposits in the fuel injector of diesel engines. The high viscosity of pure vegetable oils (27.2 and 53.6 mm²/s) makes direct use of them impossible. To solve this issue, vegetable oils have to be catalytically changed into biodiesel by transesterification or esterification process to reach a viscosity of 3.59 to 4.63 mm²/s^{7,11}.

Transesterification is the main conventional process to convert vegetable oil to their (m)ethyl esters in the presence of a catalyst^{11,29}. Various esters such as methyl,

ethyl, butyl, and 2-propyl can be obtained with the help of a catalyst, mainly potassium and/or sodium carbonate and alkaline metal alkoxides and hydroxides (sodium and potassium)^{2,11}. Among them, alkaline metalalkoxides are generally preferred owing to their highest activity and high-yield production in a short reaction time². In this process, triglyceride (oil) and alcohol react and consequently form methyl or ethyl-esters as the main product and glycerol as a by-product (**Fig. 8**)³⁵.

Due to the high dependence of the transesterification process to the presence of a catalyst, these compounds have an important role in biomass transformation to produce biofuels. Due to laborious preparation and high cost, catalysts occupy a significant percentage of overall process cost, hence, the development of cost-effective and stable catalysts to enhance the industrial production of biofuels is essential for economic viability. In this context, to reduce the required time and increase the efficiency of the reaction, other catalysts such as enzymes (e.g., lipases and esterase), acids (e.g., sulfuric and hydrochloric acids), and bases can be utilized^{7,8}. The choice of a catalyst depends on quality and type of the initial oil. For instance, the acidic oils require a basic catalyst for neutralization of their free fatty acids contents⁷.

The second method to produce biodiesel is esterification. During esterification, free fatty acids react with low molecular weight alcohol such as ethanol or methanol, to produce ester (i.e., biodiesel) and water. Oils with high free fatty acid content, resulting from the refining process of animal fats obtained from slaughterhouses or oils extracted from sewage, are the prime examples of these acidic oils⁷.

Apart from the transesterification method, micro-emulsification, thermal cracking and non-catalytic supercritical methanol methods have been applied to pure vegetable oils, as reported by Yusuf et al.²⁸ and Demirbas¹¹. Regardless of the used method, the final biodiesel product should have physical properties close to those described by Demirbas¹¹ (**Table 5**).

Table 5. Physical characteristics of biodiesel

Physical parameters	Range
Kinematic viscosity range (mm ² /s, at 313 K)	3.3–5.2
Density range (kg/m ³ , at 288 K)	860–894
Boiling-point range (K)	>457
Flash-point range (K)	420–450
Distillation range (K)	470–600
Vapor pressure (mm Hg, at 295 K)	<5
Solubility	Insoluble in water
Reactivity	Stable, but avoid strong oxidizing agents
Appearance, odor	Light to dark yellow, clear Light musty/soapy odor

There are several factors affecting the (m)ethyl ester yield efficiency and quality of biodiesel, like time and temperature of incubation, the type of catalyst and its concentration, the molar ratio of alcohol/vegetable oil, the purity of the reactants, nature, and composition of biomass and the methodology used^{11,28,29,35}. The amount of oil content and its saturation level are critical factors in the quality of the final biodiesel product. The highly unsaturated fatty acids need to be modified by hydrogenation since they increase polymerization risk in engine oil and cause oxidative stability issues for fuel²⁹. Another important factor, which needs to be taken into account, is the presence of water in oil and alcohol. Both of these two items must be anhydrous since the presence of water may lead to soap production from the existing

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free fatty acids. This undesirable by-product reduces the efficiency of the process and complicates the purification of glycerol⁷.

Biogas

The digestion of bio-wastes under anaerobic condition results in the formation of a product in the gas phase, which is called biogas^{11,36}. It is a clean form of energy, which is manufactured using a mix of anaerobic microbial species, fermenting organic materials under controlled conditions³⁷. Biogas is a mixture of carbon dioxide, methane, sulfur components, nitrogen, and hydrogen (**Table 6**). However, the main constituent (i.e., methane) is an inflammable gas with no taste, color or odor^{36,37}. The composition and yield in final methane varies depending on the type of feedstock, conditions in the digestion system and retention time³⁸.

Table 6. Composition of biogas

Composition	Volume (%)
Methane	55-65
Carbon dioxide	35-45
Hydrogen sulfide	0-1
Nitrogen	0-3
Hydrogen	0-1
Oxygen	0-2
Ammonia	0-1

Biogas, as an energy source, has many applications and advantages. It is traditionally used for internal combustion engines to produce electricity and heat. However, its potential use in fuel cells could increase its electric efficiency^{1,36,37,39}. It can also be used as a fuel a) for water pumps and agricultural engines; b) for liquefied petroleum gas and gasoline engines³⁷; c) for boilers¹, incubators and coolers^{37,38}; d) for vehicle transportation^{38,39}; and e) for heat generation^{36,39}. The

biogas is also used as a prime source of energy for cooking and lighting. Cooking accounts for a considerable portion of household energy consumption, especially in developing countries. Lighting is known as the second most common application for biogas, in particular in the areas where the electrical grid connection does not exist. In these regions, biogas can be adapted for use in gas mantle lamps³⁹.

Due to the significant advantages of biogas over other forms of gas, it is becoming a popular source of energy in both developing and developed nations. The process which is used for the production of biogas (anaerobic digestion, AD) is considered the most energy-efficient and economical method although it has low carbon efficiency and leads to large amounts of residues. It can drastically reduce greenhouse gases and therefore is accounted as an environmental treatment for recovery of clean energy from disposable residues^{38,40,41}. It can also recycle plant nutrients and increase agricultural productivity^{37,39}.

Due to these applications and benefits, there is a great interest in the production of biogas worldwide. For instance, in 2007, the biogas production in Europe reached 6 million tons of oil equivalents with a yearly increase of more than 20%. Germany is the largest biogas producing country around the world and has the strong development of agricultural biogas plants on farms. It operates about 4,000 agricultural biogas production units on German farms opened in the last decade³⁸.

Any type of biomass containing proteins, fats, carbohydrates, hemicelluloses or cellulose as principal components can be used as biogas substrate³⁸. This includes various raw materials such as sewage sludge, human excreta, animal manure, organic fraction of municipal solid waste and the residues from crop and forest^{37,39}. Algae could also be accounted to be a raw substance for production of biogas, which is gaining

particular interest because some of them can largely grow up without any oxygen supply requirement³⁷. Only strong lignified organic substances such as wood are not suitable as biogas sources due to their slow anaerobic decomposition³⁸. It is annually estimated that about 1,680 million dry metric tons of crop residues are produced in developing countries. This can be regarded as a significant portion of the source required for biogas production³⁹. Among all agro-residues, food and food-processing wastes are the primary resources for this technology³⁹. Food waste is approximately composed of 25% and 42% of domestic household and commercial waste, respectively⁴⁰.

Anaerobic digestion of food waste is regarded as a highly suitable method compared to other thermochemical bioconversion methods like gasification or combustion⁴². Surendra et al.³⁹ stated that food waste is the best source for biomethane production due to the high amounts of moisture (>80%) and volatile solids (95% of total solids). Food wastes are low in nitrogen content (except meat waste) but rich in organic matter which is readily fermentable³⁹.

The proximate composition of food-derived residues can considerably vary depending on their original source. Large ranges of moisture content (74–90%), volatile solids to total solids ratio (80–97%), and carbon to nitrogen ratio (14.7–36.4) are observed. There is a wide range of agro-substrates which can be used for the production of agricultural biogas, such as beet pulp, fruit, vegetable pomace, maize silage, maize, sunflower, grass, and sudangrass^{36,43}. The net energy yield per hectare is the most important factor for choosing crops. The highest gross energy belongs to maize and forage beets which make them as a suitable ideal source of biogas³⁸. Shortly, it is predicted that biogas production from energy crops will be increased and therefore, requires to be based on a

wide range of energy crops that are grown in versatile and sustainable crop rotations³⁶.
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As previously mentioned, anaerobic digestion is widely used for biogas production. This complex process is a biological process that converts the organic substance into energy-rich biogas under anaerobic conditions^{32,35}. This conversion is carried out by a particular ecosystem of microorganisms through a series of metabolic stages, which is divided into four phases namely, hydrolysis, acidogenesis, acetogenesis and methanation^{38,39}. In the first step, the complex compounds containing carbohydrates, lipids, and proteins are converted into their soluble monomers/oligomers such as fatty acids, amino acids, sugars or even glycerol by hydrolysis.

This step is also called a liquefaction stage. This process is facilitated by fermentative or hydrolytic bacteria which release extracellular enzymes such as xylanase, cellobiase, cellulase, amylase, protease, and lipase^{38,39}. Most of these bacteria are strict anaerobes belonging to the genera *Bifidobacteria*, *Clostridia*, and *Bacterioides*³⁸. Afterwards, the process of acidogenesis is performed by acidogenic bacteria, which ferment the soluble compounds. The output of this step is a mixture of hydrogen, alcohol, carbon dioxide, and low molecular weight volatile fatty acids such as propionate and butyrate^{38,39}. During the acetogenesis stage, alcohols and volatile fatty acids are anaerobically oxidized by hydrogen-producing acetogenic bacteria into acetate, CO₂ and H₂.

Acetate can also be formed from H₂ and CO₂ by hydrogen-oxidizing acetogenic bacteria. In the final stage, the groups of methanogenic strains produce a mixture of methane and carbon dioxide from acetate, H₂, and CO₂. Only a few species can degrade acetate into methane and carbon dioxide, such as *Methanosarcina barkeri*, *Methanococcus mazei*, and *Methanotrix soehngenii*^{38,39}.

Fig. 9 shows the main steps of the bio-methanation process³⁹.

The physical characteristics and chemical composition of final biogas are highly dependent on several factors including the type of process, nature and physicochemical properties of the organic substance, operation conditions (pH, temperature, carbon/nitrogen ratio, retention time) and origin of the substrates^{36–38,42}. These parameters strongly affect the design, performance, and stability of the digestion process and must be set up within a desirable range for an efficient production^{36,42}. Any drastic change in controlled condition for operation can adversely affect the biogas production. For instance, carbohydrates and proteins have a faster conversion rate compared to other components. However, they yield a lower quantity of biogas. Fat provides the highest yield; however, due to its poor bioavailability, it has a longer retention time. - The carbon/nitrogen ratio in the substance used must be well balanced (between 15 and 30) in order to avoid ammonia accumulation during processing³⁸. The time and frequency of harvest are also considered as effective parameters which notably affect biogas quality and its final yield³⁸.

Innovative technologies to improve the production of biofuels

Novel fractionation technologies

Lignocellulose-based biomass has considerable potential as a raw material in biofuel production⁴⁴ and its separation into cellulose, hemicellulose, and lignin is one of the most crucial steps of this production. Unfortunately, expensive, wasteful and energy-consuming pretreatment processes are still employed. Therefore, novel technologies that allow a more efficient separation with low resource consumption and the generation of a

low number of residues are required⁴⁵. At the end of biomass separation, the particles are expected to be small in size to increase reactive surface area, in order to improve the productivity since the hydrolysis process is directly influenced by the porosity of lignocellulose-based biomass.

Fractionation techniques have been employed in biofuel production for decades, but new improvements are highly required. Recently, currently available fractionation technologies are significantly improved and emerged as an effective way to minimize overall cost and increase the separation yield of lignocellulose. Different hybrid fractionation techniques are employed for the pretreatment of biomass for biofuel production.

Dry fractionation

Novel dry fractionation processes were recently shown to have significant advantages by decreasing the use of water, solvents, and chemical reagents as well as meeting other principal requirements for more efficient biofuel production. These separation techniques are essential to generate biomass with more appropriate composition and an increased rate of accessibility by enzymes or microorganisms during further fermentation steps. The use of this processes in combination with other processes result in a more efficient fractionation. Chuetor et al. (2015)⁴⁶ separately combined ultrafine milling with turbo-fractionation (size and density-dependent) and with electrostatic fractionation technologies in order to produce fractions from rice straw to be employed in the bioethanol industry. The specific energy requirement of both techniques to reduce particle size was between 12.5 and 22.4 Whkg⁻¹, which indicates that energy consumption was almost negligible compared to other conventional techniques (i.e., knife and ball milling or thermal treatments using steam). The processing time

was considerably shortened, and fractions presented a proper structure, size, and composition. Compared to untreated biomass, the glucose yield, and ethanol production during 72 h of fermentation was increased by 83-103% and 75-95%, respectively.

Piriou et al. 2018⁴⁷ developed an efficient dry fractionation process for separation of lignocellulosic biomass. The fragmentation of the biomass was conducted using a vibrating mill and a rotary ball mill. After fragmentation, an additional step of triboelectric static charging was employed in a dynamic fluidized air bed for the deviation of the path in the electric field of the charged particles in order to sort them efficiently. The sorted particles were collected since they were attached to the electrode. In general, dry processes are good candidates for biomass fractionation since the excess use of water is eliminated. However, novel, efficiently-developed ionic liquids with low cost have also been used recently for the fractionation of biomass.

Novel ionic liquids

Ionic liquids have been used in biofuel production for decades. Nevertheless, their production methods and process yield became less effective and favorable for a biofuel production with expected productivity levels especially for industrial scale. Due to the recent advances in chemical sciences, previously-used ionic liquids (ILs) were recently replaced with low-cost ILs as green solvents have been employed for the pretreatment of lignocellulosic biomass⁴⁸. The ILs are mostly designed at low cost, and for lignocellulose delignification, it is essential to avoid carboxyl, hydroxyl, and aromatic groups in the structure since the delignification capacity, and pK_a values of the conjugate acids of the anions are linearly correlated⁴⁹.

George et al. 2015⁵⁰ designed a series of protic ILs containing hydrogen sulfate anion. The developed ILs could enhance enzymatic saccharification yield, and triethylammonium hydrogen sulfate was the most efficient IL at increasing digestibility of the biomass, while providing better thermal stability with less residual generation. Most interestingly, due to their efficiency and low cost, some of the tested ILs could be replaced with industrially-used chemicals like ammonium hydroxide solution. Brandt-Talbot et al. (2017)⁵¹ also tested triethylammonium hydrogen sulfate ($\$1 \text{ kg}^{-1}$) to fractionate the grass *Miscanthus x giganteus* into a cellulose-rich pulp and lignin. With IL treatment, enzymatic saccharification of the pulp could lead to the release of 77% of the glucose from the biomass. Besides high sugar yields, ILs could be repeatedly used (up to 4 times, with 99% recovery each time).

The efficiency of ILs depends on the biomass to be fractionalized since each IL presents a different chemical affinity to a different biomass, hence, ILs should be carefully designed to show effectiveness against a varied type of biomass. In this context, An et al. 2015⁵² developed cholinium ILs to be effective for fractionation of grass lignocelluloses and eucalyptus biomass and obtained a glucose yield of 58-75%, however, the same IL was inefficient for pine biomass⁵². Moreover, ILs could be recycled 8 times with total recovery of 75%.

In some cases, standalone pretreatment of biomass using IL is not efficient and its efficiency can be enhanced by combining with alkali-based treatments. Heggset et al. 2016⁵³ compared the efficiency of 1-ethyl-3-methylimidazolium acetate (EMIM-OAc) as an IL (100 °C for 6 h) and alkali-based treatment (NaOH/urea) (-18 °C for 24 h) for Norway Spruce chips fractionation. Both methods could enhance the enzymatic digestibility of

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glucan and mannan in the biomass compared to untreated material. Interestingly, combining the two methods increased monosugar yield between 20-50%. Similarly, Nargotra et al. 2018⁵⁴ combined IL (ionic liquid 1-butyl-3-methyl imidazolium chloride) with alkali treatment (NaOH). The enzymatic digestibility of sunflower stalk biomass was significantly enhanced and the combination of two treatments resulted in a higher sugar yield (163.42 mg/g biomass) than only IL treatment (79.6 mg/g biomass).

Organosolv processes

Organosolv processes was found to be effective for the fractionation of lignocellulosic biomass and has been used in biofuel production. Suriyachai et al.⁵⁵ developed a one-step formic-acid catalyzed organosolv process for sugarcane bagasse fractionation. A glucose recovery of 84.5% was obtained while the fractionalized biomass showed a decreased crystallinity. Kubota et al.⁵⁶ employed an organosolv process for *Miscanthus x giganteus* and could obtain cellulose-enriched fibers (fibers containing 78% cellulose) without using any toxic solvents. Grande et al. 2015⁵⁷ developed an OrganoCat process consisting of a biphasic system containing water, solvent (2-methyl tetrahydrofuran (2-MeTHF)) and catalyst (oxalic acid). With this system, biomass at 100 gL⁻¹ could be fractionated in 3 h without formation of by-products and water, and organic phase could efficiency be recycled 4 times leading to an economic advantage over other methods.

Although these processes are well-established and excellent for delignification, they usually offer poor biomass deconstruction. Currently, organosolv processes don't meet specific requirements for industrial biofuel productions and combination with other methods to develop hybrid models is highly required. Matsakas et al.

2018⁵⁸ developed a novel hybrid organosolv technique combined with stream explosion method for fractionation of birch biomass. Employing explosive discharge at the end of process, pretreated solids presented high cellulose (77.9% w/w) and low lignin (7% w/w) content. The ethanol concentration obtained in this study was claimed to be the highest in literature for birch bioprocess. However, it is essential to adapt this technique to produce ethanol using other biomass. In this context, Matsakas et al. 2019⁵⁹ adapted this technique on spruce biomass and similarly, they obtained the highest level of ethanol reported for spruce.

Microfluidic technology

Microfluidic platforms (lab-on-a-chip concept, micro-reactors) have been utilized to elucidate different biological phenomena. Recently, microfluidic technology has proven to be an interesting tool for the biofuel industry since it allows the manipulation of biofuel-producer microorganisms as well as essential molecules used in biofuel technology⁶⁰. Microfluidic technology can be used in the whole process of biofuel development. One of the principal advantages of this platform came forward with the development of microchip-based electrophoretic sequencing.

Since this system provides faster processing times and reduced reagent consumption, it is convenient to obtain next-generation biofuel producer microorganisms for further use in either academic research or in industrial productions⁶¹. Pacheco et al. 2013⁶² developed a 96-well microplate as a microreactor platform for microalgae screening. This simply-designed system presented essential data for the optimization studies of some basic growth kinetics of microalgae used in biofuel production, while it allows substrate and space savings. Seguel⁶³ manufactured a 3D-printed microfluidic device for

microalgae *Dunaliella* sp. growth kinetics. Although the microdevice did not meet the expectations, it was possible to analyze important parameters such as microalgae damage test, surface retention, cell density, CO₂ and nutrition solubility as well as specific growth rate.

Catalyst optimization in microfluidic systems

Microfluidic technology is also a great tool for rapid catalyst optimization in biofuel production. Zhou and Lawal, 2014⁶⁴ developed a microreactor system, mimicking monolithic reactor, for green diesel production from hydrodeoxygenation of microalgae (*Nannochloropsis salina*) oil, using NiMo/ γ -Al₂O₃ catalyst. The microreactor system allowed a proper mass transfer as well as good yield of hydrocarbon and microalgae oil conversion rate. Increasing hydrogen to oil ratio (1000 SmL/mL), residence time (1 s), temperature (360 °C), and pressure (500 psig H₂) could enhance catalyst activity. C₁₃ and C₂₀ hydrocarbon yield of 56.2%, carbon yield of 62.7% were obtained together with almost total microalgae oil conversion (98.7%). They used the same microreactor system for biodiesel production⁶⁵ while comparing three catalysts (1% Pt/Al₂O₃, 0.5% Rh/Al₂O₃ and presulfided NiMo/Al₂O₃). They evaluated the principal conditions that lead to coke formation during the catalytic treatment of biomass, which causes significant catalyst deactivation and found that accumulation of coke decreased in the order NiMo > Pt > Rh. These studies showed that microreactor systems provide a rapid and efficient catalyst system for biofuel production. Transesterification processes are common in biodiesel produced using microalgal biomass.

Conventional transesterification processes are inefficient for obtaining a good quality fatty acid. Therefore, Liu et al. 2018⁶⁶ developed a microreactor for the rapid analysis of fatty acid profiles for continuous

biodiesel production. During transesterification, the mass transfer was significantly improved, and it took only 10 min to have an accurate fatty acid analysis with a reduced microalgal cell (a few milligrams).

Microfluidic platforms are excellent candidates to replace conventional benchtop methods that are mostly laborious and time-consuming. Lim et al. 2014⁶⁷, manufactured an integrated microfluidic system consisting of microchannels, micropillar array, cell chamber, output reservoir, and filtration unit in order to perform essential analyses such as microalgae culture, lipid accumulation and extraction for biofuel production in a single device. Lipid extraction efficiency was 13.6% higher than the Bligh-Dyer method with less isopropanol use comparing to the conventional method. Wang et al. 2016⁶⁸ conducted the synthesis of triglycerides from microalgae oil in a microreactor system packed with immobilized lipase. Compared to the batch reactor, they obtained a significant reduction (87.5%) in reaction time with 2.25-fold more lipase reuse time. The adaptation of this bioconversion technology to different biofuel production has high potential to be a cost-effective approach in the biofuel industry.

Droplet-based microfluidics

Microdroplets generated in a microfluidic platform also bring a great advantage in the rapid and cheap analysis of several parameters influencing biofuel production. Large-scale biofuel production is performed via fermentation of sugars from plant biomass, nevertheless, recently, biofuel production from photosynthetic organisms have drawn significant attention. Hence, it is important to select appropriate organisms that give the highest yield.

In this context, Abalde-Cela et al. 2015⁶⁹ developed a microdroplet system involving encapsulation of genetically-modified cyanobacteria in droplets, pico-

injection of components into the system and fluorescence detection along the microchannel. It was possible to carry out simultaneous screening of strains with different levels of ethanol production. Similarly, Sung et al. 2016⁷⁰ designed a PDMS-based microfluidic device for microalgal cell (*Chlorella vulgaris*) growth and CO₂ transfer into each microdroplet for the bioconversion of biomass by microalgal cell were significantly enhanced and comparing to flask culture, the cell growth was improved. More recently, Li et al. 2018⁷¹ produced a microfluidic platform to produce gelatin hydrogel microdroplets for high-throughput sorting of microalgal clones. The system allowed the growth of cells, metabolite production, selection of microalgae with high metabolite production used in biofuels, and cell recovery.

Genetic/metabolic engineering

Genetic engineering of plants

Conversion of cellulosic biomass as a renewable source is an essential step for biofuel production. However, due to the several limiting factors, these processes can be of high cost. Therefore, new techniques to reduce the number of steps required in pretreatment processes while increasing the yield and decreasing overall cost are very necessary⁷². The application of genetic engineering in plants is shown to be an effective method and has been employed with increased efficiency over the years. Most of the plant biomass consists of cell walls, and the content and properties of this biomass are the main factors to reach an economically-viable production with increased productivity.

Lignin reduction

The lignin biosynthesis pathway has been well-studied, and the modification of lignin structure has been investigated in the last few years to improve saccharification yield. New advances in genetic

engineering also allowed the discovery of new methods to manipulate lignin composition⁷³. Nevertheless, lignin engineering is a great challenge since the applied technique can cause a significant loss in integrity in vessels as well as tissues that are responsible for the transportation of water and nutrients⁷⁴. Yang et al. 2013⁷⁵ developed a new approach that decreases lignin content while maintaining the structure of vessels by overexpressing of transcription factors in native tissues.

This strategy allowed the reduction of lignin content and enhanced the polysaccharide deposition and consequently resulted in higher sugar yields for further enzymatic treatments. Smith et al. 2013⁷⁶ successfully designed a new miRNA to reduce lignin biosynthesis by silencing CCR1 (cinnamoyl-CoA reductase 1) using pAtCesA7 promoter without disturbing vessel integrity. In a more recent approach, Eudes et al. 2015⁷⁷ altered the Shikimate and phenylpropanoid pathways to reduce the availability of metabolites that play key roles in lignin production pathway. Lignin modification carries great importance since it is associated with the pretreatment need for biofuel production.

Altering wall sugar component

Plant cell wall mainly consists of carbohydrates, and a considerable amount of pentose sugars are present in the wall, and these pentose sugars are difficult to be fermented efficiently. Hence, new approaches to decrease the pentose level in the wall is essential. Altering nucleotide sugar conversion pathways can be another alternative method to increase hexose/pentose ratio⁷³. Rautengarten et al. 2014⁷⁸ used a new technique to characterize six bifunctional UDP-rhamnose (Rha)/UDP-galactose (Gal) transporters from *Arabidopsis* in order to identify important alterations during the biosynthesis of

Rha- and Gal-containing sugars, which resulted in increased β -1,4 galactan deposition.

Inhibition of endogenous pathways

Besides carbohydrates, lignocellulosic biomass contains a significant amount of acetyl and methyl esters, which can block the access of some enzymes to access to polysaccharides. Furthermore, these esters were found to present inhibitory effect on further fermentation processes during biofuel production⁷⁹. Genetic engineering has been used to reduce lignocellulosic acetyl groups by altering the biosynthesis of acetylated polysaccharides⁸⁰. Studies performed with *Arabidopsis* revealed that downregulation of genes encoding proteins involved in Reduced Wall Acetylation process could decrease the acetylation levels by 25%⁸¹. Unfortunately, the investigations are limited to *Arabidopsis* and new studies with other plants are required to employ these genetic engineering techniques for large-scale biofuel production.

Biomass increase

Overall plant biomass can be increased with genetic engineering, more specifically by modification of plant growth regulators. According to the studies with transgenic trees, increased gibberellin biosynthesis by overexpressing a responsible regulatory gene was found to provoke biomass growth, resulting in more biomass per unit area⁸². Different genetic engineering techniques have also been employed in order to improve some factors (i.e., carbon allocation, uptake of CO₂, N₂ and other essential sources, efficient utilization of O₂, water, and other nutrients, respiration, and even circadian clock) to increase the overall biomass⁷².

Genetic engineering of microalgae

Biofuel obtained from microalgae was proven to significantly reduce the amount of CO₂ and sulfur emissions compared to conventional biomass⁸³. Due to the great advantage of microalgae over other biosources, including plants, genetic modifications are important tools to enhance the quality and productivity of next-generation biofuels. Over-expressing some genes in order to alter specific metabolic pathways in microalgae for enhanced biofuel yield can be achieved using genetic and metabolic engineering. It has been reported that triggering triacylglycerol (TAG) accumulation in microalgae can significantly benefit biofuel production. Kaye et al. 2015⁸⁴ could enhance the biosynthesis of polyunsaturated fatty acids in *Nannochloropsis oceanica*, which is a great candidate in the biofuel industry, by overexpression of endogenous Δ 12 desaturase (NoD12). This overexpression using native genes and promoters significantly enhanced conversion of these fatty acids in the TAG. Kamennaya et al. 2015⁸⁵ engineered the cyanobacterium *Synechocystis* sp. PCC6803 to increase the number of copies of the endogenous bicarbonate transporter BicA, which is required for a more efficient CO₂ use. Under CO₂ pressure, this modified strain was able to produce additional BicA, which resulted in a biomass and growth rate twice more than the wild-type. Chien et al. 2015⁸⁶ genetically engineered *Chlorella* sp. by codon-optimization of several genes. The expression of genes encoding enzymes of the biosynthetic Kennedy pathway, which is a metabolic pathway for the production of TAG, resulted in increased TAG levels (20-46 wt%) and total lipid storage (35-60 wt%) compared to the wild-type. The malic enzyme has a critical role in pyruvate metabolism and carbon fixation in microalgae. Xue et al. 2015⁸⁷ overexpressed the gene encoding malic enzyme in *Phaeodactylum tricornutum* and obtained an increase in malic enzyme activity. Malic enzyme overproduction

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significantly increased total lipid content (2.5-fold) while the growth rate was maintained. The cell shape of microalgae became thicker and shorter, indicating a high-loaded oil inside.

CRISPR/Cas9 gene editing machinery**CRISPR/Cas9 technology applied to algae**

The discovery of CRISPR (interspaced short palindromic recurrence grouped regularly) / Cas9 (nuclease 9 associated with CRISPR) has significantly changed the field of genome engineering and paved the way for a wide variety of applications of different industrial branches⁸⁸. CRISPR-Cas9-mediated genome editing has emerged as a novel tool in genetic engineering to improve essential traits in microorganisms to make the product viable for industrial applications.

The CRISPR/Cas 9 technology is based on the genome edition, allowing to insert, eliminate or alter a desired genetic material in specific places of the genome. This system consists of two essential molecules: i) the Cas9 endonuclease DNA and ii) a single guide RNA (gRNA). While the previous molecule acts as a pair of "molecular scissors" that unwind and consequently cut the target DNA at specific loci, the gRNA has 20 bases long to make sure that the desired part of the DNA is being attacked⁸⁹. The Cas9 RNA-guided enzyme originated from the CRISPR-Cas adaptive bacterial immune system and is transforming the science of molecular biology by providing an advanced genomic engineering tool.

This technique is based on the principles of Watson-Crick base pairing and was adopted in some laboratories and fields due to its diverse applicability^{88,90}. Recent applications of CRISPR/Cas9 are creating new opportunities to investigate the function of genes and reveal important biological knowledge such as microbial consortium engineering⁹¹, establishing CRISPR-Cas9

systems as potent and programmable antimicrobials⁹² designing the vaccination of microorganisms against invasive genetic elements⁹³ and controlling gene expression in an inducible and reversible way^{94,95}.

Microalgae-based bioresources are considered the third-generation biofuel feedstocks and genome editing tools like CRISPR/Cas 9 are important candidates to produce next-generation biofuels. Due to the novelty of the technique, there is a limiting number of studies performed with genome editing of microalgae using CRISPR tool. Although genome editing has been well established in some organisms, the application in microalgae was shown to be a challenging process. The first study with CRISPR/Cas9 system was conducted in *Chlamydomonas reinhardtii*⁹⁶. In this study, the transient expression of Cas9 and single guide RNA genes was successfully carried out.

However, Cas9 toxicity was observed when Cas9 was produced constitutively in microalgae. For being the first study in the application of CRISPR/Cas9 in microalgae, effective methods were required for proper gene editing. After this study, Shin et al. 2016⁹⁷ employed this powerful tool in *Chlamydomonas reinhardtii*. The induced mutations were obtained at three different loci (MAA7, CpSRP43, and ChIM) and mutagenic efficiency was enhanced up to 100-fold comparing to the previous study. The improvement of the knockout effectiveness of Cas9 ribonucleoproteins could pave the way for the new industrial applications of microalgae for biofuel production. Wang et al. 2016⁹⁸ also engineered the genome of model microalgae *Nannochloropsis* spp. by CRISPR/Cas9 using nitrate reductase. The isolated mutants could maintain metabolic activities normally under NH₄Cl but could not survive under NaNO₃.

CRISPR/Cas9 technology applied to crops

CRISPR/Cas9 technology has also been employed for crops to increase biofuel production. Park et al. 2017⁹⁹ applied CRISPR/Cas9 to the switchgrass (*Panicum virgatum*), as an important crop for bioenergy production, by targeting an essential enzyme that involves in the biosynthesis of monolignol. Among three tested 4-Coumarate: coenzyme A ligase (4CL) genes, Pv4CL3 was selected for CRISPR/Cas9 treatment due to its overexpression in lignified stem tissues of the plant. Among 39 generated transgenic plants, four plants presented tetra-allelic mutations, and the knockout of Pv4CL1 caused a reduction in cell wall thickness (8-30% reduction in lignin, 7-11% increase in glucose release, 23-32% increase in xylose release). This study was essential for the further application of CRISPR/Cas9 technique in plants to improve biofuel production.

Omics technologies

A way to get around the difficulty related to lignocellulosic material bioconversion has recently been explored through omics approaches. Co-cultures of bacteria which can directly ferment lignocellulosic biomass have been reported to display increased rates of cellulose hydrolysis and higher ethanol titers than observed in monocultures. To this purpose, metagenomics, transcriptomics, proteomics and metabolomics are useful tools to better understand microbial communities, enzyme interactions, and how lignocellulose breakdown occurs^{100,101}. The establishment of microbial consortia in naturally degrading lignocellulosic compound ecosystems has proven its value to propose synthetic microbial ecosystems with genetic content related to a desirable set of biochemical functions. Comprehensive and consistent knowledge of a biological system, and of the interactions which occur in, is a first required step to conceive synthetic biological systems¹⁰². For biogas production,

omics tools have been used to evaluate the perturbations resulting from the application of variable biotic and abiotic factors (temperature, sludge retention time and organic loading rate) to the system¹⁰³⁻¹⁰⁵. Applied to algae, the omics approach is seen as an opportunity to define control points governing metabolic flux, and to propose rational algal strain-engineering targets^{106,107}.

Microbial tolerance during biofuel production

Biocatalysts have been widely used for biofuel production since they can efficiently degrade heterogeneous polymers into simpler form while allowing the fermentation occurs simultaneously to produce biofuel. However, microbial tolerance against increased final product concentration is usually limited since biofuels¹⁰⁸, as natural antimicrobials, can disrupt the cellular macromolecules, hence, the techniques to avoid chaotropic effect on biocatalysts caused by final product should be employed for a continuous biofuel production¹⁰⁹.

As a good example, fermentation with *Clostridia* is used to produce biobutanol together with acetone and ethanol¹¹⁰⁻¹¹². Before fermentation, pretreatments of lignocellulosic materials are required to produce the highest possible fermentable sugars from lignocelluloses with a minimal risk of contamination by inhibitors. Thereafter, cellulolytic enzymes convert the substrates into a fermentable hydrolysate. The improvement of the yield of fermentation is limited by the tolerance of *Clostridia* to butanol^{113,114}. For the latter, strain engineering to obtain a hyper-butanol producer is being investigated.

One of the most common method is the product removal via different separation techniques to recover highly-purified biofuel¹¹⁵. Comparing to conventional batch processes, new separation systems integrated with

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fermentation process that allows *in situ* recovery are shown to result in a 25-times more biofuel production in 21 days of continuous process¹¹⁶. Beside final product inhibition, pretreatment of lignocellulosic biomass could also generate by-products that show inhibitory effect on biocatalysts. Salvachúa et al. 2011¹¹⁷ showed that inoculation of white-rot fungi could decrease the inhibitory effect of by-products and allow the complete fermentation of glucose into ethanol using *Saccharomyces cerevisiae*.

Immobilization techniques have been widely used for decades to provide an additional protection to biocatalysts during fermentation¹¹⁸. Encapsulation of biocatalysts using polymeric matrices were also found to be effective to decrease or completely eliminate the inhibition caused by final biofuel. As previously mentioned, biofuels show chaotropic activity against biocatalysts, therefore, immobilization material should be kosmotropic (order-making) in order to stabilize macromolecular systems of the used biocatalysts. In this context, hydrophilic polymers such as agarose, calcium-alginate conjugate, and carrageenan can be used to encapsulate biocatalysts¹⁰⁸. Liu et al. 2014¹¹⁰ immobilized *Clostridium acetobutylicum* using adsorption technique on a novel macroporous resin, KA-I, to produce biobutanol. Biocatalysts showed improved butanol tolerance and high fermentation yield. Immobilization could allow the use of biocatalysts repeatedly for the continuous production of biofuel.

Beside these techniques, genetic and metabolic engineering techniques can be employed to create more robust strains with high tolerance against inhibitions. Moreover, it is possible to identify the main factors that contribute to the final product inhibition and

consequently, new strains with improved tolerance to a wide range of alcohols can be selectively produced¹¹⁴.

Conclusions

Plant/agricultural materials in particular low value/waste biomass present great potential for production of various types of biofuels including bioethanol, biomethanol, biodiesel and biogas. They have superiority in terms of environmental effects, economic potential and sustainability as compared to other fuel resources.

Therefore, the industry sectors have been shown growing interest to utilize such agro-waste residues. Although various biomass sources are introduced for biofuel production (e.g., food, livestock, forestry, fishery and plantation), the choice of biomass type for production of biofuel is crucial. Starchy/sucrose-containing crops are considered as most suitable source for bioethanol, whereas, polycarbohydrate complexes such as lignocellulosic materials are less popular due to their complex processing. For biodiesel production, oil seeds biomass has preference due to their high energy yield, while in case of biogas, food-processing waste are chosen as best source of biomass in this regard. However, there is a need to optimize the processing conditions according to the matrix used as well as the different stages of the process, with distillation being a key step. There is a need for innovative strategies to make the process more efficient. In this sense, new energy saving strategies have been used, which have shown promising results as alternatives to conventional distillation to obtain ethanol, either zeotropic or anhydrous, from fermented broths. Additional research should focus on the development of economically viable energy-saving distillation systems, the impact of processing variables on bioactive extraction, the

expansion of such operations and the characterization of bioactive compounds and related biological benefits.

Conflicts of interest

There are no conflicts to declare.

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