Use of fungi to aid the implementation of biofuels amid the current energy crisis

A writing assignment by Julia Klein
Student No. 5867614
As a part of Environmental Biology Master (Fungal Track),
Utrecht University, Leuvenlaan 4, Utrecht Science Park, The Netherlands
Contact: j.c.r.klein@students.uu.nl

Supervised by Prof. dr. H.A.B Wösten,
Faculty of Science, Microbiology, Utrecht University
Padualaan 8, 3584 CH Utrecht, The Netherlands
Contact: h.a.b.wosten@uu.nl

Content

Layman's summary

Biofuels are a green alternative to the use of fossil fuels. Fossil fuels have a severe impact on the environment and their supply is running out. These problems are now highlighted by the Ukraine-Russia war, which has significantly increased energy prices. Biofuels, made from lignocellulosic biomass, are a renewable energy source which can be a solution to these problems. Biofuels are a biological alternative which can be produced locally, reducing foreign oil dependence. In addition, biofuels will also increase rural job opportunities and benefit local economy. Moreover, biofuels can readily be applied in current engines with minimal changes or by blending biofuels with conventional fuels. The first generation of biofuels were made from crops; the second generation from non-edible sources and waste streams; the third generation from algae; and the fourth generation biofuels use genetically modified microorganisms. However, biofuels can currently not compete with fossil fuel prices. The difficulty regarding biofuel production is, among other things, breaking the lignocellulosic structure of the biomass. Fungi could help with increasing this efficiency. Fungi can help break down the lignocellulose structure with lower environmental impact than other current methods. Also, fungi themselves can form the biomass which can be used for biofuel production or their derivatives can be applied for higher efficiency. Furthermore, fungi can be genetically modified to express certain characteristics that are beneficial. Given the versatility that fungi offer within this process, fungi could be the solution to produce biofuels at a lower cost to implement them in society.

Abstract

The combustion of fossil fuels has detrimental effects on the environment. As fossil fuels are a finite source, the world now faces the problem of energy insecurity. A renewable alternative that is consistently available is highly sought after. Biofuels, produced from lignocellulosic biomass, can pose a solution here. Biofuels know four generations. Since first generation biofuels compete with food sources, they are not suitable substrates. Therefore, higher generation biofuels have been investigated. Second generation biofuels are more difficult to produce due to the recalcitrant structure of lignocellulose. Third generation biofuels made from algal biomass are not economically viable yet and raise biosafety matters when genetic engineering is involved. Fourth generation biofuels make use of genetic engineering of microorganisms. Currently, biofuels are not able to economically compete with fossil fuels. The incorporation of fungi or novel fungal derivatives in multiple parts of biofuel production can increase production efficiency and lower production costs. For biodiesel production, oleaginous fungi or yeasts can be used as biomass. Fungal lipases can also be used during enzymatic transesterification as an environmental friendly option to chemical transesterification. For bioethanol production, fungi can be applied during pretreatment, enzymatic hydrolysis and fermentation. Properties of plant pathogenic fungi can be explored to increase lignocellulosic breakdown. Moreover, fungi can be genetically engineered to express beneficial characteristics. Since fungi know a wide genetic variety and genetic tools are widely available for genetic enhancement, this is a promising prospect. Altogether, fungi can advance biofuel production for better implementation in modern society.

Introduction

In late February 2022, Russia invaded Ukraine. While economic growth was expected in the post-COVID era, the Russian invasion in Ukraine has caused a complex economic disruption (Benton et al., 2022; Ozili, 2022). The energy markets is hugely impacted after already being strongly affected by the COVID-19 pandemic (Benton et al., 2022; Esfandabadi et al., 2022). Russia plays a significant role in this market since it is a main exporter of fossil fuels including oil, gas and coal. The EU relies heavily on energy import from Russia as 60% of its total energy need is accounted for (Benton et al., 2022). Due to the Ukraine-Russia war, there is a disruption in the global energy market due to trade sanctions with Russia. Therefore, energy prices have been on the rise. For example, crude oil prices in 2022 have increased 42% compared to 2021. Moreover, natural gas and coal prices are estimated to have increased 50% and 80% in 2022, respectively (World Bank, 2022). These prices could pull back in 2023 if sanctions on Russian trade will not broaden. However, these prices will settle higher than before the COVID-era and Ukraine-Russia war. On top of this, with the growing world population estimated to reach 9.2 billion by 2050 (Alexandratos & Bruinsma, 2012), there is an increasing demand for energy. For instance, the worldwide energy demand is expected to increase from 6.6×10^{20} J in 2020 to 8.6×10^{20} J by 2040 (Guo et al., 2015). Accordingly, world energy-related carbon dioxide emissions are calculated to increase from 35.6 metric tons in 2020 to 43.2 metric tons by 2040 (EIA, 2016).

Since the Industrial Revolution in the late 1900s, the world has been heavily relying on burning fossil fuels such as petroleum and natural gas (Yang et al., 2021). However, harmful effects of burning fossil fuels were not foreseen. Atmospheric CO_2 levels have been stable for thousands of years due to the Earth's natural carbon cycle. This has been disturbed by burning fossil fuels such as coal, petroleum and natural gas. CO_2 is the most important greenhouse gas; its concentration has increased from 280 ppm of pre-industrial times to 379 ppm in 2005 (Solomon et al., 2007). These increasing atmospheric CO_2 levels have many drastic environmental consequences such as warmer oceans, less snow coverage, more intense droughts and rising sea levels (Solomon et al., 2007). Another reason why the heavy reliance on fossil fuels is injudicious, is the fact that fossil fuels are a finite source (Bentley, 2002). The natural carbon cycle produces fossil fuels many times slower than at the rate at which humans use them (Solomon et al., 2007). Therefore, there is a decline in production of conventional oil products. However, other oil sources may not be able to substitute conventional oil sources fast enough to prevent an oil shortage (Bentley, 2002). The aforementioned arguments, combined with the expected increase in future energy demand, show there is a need for clean and renewable energy source to sustain future generations.

Renewable energy is defined as clean and consistently available energy that can be produced within a relatively short time and is therefore readily available for use without having a drastic environmental impact (Demirbas, 2008). Several renewable energy sources have come about such as geothermal energy, hydroelectric power, solar energy and wind power. Currently, only 12% of the world's energy needs are met by such renewable sources, while 88% is provided by nuclear power and fossil fuels such as coal, conventional oil and natural gas (Pimentel & Patzek, 2008). The United States of America is one of the world biggest energy consuming countries as it accounts for 25% of the world's energy consumption. Yet, only 6.8% of their energy needs are met by renewable energy sources (Pimentel & Patzek, 2008). Although policy making towards renewable energy sources is slow, it has become evident that an energy transition towards renewable energy is necessary to sustain future generations (Stokes & Breetz, 2018).

The transportation sector accounts for 28% of the global energy consumption. Moreover, the transportation sector is mainly dependent on oil as it accounted for 90% of energy consumption in 2016 in comparison other sources such as natural gas which only accounted for 4% (Dewangan et al., 2018). Several environmental or economic problems have driven the transition towards renewable energy sources. One example is foreign oil dependence, which is one of the main drivers in the current energy crisis due to the Ukraine-Russia war (Benton et al., 2022; Stokes & Breetz, 2018). Thus, it is evident there is a need for alternative, renewable fuel sources in the transportation sector.

A renewable energy source which can substitute conventional fuel in the transportation sector is biofuel. Biofuels are made by the conversion of biomass (Alalwan et al., 2019; Demirbas, 2008). Depending which substrate is used during their production process, biofuels are classified into the first, second, third, or fourth generation of biofuels (Alalwan et al., 2019; Osman et al., 2021). With different kinds of substrates and production processes, different kinds of biofuels can be produced. As such, biodiesel could substitute for diesel and bioalcohols, such as biomethanol, bioethanol and biobutanol, could substitute for gasoline (Alalwan et al., 2019; Demirbas, 2008).

The query to be answered is to which extent biofuels can be a renewable energy source for conventional fuels in the transportation sector. To answer this query I will first discuss what exactly biofuels are and which biofuels currently exist. Subsequently, the production process of different biofuels will be discussed and the advantages and disadvantages of these processes will be identified. Finally, it is discussed how these processes can be optimized using fungi. Fungi have various applications within these processes which could ensure that biofuels can be produced more cheaply and on a larger scale. This paper will mainly cover the well-known liquid biofuels such as biodiesel, bioethanol and biobutanol, but will not delve deeply into other biofuels such as biohydrogen and biomethanol. Since biodiesel and bioethanol have been extensively studied and are the biofuels which account for most of the biofuel production for commercial use, they will be discussed in depth.

Chapter 1. The impact of different generations of biofuels

Two main drivers towards the use of biofuels are climate change and the foreign oil dependence, which is now highlighted due to the Ukraine-Russia war (Esfandabadi et al., 2022). As aforementioned, biofuels are renewable fuels made from the conversion of biomass. Biofuels can be divided into primary and secondary biofuels. While primary biofuels refer to direct burning of firewood, plants or crop or animal waste, secondary biofuels refer to derived products of the conversion of different substrates (Rodionova et al., 2017). The secondary biofuels are classified by solid, liquid or gaseous form (e.g. bio-char, biodiesel and biohydrogen) (Demirbas, 2009). Here, liquid biofuels will be discussed as substitute for petroleum in the transportation sector. The emphasis will be on biodiesel and bioethanol. Biodiesel, fatty acid esters, can be produced from various oleaginous substrates to substitute for diesel fuel. Bioethanol, liquid ethyl alcohol, can substitute for gasoline or be applied by blending with gasoline. These biofuels can be made from various substrates (see Table 1 for the most common ones). In fact, over 350 substrates have been used for the production of biodiesel alone (Atabani et al., 2012). Based on which substrate is used for biofuel production, biofuels can be categorized into four generations of biofuels.

1.1 First generation biofuels

First generation biofuels are produced from edible biomass sources, such as crops including sugarcane, corn and rapeseed (See Table 1). Worldwide, around 45 to 50 billion liters of first generation biofuels are produced annually for commercial use (Osman et al., 2021). For biodiesel, oleaginous crops are used for transesterification to produce biodiesel. For bioethanol, crops can be categorized into sugar-containing crops and starch-containing crops (Alalwan et al., 2019). Eventually, sugar monomers will be used for fermentation to produce bioethanol. Sugar-containing crops are preferred due to low conversion costs (Osman et al., 2021).

First generation biofuels showed great promise as they produce significantly lower greenhouse gas emissions (GHG) during combustion. For example, bioethanol derived from sugarcane produces 71% less GHG emissions compared to gasoline (Koizumi, 2014). Also, first generation biofuels can be blended with conventional fuels, transported with current infrastructure and applied in current engines (Naik et al., 2010). However, the use of edible crops for biofuel conversion has received criticism. Agricultural arguments include the use of cropland and water resources with the subsequent loss of biodiversity, soil erosion, environmental pollution by fertilizer and pesticide use (de Fraiture et al., 2008; Pimentel & Patzek, 2008). The main argument against the use of edible crops, however, is food security. Food security entails the continuous access to sufficient amounts of nutritious foods and clean water (Alexandratos & Bruinsma, 2012).

Food security is part of Sustainable Development Goal 2 ‘Zero Hunger’, which means to obtain worldwide food security and improve quality of nutrition with sustainable agriculture (https://sdgs.un.org/goals/goal2). With the growing world population and destabilizing agricultural conditions due to climate change, food insecurity is becoming a bigger problem. For example, food consumption is expected to increase from 2770 kcal/person/day to 3070 kcal/person/day by 2050 (Alexandratos & Bruinsma, 2012). Thus, there is a great pressure on the agricultural sector. Food insecurity is also highlighted by the Ukraine-Russia war. Apart from being the main exporter of fossil fuels, Russia is also the main exporter of food (wheat, sunflower oil) and potassic and nitrogenous fertilizers. Ukraine is a main exporter in food such as sunflower oil, maize and wheat (Benton et al., 2022). The restricted trade in food sources has caused a surge in food prices, restricting food access (Benton et al., 2022; Esfandabadi et al., 2022). As the use of crops for biofuel production further drives food insecurity, a conflict between Sustainable Development Goal 2 and 7 is identified. Sustainable Development Goal 7 ‘Affordable and Clean Energy’ aims to secure renewable energy which is affordable and reliable (https://sdgs.un.org/goals/goal7). Biofuels were introduced to achieve this goal with climate change as a main driver (Esfandabadi et al., 2022). The Ukraine-Russia war has increased both food insecurity and energy insecurity. Crops are needed to tackle both problems in a sustainable manner. Some countries have considered adjusting to lower biofuel mandates. However, this would in turn affect goals set to tackle climate change. To keep high biofuel mandates to tackle climate change while not endangering food security and risking food shortages, a transition towards higher generation biofuels is needed (Esfandabadi et al., 2022).

1.2 Second generation biofuels

Second generation biofuels are produced from non-edible sources including jatropha, waste cooking oils and even municipal sewage sludge (MSS) (see Table 1). These biofuels have a neutral to negative carbon impact (Alalwan et al., 2019). Second generation biofuel do not directly endanger food security as they are non-edible plants or come from residual waste from several sectors. However, second generation biofuels compete with food crops when they are specifically cultivated for biofuel production. In this sense, crops cultivated for either food or biofuel compete for arable land. For example, jatropha needs good soils and high input for biodiesel production or yield levels will vary drastically (Carriquiry et al., 2011). Thus, it is highly dependent on the geographical location, climate, soil condition and local agricultural practices which feedstocks can be cultivated for biofuel production (Atabani et al., 2012). Another disadvantage of using non-edible crops and waste for second generation biofuel production, are the technological difficulties of fuel production compared to first generation biofuels (Naik et al., 2010). As non-edible plants often contain more lignocellulose, the conversion is more complex. This complexity causes the price of second generation biofuels to surge above current conventional fuel prices. Therefore, production of second generation biofuels is not fully economically viable depending on the substrate. For example, second generation bioethanol is about 1.1-2.9 times more costly than gasoline. Biodiesel derived from jatropha could compete with diesel prices if low costs were to be achieved on commercial scale production (Carriquiry et al., 2011). Thus, further developments are needed before second-generation biofuels become an economically viable solution.

1.3 Third generation biofuels

Third generation biofuels are produced with algae. Algae include both macroalgae such as kelp, and microalgae such as the green, golden, and brown algae and diatoms (Hannon et al., 2014; Osman et al., 2021). Microalgae pose an attractive alternative to biofuel production as they have several advantages over plant biomass. Firstly, unlike first or second generation biofuels, microalgae rapidly divide to create biomass (Hannon et al., 2014). This biomass can be used for both bioethanol as biodiesel. For example, one strain of *Nannochloropsis* sp. had 60% lipid content after nitrogen starvation which can be used for biodiesel production (Rodolfi et al., 2009). Secondly, microalgae do not require vast amounts of arable like crops and does therefore not compete with crops for arable land. Algae can be grown in photobioreactors or open ponds systems. While conditions in photobioreactors can be more easily controlled, they are more costly than open pond systems. In addition, if third generation biofuels are to be upscaled, new facilities to culture algae will have to be manufactured. For example, to meet the US oil demand, 30 million acres of land will have to be attributed to such facilities. This will be accompanied by significant water use and unsustainable quantities of nutrients such as phosphorous, needed for algal growth (Hannon et al., 2014). The growth, harvesting and extraction costs need to be lowered before large scale production becomes a viable option. However, advantages of culturing microalgae for biofuel production include production of co-products (e.g. waste water remediation) and the genetic diversity of algae, which can also be engineered to express preferred properties to optimize biofuel production. This genetic engineering is what characterizes fourth generation biofuels and can be applied in several ways in different processes.

1.4 Fourth generation biofuels

Fourth generation biofuels make use of genetic engineering to overcome obstacles identified in previous biofuel generations. The genetic engineering of microorganisms can aid in producing biofuels more efficiently (Aamer Mehmood et al., 2021). There has been an increase in creating cell factories with the purpose to produce biofuels or other chemical compounds. Microorganisms, including bacteria, yeasts, cyanobacteria and microalgae, can be metabolically engineered to increase yield from the cell factories with reduced carbon emissions (Aamer Mehmood et al., 2021; Osman et al., 2021). For example, *Saccharomyces cerevisiae* is often used in studies due to its well-characterized genome and available tools to edit and cultivate it (Aamer Mehmood et al., 2021). Metabolic engineering removes multiple costly steps from the production process as specific traits can be introduced, such as nonnative enzymes, to more easily produce biofuels (Lü et al., 2011). However, the consequences to the native metabolism when introducing nonnative enzymes are not yet fully understood. Also, working with genetically modified organisms requires sufficient safety measures to be implied before large scale production to ensure environmental safety (Aamer Mehmood et al., 2021). Biosafety concerns are raised especially when genetic engineered algae are cultivated in open pond systems (Kumar, 2015). Thus, production of biofuels using genetic engineered microorganisms shows promising possibilities but requires strict regulations.

1.5 Environmental impact of biofuels

Climate change is one of the main drivers towards an energy transition from petroleum derived fuels to biofuels (Esfandabadi et al., 2022). The fuel oxygen content is the main difference between petroleum derived fuels and biofuels. While petroleum derived fuels essentially contain no oxygen, biofuels contain between 10-45% oxygen content. In oxygenated fuels, combustion is more efficient and emissions are reduced. However, oxygenated fuels have less energy content resulting in more fuel consumption (Demirbas, 2009). The highest reduction in GHG emissions was 71% for sugarcane derived bioethanol and 83% for waste vegetable or animal oil biodiesel (Koizumi, 2014). Yet, combustion of biodiesel in compression-ignition engines resulted in increasing NO_x levels upon increasing the biodiesel percentage in diesel blends. In contrast, CO, CH, SO_x and particulate matter emissions decreased. Engines can be adapted to decrease NO_x emissions (Demirbas, 2009). Vegetable oil biodiesel did not differ performance-wise (i.e. brake power) from diesel. Bioethanol even increases engine torque and power (Demirbas, 2009). Lastly, for the production of most biofuels, especially waste vegetable oil biodiesel, there is a great net energy return. This indicates that less energy is needed to deliver the wanted energy (i.e. waste vegetable oil biodiesel). The greater the net energy return, the fewer GHG emissions released (Demirbas, 2009; Koizumi, 2014).

An obstacle towards producing biofuels at large scale are low production costs to compete with fossil fuel prices. For example, only bioethanol derived from sugarcane was found to compete with fossil fuels. However, in many calculations revenue from co-products are not taken into account. Revenue from co-products such as chemicals or secondary metabolites can reduce the costs of biofuels and make them more compatible (Aamer Mehmood et al., 2021; Koizumi, 2014).

1.6 Economic impact of biofuels

Apart from environmental benefits of biofuel use such as reduced GHG emissions, there are several economic benefits. Energy security can be obtained with this sustainable and renewable approach. In addition, biofuels can be produced locally with local biomass which reduces the foreign oil dependence (Benton et al., 2022; Demirbas, 2009). Production of different kinds of biofuels from different substrates will create an international competitive market. Furthermore, feedstock agriculture and biofuel production facilities will in turn increase rural job opportunities, income and gross output. Local economy will also benefit as local services and supplies will be purchased (Demirbas, 2009).

Table 1. Different generations of biofuels and their most common substrates. The different kinds of common substrates are provided per generation of biofuel for both biodiesel and bioalcohol (bioethanol and biobutanol). This table is compiled with data from (Alalwan et al., 2019; Rodionova et al., 2017).

Biofuel	**First generation**	**Second generation**	**Third generation**	**Fourth generation**
Biodiesel	Edible sources (rapeseed, peanut, coconut, palm, canola, milkweed seed, mustard, olive, linseed, rice bran, safflower, sunflower, soybean) Animal fat	Non-edible sources (apricot seed, pongamia oil, castor bean, cotton seed oil, fish oil, jojoba, rubber seed, neem oil, okra seed oil, wild safflower seed oil, sugar apple seed oil) Waste vegetable or cooking oil Waste animal fats Insect oil Municipal sewage sludge (MSS)	Oleaginous microorganisms (algae, yeast)	Genetically engineered microorganisms (microalgae, cyanobacteria, yeast, fungi)
Bioalcohol Bioethanol Biobutanol	Edible sources (wheat, barley, beet root, corn, fruits, palm juice, potato, rice, sugarcane, sugar beet, sorghum)	Non-edible sources (jatropha, cassava, miscanthus, straw, grass, wood) Lignocellulose waste (agricultural residues, forest residues, energy crops, cellulosic waste)	Algae Microbes	Genetically engineered microorganisms (microalgae, cyanobacteria, yeast, fungi)

Chapter 2. Biofuel production and its challenges

Different biofuels require different production processes. In addition, depending on the substrate and thus the generation of biofuel, the production process differentiates. The eventual production cost of the biofuel is determined by the used substrate and production process (Alalwan et al., 2019; Demirbas, 2009). For example, 75% of production cost of biodiesel is represented by the substrate alone (Atabani et al., 2012). Here, the various aspects of biodiesel, bioethanol and biobutanol production will be discussed, including the factors influencing the production process. Biobutanol is included as it is an alternative to other biofuels as it has several advantages over bioethanol.

2.1 Biodiesel production

Biodiesel can be produced via four methods including pyrolysis, dilution, micro-emulsion and transesterification. As transesterification is the most simple and inexpensive method, is it most widely used (Atabani et al., 2012). Therefore, this method is discussed here. Transesterification is the process wherein triglycerides from substrates combined with alcohol, often methanol or ethanol, are converted to biodiesel and glycerol as a byproduct (see Figure 1) (Alalwan et al., 2019). Transesterification on itself is not very efficient. Therefore, a catalyst is employed which can improve biodiesel production by 98% (Basha & Raja Gopal, 2012). Various chemical or biological catalysts are available for acid or alkali transesterification or via enzyme-mediated transesterification.

2.1.1 Acid or alkali transesterification

Acid and alkali catalysts can either be homogenous or heterogenous. Homogenous catalysts occur in the same phase as the reactants (e.g. liquid or gaseous) while heterogenous catalysts do not (e.g. solid) (Talha et al., 2016). Examples of acid catalysts are sulfuric acid and ferric sulfate, while sodium hydroxide and potassium hydroxide are examples of alkali catalysts. These are the most used homogenous alkali catalysts as they are inexpensive and extensively available (Atabani et al., 2012; Talha et al., 2016). Heterogenous alkali catalysts are sometimes preferred due to easier separation properties. However, with alkali catalysts soap formation can occur which is why acid catalysts are used. Yet, acid catalysts are harder to separate from the fuel and can thus contaminate the product (Talha et al., 2016).

2.1.2. Enzyme-mediated transesterification

Enzyme-mediated transesterification is performed by lipases. The ester bonds of triglycerides are broken up by lipases (see Figure 1) (Marchetti et al., 2007). Lipases for biodiesel production are derived from bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae, Pichia pastoris*) and fungi (*Aspergillus oryzae*) (Borrelli & Trono, 2015). They can be employed during transesterification as free lipases or immobilized lipases. Lipases can be immobilized by adsorption, covalent linkage, crosslinking, encapsulation and entrapment. Adsorption by a textile membrane carrier of lipase derived from *Candida* spp. 99-125 is a cost-effective option for industrial scale (Lu et al., 2008; Tan et al., 2010).

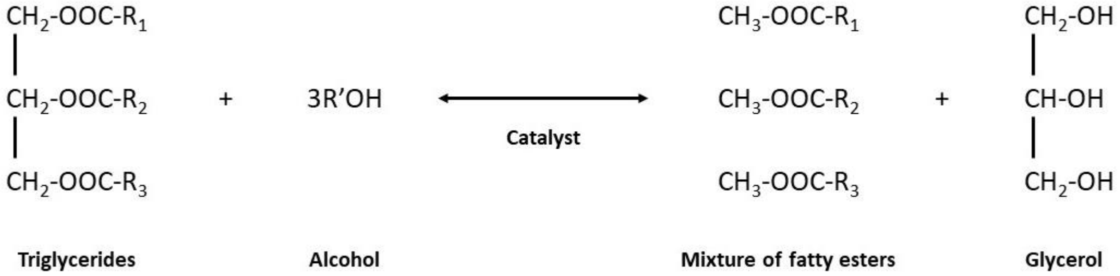

Figure 1. Transesterification reaction. Triglycerides and alcohol, often methanol or ethanol, are converted to fatty esters and glycerol as a byproduct catalyzed by either a chemical or enzymatic catalyst. ‘R’ indicates a chain of carbon atoms. Adapted from Alalwan et al. (2019).

By reusing the immobilized enzymes, production costs are reduced. Unlike enzymatic catalysts, chemical catalysts are hard to separate from the product and result in expensive waste water which requires expensive treatment (Atabani et al., 2012). Furthermore, lipases do not cause soap formation and product purification is easy. Moreover, lipases are more environmentally friendly as they use less energy and can be reused when immobilized. Yet, lipases may not be widely used since they are expensive (exact cost not publicly known) and have a long reaction time compared to the abovementioned catalysts (Marchetti et al., 2007; Talha et al., 2016). Examples of common lipases used for biodiesel production include Novozyme 435, Lypozyme TL MI, Lipozyme RM IM and Lipase PS-C (Reddy et al., 2018).

2.1.3 Factors influencing biodiesel production

Several factors can affect biodiesel production during transesterification. Firstly, the molar ratio of alcohol determines the amount of fuel yield and its purity (Atabani et al., 2012). Increasing the alcohol to triglyceride ratio will increase yield and purity up to a certain point before the reaction equilibrium shifts. The optimal alcohol to triglyceride ratio is also determined by the type of catalyst used. For example, alcohol to oil ratio when employing an alkali catalyst was reported at 6:1 M (Musa, 2016). In contrast, alcohol to oil ratio when using an acid catalyst was set at 7:1 M (Leung & Guo, 2006). Following, the catalyst type and concentration used during transesterification can determine the fuel yield. As discussed, alkali, acid and enzyme catalysts can be chosen for transesterification. It is reviewed that alkali is able to catalyze reactions faster than acids (Basha & Raja Gopal, 2012). Next, biodiesel production is affected by high concentrations of free fatty acids (FFA) and water. FFA can neutralize alkali catalysts by increasing acidity and water can cause soap formation which in turn inhibits both the catalyst and glycerol separation (Atabani et al., 2012). For example, for optimal transesterification of beef tallow water content should be below 0.06%, w/w and FFA content below 0.5%, w/w (Ma et al., 1998). Also, reaction temperature is of importance as it creates a balance between either higher yield or quick reaction time. If the reaction time is too short not all glycerides are converted, but if the reaction time is too long this can lead to soap formation. As the added alcohol should not evaporate, reaction temperature has to take this into account. Lastly, balance exists between yield and soap formation depending on agitation speed necessary during transesterification (Atabani et al., 2012; Leung & Guo, 2006). Therefore, all these factors need to be assessed carefully for biodiesel production.

2.2 Bioethanol production

Bioethanol production is a three-step process in which lignocellulosic biomass is converted into ethyl alcohol (Alalwan et al., 2019). Lignocellulose exists of cellulose, hemicellulose and lignin. Cellulose is a linear homopolymer made up of glucose monomers linked by β-1,4 glycosidic bonds. Hemicellulose is a branched homopolymer or heteropolymer made up of various sugar monomers including D-xylose, D-arabinose, D-glucose, D-galactose, and D-mannose combined with organic acids. Lignin is a complex three-dimensional structure made up of ρ-coumaryl, coniferyl and sinapyl alcohol (see Figure 2) (Kumari & Singh, 2018). These three component are heavily interlinked with one another by covalent and hydrogen bonds. Moreover, lignin is highly resistant to solubilization (Kumar & Sharma, 2017). To increase accessibility to cellulose and hemicellulose which are needed for bioethanol production, pretreatment is necessary (see Figure 2E). In the second step of enzymatic hydrolysis, sugar monomers are generated which are subsequently used for fermentation (Kumar & Sharma, 2017; Kumari & Singh, 2018; Vasić et al., 2021).

2.2.1 Pretreatment methods

Pretreatment of lignocellulosic biomass is necessary to create enzymatic accessibility to cellulose and hemicellulose from the complex structure for subsequent hydrolysis and eventual high yields (Kumar & Sharma, 2017; Kumari & Singh, 2018). This step is often the most expensive step in the production of bioethanol (Kumar & Sharma, 2017). Therefore, the most cost-effective method with high yield is sought after. Pretreatments can be subdivided into four categories, namely physical, chemical, biological or combined methods.

Physical methods break the lignocellulosic biomass and thereby increase its surface area. Physical methods include milling, extrusion, freezing, steam explosion, hydrothermal and microwaveassisted methods (Alvira et al., 2010; Chang et al., 2011; Li et al., 2016; Senturk-Ozer et al., 2011; Zhang et al., 2018; Zhu et al., 2009). Secondly, chemical pretreatments use acid, alkaline, ionic, organic solvent and oxidative methods to dissolve hemicellulose or lignin and thereby increase the surface area (Bali et al., 2015; Elgharbawy et al., 2016; Kautto et al., 2014; Sheikh et al., 2015; Solarte-Toro et al., 2019). Thirdly, biological methods make use of microorganisms or their derived bioactive compounds to reduce the recalcitrant lignocellulose structure. Biological methods include the use of fungi, microbial consortia or enzymes (Romano et al., 2009; Wan & Li, 2012; Zhang et al., 2011). The advantages and disadvantages of these various methods are compiled in Table 2. Various methods can also be combined to overcome disadvantages such as long incubation time or the formation of inhibitors. For example, fungal pretreatment can be combined with dilute acid pretreatment to shorten incubation time while remaining environmental friendly (Kumari & Singh, 2018).

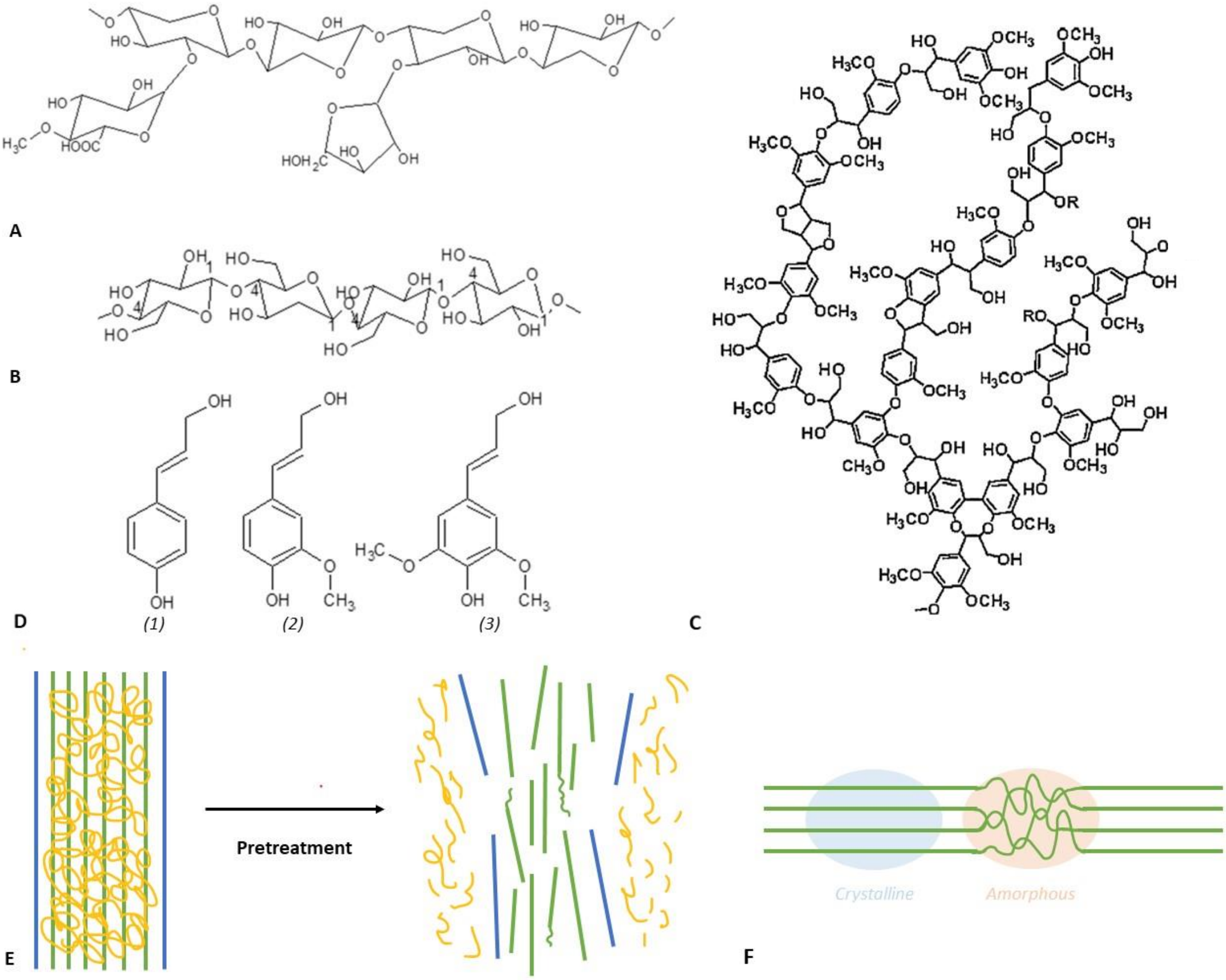

Figure 2. Components of lignocellulose and the effect of pretreatment. Molecular structure of cellulose (A), hemicellulose (B), lignin (C). The building blocks of lignin consist of (1) ρ-coumaryl (2) coniferyl and (3) sinapyl alcohol (D). E) Simplified cross section of lignocellulose with cellulose in green, hemicellulose in orange and lignin in blue. The recalcitrant structure of lignocellulose is broken up by pretreatment, which releases the individual components. F) Cellulose (green) can either occur in crystalline (light blue) or amorphous (soft red) form. The amorphous form is not as easily hydrolyzed as the crystalline form which is linked by non-covalent hydrogen bonds. Compiled and adapted from Alalwan et al. (2019) and Kumari & Singh (2018).

Table 2. Advantages and disadvantages of various bioethanol pretreatments. Pretreatments are divided into three categories wherein several pretreatment methods are included.

Category	Method	Advantage	Disadvantage	Reference
Physical	Milling	High recovery yield	High energy requirement	(Zhu et al., 2009)
	Extrusion	Low energy requirement	Difficult applicability for larger quantities	(Senturk-Ozer et al., 2011)
	Freezing	Improved subsequent hydrolysis; low environmental impact; less chemicals required	High costs	(Chang et al., 2011)
	Steam explosion	High lignocellulosic breakdown; high energy efficiency; low environmental impact	Partial lignin removal; partial hemicellulose degradation; generation of toxic chemicals	(Alvira et al., 2010)
	Hydrothermal	No chemicals required	High energy requirement; high water consumption;	(Zhang et al., 2018)
	Microwave irradiation	Improved subsequent hydrolysis	High cost; high energy requirement; no large-scale equipment	(Li et al., 2016)
Chemical	Acid	High lignocellulosic breakdown; amorphous cellulose conversion	High cost; recycling restrictions; formation of inhibitors	(Solarte-Toro et al., 2019)
	Alkaline	High lignocellulosic breakdown	Long treatment time	(Bali et al., 2015)
	Ionic liquids	Improved subsequent hydrolysis	High cost; recycling restrictions	(Elgharbawy et al., 2016)
	Organic solvent	Production of pure lignin fraction; production of byproducts	High cost; high energy requirements; low recovery yield	(Kautto et al., 2014)
	Oxidative	High lignocellulosic breakdown; improved subsequent hydrolysis; short treatment time	Formation of inhibitors	(Sheikh et al., 2015)
Biological	Fungi	High lignocellulosic breakdown; low energy requirements; low cost; low environmental impact	Long treatment time; Loss of cellulose and hemicellulose; contaminations	(Wan & Li, 2012)
	Microbial consortia	Low cost; low energy requirements; improved subsequent hydrolysis	Long treatment time; no large-scale application	(Zhang et al., 2011)
	Enzymes	High lignocellulosic breakdown; no formation of inhibitors	Varying yields; factors influencing enzyme activity	(Romano et al., 2009)

2.2.2. Enzymatic hydrolysis

Enzymatic hydrolysis, also known as saccharification, is the second step in bioethanol production. After pretreatment, cellulose and hemicellulose are no longer interlinked with lignin. During enzymatic hydrolysis, cellulose and hemicellulose are hydrolyzed into fermentable sugar monomers such as glucose and xylose (Vasić et al., 2021). Therefore, it is important that these polymers are not degraded during pretreatment and that inhibitors are not formed (Kumari & Singh, 2018). Different enzymes are employed based on which substrate is used. Examples of enzymes employed include cellulase, amylase, xylanase and β-glucosidase (Vasić et al., 2021). Factors that influence the efficiency and efficacy of hydrolysis include the crystallinity of cellulose, the particle size of the substrate and enzymatic accessibility (Kumar & Sharma, 2017; Vasić et al., 2021). Hydrolysis is easier with crystalline fibers as they are linked by non-covalent hydrogen bonds compared to the amorphous form or cellulose (see Figure 2F). Particles of certain size are required to gain high yield with efficient reaction time. Lastly, enzymatic accessibility is determined by available surface area and porosity also influencing yield.

2.2.3. Fermentation

Fermentation is the final step in bioethanol production. During fermentation, microorganisms such as *S. cerevisiae* are employed to ferment the sugar monomers generated by enzymatic hydrolysis. Depending on the substrate and microorganism, fermentation can occur in either a continuous, batch or a fed-batch process (Vasić et al., 2021). The continuous process consistently provides substrate and nutrients to the microorganisms and continuously extracts product. The batch process essentially is a closed culture system unlike the fed-batch process which systematically adds nutrients to feed the culture. Initially, water and the hydrolysate are mixed to form a broth which is fermented under anaerobic conditions at determined temperatures. While *S. cerevisiae* is able to ferment glucose, it is not able to ferment other sugar monomers. Other yeast such as *Pichia stipites* and *Candida parapsilosis* naturally ferment xylose. Apart from these characteristics, high alcohol and chemical inhibitor tolerance are preferred characteristics for fermentation (Vasić et al., 2021).

2.3 Biobutanol production

Biobutanol, butyl alcohol, knows several advantages over bioethanol. Firstly, biobutanol has a higher blending percentage with gasoline. Therefore, no alterations to existing engines are needed. In addition, it can be transported via existing pipeline infrastructure. Lastly, it shows even less GHG emissions than bioethanol while having increased energy content resulting in less fuel consumption (Anandharaj et al., 2020; Swana et al., 2011). Thus, biobutanol shows promising characteristics over other biofuels.

Biobutanol production resembles bioethanol production. While pretreatment (also see Table 2) and enzymatic hydrolysis are performed similarly, the fermentation step differs. Biobutanol production makes use of so-called ABE fermentation wherein three products, namely acetone, butanol and ethanol, are purified after three distillation and other processing steps. Of the eukaryotes, *S. cerevisiae* was determined the best for biobutanol production apart from prokaryotes such as *Clostridium beijerinnkii* and *Clostridium acetobutylicum*. These microorganisms are able to generate acetone:butanol:ethanol in a ratio of 3:6:1 (Anandharaj et al., 2020; Swana et al., 2011). However, biobutanol and its production is not yet commercialized. While bioethanol production has been developing for many years and biotechnological processes are properly researched, this is not the case for biobutanol (Swana et al., 2011). For commercialization, proper substrate selection (e.g. higher generation of non-edible substrates) can lower the production cost. Moreover, strain selection for properties including high butanol transformation and high butanol tolerance are of importance for sufficient yield. *Clostridium* species have been investigated in this regard and were able to produce butanol from sugars, acids and organic compounds in the presence of several solvents. Genetic modifications and heterologous expression of *Clostridium* genes in *S. cerevisiae* could further enhance these traits (Anandharaj et al., 2020).

Chapter 3. Use of fungi for optimization of biofuel production

As mentioned previously, filamentous fungi and yeast can be employed throughout the production process of several biofuels. Microorganisms pose a more green solution compared to for example physical or chemical pretreatment for bioethanol production. Moreover, fungi can be genetically enhanced to express certain traits beneficial to the production process of biofuels. In this chapter, the use of fungi and the genetic engineering of fungi for several applications throughout the production process will be discussed for biodiesel and bioethanol.

3.1 Optimization of biodiesel production

Fungi can be used for biodiesel production in two ways. Firstly, fungi can be used to produce lipids which can be directly used for biodiesel production. Secondly, they can be used and genetically enhanced to produce lipases. Lipases are used during enzyme-mediated transesterification. These examples will be highlighted in this paragraph.

3.1.1 Use of oleaginous fungi and yeasts

Oleaginous fungi and yeasts with high lipid content can be used for biodiesel production. While oleaginous plants can also be used, they would raise the issue of food security or compete with food crops for arable land (Esfandabadi et al., 2022; de Fraiture et al., 2008; Pimentel & Patzek, 2008). Moreover, non-edible oleaginous plants contain low oil content. While several methods to genetically enhance plants to generate higher oil content have been investigated, upscaling from laboratory conditions to cultivating genetically modified (GM) crops raises biosafety issues (Salehi Jouzani et al., 2018). Genetically engineering algae also raises the issue of biosafety. In addition, due to high production costs, insufficient biomass production and multiple regulations, biotechnological advances for GM algae lack behind (Shokravi et al., 2021). As fungi and yeasts have been well-researched for several decades, they may pose an attractive alternative.

Fungi and yeasts can be cultivated with higher oil content than most plants (Sitepu et al., 2014). The first step is the screening for oleaginous fungi and yeasts. Athenaki et al. (2018) and Sitepu et al. (2014) have summarized oleaginous fungi and yeasts which have been identified for biodiesel production. For example, biodiesel can be produced by oil derived from yeast *Yarrowia lipolytica* when grown on waste cooking oil. With this method, 0.22 grams of oil was produced intracellularly per gram of fungal biomass. Moreover, the composition of the oil showed suitable amounts of saturated and unsaturated fatty acid esters (Katre et al., 2018). This method is an example of direct transesterification of oleaginous fungal biomass. Compared to transesterification of the extracted oil which is a three-step process, direct transesterification of fungal biomass required less equipment, less energy and less solvent (Katre et al., 2018). Thus, direct transesterification is more efficient while also having a lower environmental impact. With this method, Chopra et al. (2016) were able to reduce transesterification process time by seven hours for the yeast *Pichia guilliermondii*. Furthermore, parameters of the cultivation process can be adapted to increase the lipid content of fungi and yeast. To add on to the previous example of *Y. lipolytica*, Bellou et al. (2016) found that lipogenesis was enhanced when the yeast was cultivated under double nitrogen and magnesium limitation resulting in increased intracellular lipid content of 47.5% w/w.

Fungi and yeast can also be genetically enhanced to increase their lipid content. Gene editing techniques for fungi have been well-established and are widely available. To this end, lipid biosynthesis was overexpressed in *Y. lipolytica*. Genes involved in lipid biosynthesis, including heterologous pyruvate carboxylase (PYC) and endogenous ATP citrate lyase (ACL1), were overexpressed. This resulted in lipid content of 45.3% w/w, which was 1.5-fold increase compared to the parental strain (Wang et al., 2015). In another study from 2018, lipid content of *Y. lipolytica* was enhanced by overexpression of heterologous wax ester synthases (WS) genes. Here, WS gene from *Marinobacter hydrocarbonoclasticus* resulted in an extracellular yield of 1.18 g/L titer of fatty acid ethyl esters (Gao et al., 2018). Thus, it seems multiple genes are involved in lipogenesis.

3.1.2 Use of fungal and yeast lipases

As mentioned above, free and immobilized lipases have several advantages over chemical transesterification. For example, they pose an environmentally friendly option which use less energy as opposed to acids or alkali. The major drawbacks of lipases are the expenses and the longer reaction time during transesterification (Marchetti et al., 2007; Talha et al., 2016). Lipases are readily extracted and applied from several filamentous fungi and yeast, including *Aspergillus niger* and *Rhizopus oryzae*. Fungi or yeasts can also be employed as whole cells. For example, whole fungal cells of *Mucor circinelloides* with high lipase activity were immobilized using polyurethane foams. The highest fatty acid ethyl ester yield was 87.3% using this method (Soares et al., 2017). Apart from selecting fungi for lipase production, they can also be genetically engineered to enhance favorable protein characteristics to lower biodiesel production costs. For example, Ahmed et al. (2020) generated mutant strains of *Rhizopus stonifer* and *Aspergillus tamarii* isolated in Egypt by chemical mutagenesis and screened for enhanced lipolytic activity. By employing lipases from both mutant *R. stonifer* and *A. tamarii* in a ratio of 3:1, a yield of 92.3% was achieved. This was produced for a cost of 190 USD per ton compared to the cost of 401 USD per ton of petroleum diesel in Egypt (Ahmed et al., 2020). Thus, fungal lipases or whole fungal cells can be employed and genetically enhanced to improve transesterification.

3.2 Optimization of bioethanol production

Fungi can be employed in several ways throughout bioethanol production. As mentioned previously, pretreatment is the most costly part of bioethanol production (Kumar & Sharma, 2017). Herein, fungi can be used to improve the breakdown efficacy. Additionally, fungi can be used for improving bioethanol production by exploring the potential of pathogenic fungi for lignocellulosic breakdown; by adding fungal derived enzymes during pretreatment and hydrolysis; by genetically engineering fungi to express beneficial properties for pretreatment, hydrolysis or fermentation.

3.2.1 Lignocellulosic breakdown by plant pathogenic fungi

Plant pathogenic fungi are known to feed on their plant host. They can be divided into three categories, namely biotrophic, hemibiotrophic and necrotrophic. While biotrophic fungi feed on live plant material, necrotrophic fungi actively invade their plant host and feed on the subsequent dead plant material. The hemibiotropic fungi are an intermediate between the aforementioned two categories and can switch from a biotrophic to a necrotrophic state. The necrotrophic fungi have a wider host range than biotrophs and have been observed to produce the more hydrolytic enzymes to degrade lignocellulosic biomass (Oliver et al., 2004). There are several well-known plant pathogens from multiple genera which have great lignocellulosic breakdown capabilities. Examples include fungi from the genera *Fusarium* and *Aspergillus*.

Fusarium species are filamentous fungi which known a wide range of plants they can infect. They are able to invade multiple parts of the plant and cause severe disease symptoms (Leslie & Summerell, 2008). *Fusarium verticilioides* is a well-known plant pathogen that produces several hydrolytic enzymes which can be used during pretreatment of bioethanol to degrade complex lignocellulose substrates. These enzymes include β-glucosidase, endoglucanase, cellobiohydrolase, endocellulase and exocellulase. Several enzymes from *F. verticilioides* were characterized by de Almeida et al. (2013). They identified a novel enzyme complex consisting of two endoglucanases, a cellobiohydrolase, a xylanase and a free endoglucanase. They were observed to remain active under wide pH and temperature ranges and were therefore believed to be suitable for biotechnological application (de Almeida et al., 2013). This was shown when de Almeida et al. (2019) produced two enzymatic cocktails derived from *F. verticilioides* grown on gamba grass, namely an endoglucanase-rich and cellobiase-rich cocktail. When combined in a ratio of 12:1, the enzymes were able to convert 43.4% glucan and 73.1% xylan into sugar monomers when applied at 40 mg/g for sugarcane bagasse. Lower total saccharification rates of sugarcane bagasse were found for four other fungal species, namely *Trichoderma sp.* (45.71%), *A. niger* (19.98%), *Cladosporium sp.* (12.86%) and *Curvularia sp.* (13.98%) (Mahamud & Gomes, 2012).

Aspergillus species are filamentous fungi which have well-established commercial biotechnological applications such as production of citric acid by *A. niger*. This fungus is also known to cause rot in several fruits and vegetables by production of hydrolytic and oxidative enzymes (Samson et al., 2014). Therefore, it can also be exploited for its potential to breakdown lignocellulosic biomass. For example, β-xylosidase derived from *A. niger* remained active at high temperatures and was able to hydrolyze ρ-nitrophenyl-β-D-xylopyranoside, ρ-nitrophenyl, β-D-glucopyranoside and ρ-nitrophenyl α-L-arabinofuranoside. It increased xylose yield by 19-fold when included in a commercial enzyme cocktail and thus shows great potential (Boyce & Walsh, 2018). *Aspergillus* genes coding for such enzymes can be recombinantly expressed. For example, an endoglucanase from *Aspergillus flavu*s was recombinantly expressed on the cell surface of *S. cerevisiae* to hydrolyze lignocellulosic biomass with greater efficiency. The whole cell showed high thermostability and maintained 60% of its activity over a pH range of 2.0-11.0. Therefore, it was concluded to be suitable for bioethanol production (Gao et al., 2017).

3.2.2 Genetic engineering of fungi for optimized bioethanol production

Fungi know a wide applicability throughout the bioethanol production process. They can be applied during pretreatment, enzymatic hydrolysis and fermentation. Moreover, fungi can be genetically engineered to improve yield or process efficiency.

During pretreatment, fungi are used as a biological pretreatment which requires less energy and have a lower environmental impact (Wan & Li, 2012). They can be genetically enhanced to increase sought after characteristics such as delignification capacity. This is what Ryu et al. (2013) did using the white-rot fungus *Polyporus brumalis*. Making use of the constitutively active glyceraldehyde-3-phosphate dehydrogenase gene promotor, transformants with 4-fold increase in laccase activity were identified. Subsequently, when red pine and tulip tree were pretreated with the transformants, 32.5% and 29.5% higher sugar monomer yield were obtained during hydrolysis compared to the control (Ryu et al., 2013).

As a last step during enzymatic hydrolysis, cellobiose and short cellodextrins are converted into glucose by β-glucosidase. This appears to be the rate limiting step in lignocellulose biomass conversion. Even though β-glucosidase appears to be of high importance, only 1% of the secreted enzymes by the model fungus *Trichoderma reesei* are β-glucosidases (Singhania et al., 2017). Thus, enzymatic cocktails used during enzymatic hydrolysis need to be supplemented with β-glucosidases. Another possibility which has been exploited is recombinantly expressing β-glucosidase in *T. reesei*. For example, Nakazawa et al. (2012) recombinantly expressed *Aspergillus aculeatus* β-glucosidase 1 in *T. reesei*. When grown on 1% Avicel, a 63-fold increase in β-glucosidase activity against cellobiose was measured compared to the parental strain. Low enzymatic dosage was needed for the hydrolysis of NaOH-pretreated rice straw, indicating good potential for industrial application (Nakazawa et al., 2012). Likewise, Treebupachatsakul et al. (2015) investigated ethanol productivity of *T. reesei* recombinantly expressing *A. aculeatus* β-glucosidase 1 compared to the enzymatic cocktail from the parental strain supplemented with Novozyme 188. Similar results were obtained for both enzymatic cocktails suggesting potential applicability of recombinant *T. reesei* (Treebupachatsakul et al., 2015).

During fermentation, fungi are used as ethanologens to convert biomass into bioethanol. As mentioned, *S. cerevisiae* is an example of a yeast used for fermentation usually not able to ferment xylose (Vasić et al., 2021). *S. cerevisiae* strain XUSEA was genetically engineered to overexpress the pentose phosphate pathway. This resulted in increased consumption of xylose, another abundant sugar monomer generated by enzymatic hydrolysis apart from glucose. Co-fermentation of both glucose and xylose by *S. cerevisiae* strain XUSEA increased ethanol yield by 2-fold (30.1 g L^{-1} ethanol) while reducing process time by half (Hoang Nguyen Tran et al., 2020). Likewise, heterologous xylose metabolizing genes were expressed in *S. cerevisiae*. This increased ethanol yield by 3.7-fold compared to the control strain (He et al., 2022). These studies show the potential and applicability of genetic engineering strategies for improving bioethanol production.

Discussion and Conclusion

Biofuels pose an environmental solution to the growing problem of energy insecurity. The heavy reliance on fossil fuel combustion has caused multiple environmental consequences due to the disruption of the Earth's natural carbon cycle (Solomon et al., 2007). As biofuels are produced from lignocellulosic biomass, they do not disrupt this carbon cycle and are therefore considered a green alternative. Apart from posing a solution to environmental pollution, biofuels can also aid in solving the worldwide growing problem of energy insecurity. Energy security entails consistently available and accessible energy worldwide. As fossil fuels are a finite source, its production has been declining over time (Bentley, 2002). The problem of energy insecurity is highlighted by the Ukraine-Russia war. For example, EU countries heavily rely on energy import from Russia, which has come to a halt by trade restrictions thus increasing energy prices (Benton et al., 2022; Esfandabadi et al., 2022; World Bank, 2022). Therefore, it is necessary that a renewable energy source is implemented to obtain energy security while reducing foreign oil dependence.

Biofuels know four generations (Alalwan et al., 2019; Osman et al., 2021). The first generation biofuels gave rise to the food-versus-fuel argument, also highlighted by the Ukraine-Russia war as both countries are suppliers of various food sources (Benton et al., 2022). Second generation biofuels are a better alternative as they use non-edible sources and waste streams. However, these are harder to process and therefore more costly (Carriquiry et al., 2011). Third generation biofuels are made by algal biomass and while showing great promise, high production costs need to be overcome before third generation biofuels become a viable option (Hannon et al., 2014). Fourth generation biofuels make use of genetic engineering of microorganisms to overcome obstacles in the production process and increase efficiency. Yet, complete understanding of genetically modified organisms and its safety remains a drawback to some (Aamer Mehmood et al., 2021). Especially genetic modification of algae combined with the use of open pond systems has raised serious concerns (Kumar, 2015).

As there are drawbacks to each generation of biofuel, an interesting and biological way to improve biofuel production is by the incorporation of fungi. Fungi make an attractive solution to producing biofuels in an economically viable way. With the use of fungi, high production costs or process time can be reduced. As seen, fungi can be applied throughout the production process of biodiesel and bioethanol in several ways. For example, for application in biodiesel production oleaginous yeast. *Y. lipolytica* was identified and genetically enhanced to improve its lipogenesis (Gao et al., 2018; Katre et al., 2018; Wang et al., 2015). Fungal derived lipases can be used for biodiesel production. Ahmed et al. (2020) showed that by overexpression native lipases of *R. stonifer* and *A. tamarii* and applying them in a ratio of 3:1, production costs of biodiesel were significantly lower than that of petroleum in Egypt. Moreover, it is estimated that 1500 tons of biodiesel can be produced annually form waste frying oil this way. For bioethanol production, fungi can be employed during pretreatment, enzymatic hydrolysis and fermentation. Properties of pathogenic fungi can be exploited for enhanced lignocellulosic breakdown or characteristics can be enhanced for improved bioethanol production. For example, obstacles of fermenting xylose were overcome by overexpressing the pentose phosphate pathway of *S. cerevisiae* and by heterologous expression of xylose metabolizing genes from *Candida tropicalis* (He et al., 2022; Hoang Nguyen Tran et al., 2020). Thus, fungi provide a biological method to improve biofuel production in multiple ways for it to become economically viable.

The aforementioned examples make use of non-edible sources, waste streams or fungal biomass, therefore not competing with food sources. As mentioned, non-edible sources and waste streams all over the world are different depending on geographical location and its climate. Therefore, different substrates will be used to locally produce biofuels (Atabani et al., 2012). However, with the wide genetic variety of fungi, research can delve into which fungi or yeasts can be used for which substrate to keep production cost low. Furthermore, with genetic engineering, solutions to problems encountered during biofuel production can be solved. As genetic strategies to engineer fungi are well developed, applications can we relatively quickly researched. Therefore, it can be argued that investments in biofuels should be made. As shown, local production of biodiesel in Egypt enabled by lipases from *R. stonifer* and *A. tamarii* cost less than have the costs of conventional petroleum (190 USD compared to 401 USD) (Ahmed et al., 2020). Biodiesel production from oleaginous fungi is cheap. A life cycle assessment study determined that biodiesel from oleaginous fungi is a viable option when lipid extraction methods become more developed as the main drawback remains here (Hosseinzadeh-Bandbafha et al., 2020). Thus, biodiesel is an attractive fuel, also considering it is compatible with existing motor engines and significantly shown decrease in emissions and good net energy return (Demirbas, 2009; Koizumi, 2014),

With the search for alternative fuels, carbon-neutral synthetic fuels (CNSFs) and battery electric vehicles (BEVs) have been developed as well with the goal to decarbonize the transport sector. Therefore, the question remains whether or not biofuels can compete with these alternatives. Hannula & Reiner (2019) determined that BEVs have readily made it onto the market and are competitive over short distances. However, over longer distances and for the use of air and sea travel, BEVs are not suited. Therefore, it is expected that biofuels and CNSFs will play a part here (Hannula & Reiner, 2019). Ternel et al. (2021) conclude that both the implementation of electrified vehicles and biofuels are necessary to reach the European Green Deal. Furthermore, it is discussed that plug-in hybrid vehicles (PHEVs) are most relevant solutions to decarbonize transportation. Yet, this is only the case for mid-sized cars for daily use. Increasing battery size of PHEVs would have opposite effects on carbon emissions. Also, whether electricity for electric vehicles is generated at low-carbon intensity or not, is of high importance for the total carbon emissions produced. For example, Europe produced about eight times higher CO_2 emissions than France for electrified transport for 150,000 km as some European countries still use coal instead of renewable sources and electricity mixes (i.e. nuclear, gas and renewable) (Ternel et al., 2021). Thus, biofuels still play a significant part in decarbonizing the transportation sector and can be employed in several transport sectors. In the long run, biofuels and electric vehicles can together help decarbonize transportation. For this implementation to occur, these renewable options need to be made an attractive alternative for consumers (Ternel et al., 2021).

In conclusion, biofuels pose an attractive renewable alternative to fossil fuels which can be used in various transport sectors (i.e. land, air and sea travel) and can therefore aid in the current energy crisis. As they can be produced locally with non-edible sources and waste streams, they do not compete with edible sources. Since the production processes of biofuels have drawbacks, the use of fungi or fungal derivatives (i.e. lipases) can increase the efficiency of biofuel production as fungi can be employed in all stages of biofuel production. This will lower production costs, as exemplified by Ahmed et al. (2020). Lowering of costs is one of the drawbacks to biofuels for them to economically compete with fossil fuels. The incorporation of fungi in biofuel production is environmental friendly and effective. Furthermore, the wide genetic variety of fungi and available genetic engineering tools create opportunities for overcoming limitations encountered. This is exemplified by Gao et al. (2018) and Wang et al. (2015) by overexpressing lipogenesis in *Y. lipolytica* and by Hoang Nguyen Tran et al. (2020) by increasing xylose consumption of *S. cerevisiae*. Thus, with the use of fungi, advancements in biofuel production can be anticipated.

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