Chapter 11
Genetic Modification: A Gateway to Stimulate the Industrial Production of Biofuels

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Abstract Recent years have seen an explosion in the use of advanced biotechnology techniques in academic and industrial activities to modulate microorganism pathways for the production of fuels or chemicals. Synthetic biology is adopted for biofuel production, and it needs scientific evidence to support the fundamentals and risk assessments. Biofuel is derived from biomass of a plant, animal waste, or microalgae. These materials can be replenished after some time; hence they are a renewable source of energy. Risk assessment is considered significant to maintain and comply with regulatory frameworks existing around the world. The use of scientific tools such as enzymes and microbes itself needs review and approval. Risk profiles are done for the toxicity, infectivity, and strategies. Recent years have seen an exciting increase in developing strategies for the use of advanced biotechnology techniques to enhance the productivity of existing biosynthetic pathways in microbes by cutting off the competing pathways. The biomass is pretreated to speed up the process of obtaining biofuel. The mutant, after genetically modifying the enzymes, produces cellulases and hemicellulases in higher levels. Functional analysis can confirm the changes in several transcription regulatory elements. Generally, successful engineering is demonstrated with an enhanced supply of amino acids.

Keywords Biofuels · Genetic engineering · Lignocellulose · Enzymes · Fungus · Artificial intelligence

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11.1 Introduction

The geographical production of fuel from fossils is a gradual process; an alternative is to obtain the fuel using biomass or living beings, which is termed biofuel. The times have changed, and there is a constantly increasing need for petroleum (Lazarus and Van 2018). The natural sources of the fuel industry are not present in all the countries. Fuel exhaustion has become a significant problem putting the countries unrest and dependent on buying fuel from the enriched countries (Pfaltzgraff and Clark 2014). The readily available biomass that is used directly to convert to biofuel is recovered from wood and grass. Biofuels produce heat energy which generates electricity after being run in a generator. No corner in the world is left to be negatively impacted by the effects of petroleum extraction, refining, transportation, and use. Therefore, biofuels are increasingly getting attention as an alternative source of energy (Nehring 2009; Zou et al. 2016).

11.1.1 Biofuels Basic Definition

Biofuel exists in all forms such as solid, liquid, and gas. Liquid and gaseous forms are easier to transport and deliver. Biofuel term is used for ethanol, biodiesel, green diesel, and biogas (Masjuki et al. 2012). The biofuel production is broadly characterized into first-generation, derived from sugars, starches, and oils, and the crops versus second-generation biofuels extracted from lignocellulosic biomass sources. Recently, the effort has been started to derive biofuels from microalgae and cyanobacteria, termed third-generation production (Rodionova et al. 2017); and fourth-generation biofuels which include the genetic modification of the microorganisms for the enhancement of biohydrogen production processes.

It is no secret that biofuels are preferred and viable substitutes over fossil fuels. However, biofuels such as ethanol create a net energy loss when compared to petrol. If we consider the food-based crops, they must be used for feeding the enormous population than used for fuel. As the demand to produce bio crops increases with the demand for organic consumables, soil erosion, deforestation, fertilizer run-off, and salinity are some of the major issues (Larson and United Nations Conference on Trade and Development 2008; Cheng and Timilsina 2011).

11.1.2 Why the Need for Biofuel?

The need for alternative and efficient methods has made researchers find new ways to produce biofuel. Sugar and sugar-derived ethanol is making a significant contribution to satisfying the need at the moment. Sugar fermentation is used for transportation fuel. Starch-derived ethanol fills the energy supply by mainly using corn

grain production. After the conversion of corn grain into ethanol, its burning causes an emission that impacts greenhouse gas emission but not in a net increase in atmospheric carbon dioxide (Lal 2005). The countries are announcing policies and goals to produce their own biofuel and be less dependent on foreign oil. Britain, the USA, and Canada are encouraging to grow primarily biofuel crops and establish cellulosic ethanol refineries major biofuel centers to produce a million gallons of cellulosic ethanol per year (Sims et al. 2010).

11.1.2.1 Bioethanol

Petroleum demand has put much industrial unease in the countries. Ethanol is readily biodegradable, but its use, just like petroleum, produces air and water-borne pollutants. Feedstock production of the crops to get bioethanol reduces the greenhouse gases like carbon dioxide from the environment, which is being used for photosynthesis (Lima et al. 2012). As the cellulosic matter is present in an abundant amount, it is more fitted than starch and sugar for ethanol because of its limited supply. Food supplies rely on starch-based crops such as sugar cane or corn that need specific climatic conditions to grow; hence, cellulose biomass that is not dependent on weather conditions is convenient to produce bioethanol in most countries (Sarkar et al. 2012). Ethanol produced from cellulose-based material has the potential to replace petroleum (In Marcel 2015).

11.1.2.2 Biodiesel

The second most demanding biofuel is biodiesel, which is obtained from soybean, palm oil, and fat of cooking oil. Algae and cyanobacteria have the potential to account for a large amount of fuel per unit area. Biodiesel is used in combination with petroleum and is widely accepted in European countries.

In this chapter, an oversimplified view of the production of biofuels through various resources, their economic analysis, and possible genetic manipulation of the crops to overcome the existing challenges are presented.

11.2 Biofuel from Different Sources

11.2.1 Biofuels from Lignocellulose Biomass

Lignocellulose biomass is universal and widespread renewable biomaterial on our planet. The bioconversion of lignocellulose comes as a feasible strategy when juxtaposed with the other alternative energies. Three components that are rigidly packed with each other in lignocellulose are cellulose, constituting 30–50% part of it, whereas hemicelluloses 20–40%, and lignin 20–30%. Due to the compact

structure, degradation into fermentable sugars and further conversion into fuels and other value-added materials become difficult (Menon and Rao 2012; Sharma et al. 2017).

Cellulose is linked with β, 1–4 linkage of glucose. Cellulase enzymes can break it down into glucose. Cellulose is not found in pure form in nature but with hemicelluloses and lignin, which act as a physical barrier for cellulase to access cellulose (Volynets and Dahman 2011). Cellulosic ethanol is associated with a high cost of bioreactors; the breakdown of lignin and its removal is required to access cellulose biomass by cellulases to obtain the biofuel from a plant source. The use of xylanases with cellulases is more effective as xylanases hydrolyze hemicelluloses and make cellulose available for biomass degradation. This co-acting releases more fermentable sugars from the biomass (Hu et al. 2011); this method is economically more expensive in comparison to ethanol from corn. Genetic engineering offers a substitution to minimize the cost production of cellulosic ethanol. The first approach can be an integration of cell wall degrading enzymes cellulases and hemicellulases in the crop instead of adding them directly in the bioreactor. Secondly, the amount of lignin can be configured, and the pretreatment process can be avoided. Most importantly, the maximization of polysaccharides can boost cellulosic biofuel production (Hu and Catchmark 2011).

11.2.1.1 The Cellulosic Ethanol Production Process

Lignocellulosic biomass harvested from the feedstock crop is transported to a refinery where it is stored. Either this biomass is treated with extreme heat or with chemicals to remove the lignin by breaking it down into the intermediates. The separation of the solid and liquid components is done by filtration, distillation, evaporation, and chromatography. After the enzymatic hydroxylation using bacteria or fungi, it is ready for the conversion to cellulosic ethanol. After the separation of sugar is done, pure ethanol is obtained (Liu et al. 2019; Zheng et al. 2009).

11.2.1.2 Factors Affecting the Production from Lignocellulose Biomass

Cellulosic ethanol production changes with country, region, agriculture, economy, and politics. These complex factors depend on the type of crop produced, its demand, and the transportation fuel used in that area. The support from the community and government regarding breeding strategy is also paramount for conversion to cellulosic ethanol as it gives a high amount of cellulosic biomass. C4 photosynthetic pathway, perennial growth, water usage efficiency, and segregated underground storage nutrients are the prototypical features of a non-consumable cellulose crop (Huang et al. 2009). These might include silver grass, switchgrass, and woody crops. Other edible crops are rice, corn, and sugarcane. The plant cell wall is the source of lignocellulosic biomass, and it determines the structural configuration of the plant; a representation is shown in Fig. 11.1. The various combinations of glucose sugars are

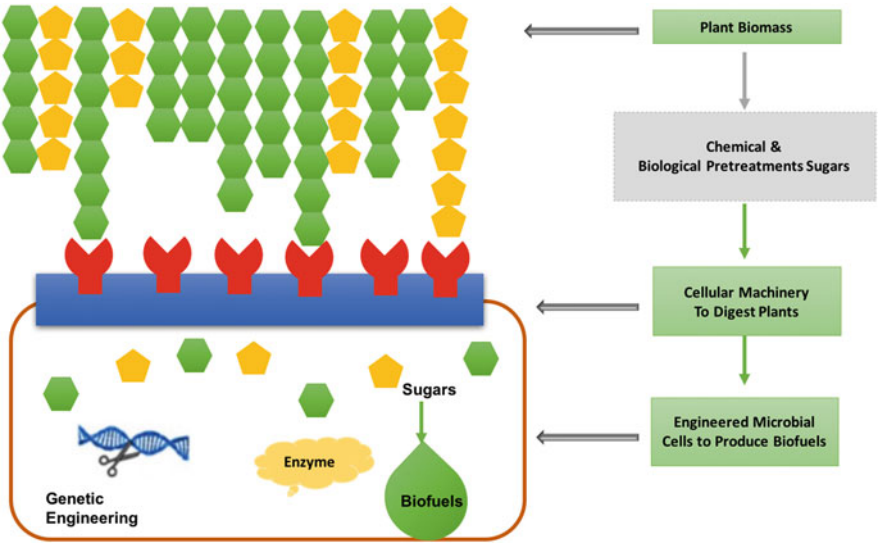

Fig. 11.1 A depiction of the biomass treatment process and genetic engineering for biofuel production

converted to ethanol (Xiros et al. 2013). The cell wall has crosslinked glycan in cellulose, and it is classified in consonance with the type of crosslinks. There are two types: Type I walls have the same amounts of glucan and xyloglucan embedded in a matrix of pectin in dicotyledonous plants; whereas Type II has glucuronoarabinoxylans and lacks pectin and structural proteins in cereals and grasses (Fig. 11.2).

Cellulose, hemicellulose, and pectin are the polysaccharides present in plant primary cell walls; the hydrolysis of their fermentable sugars provides bioethanol production. In trees, the secondary cell wall has three layers, distinguished based on different arrangements of cellulose microfibrils, with only the outermost layer containing the helices. The secondary cell wall of a plant has cellulose, hemicellulose, and lignin, mostly in which cellulose is embedded in lignin in the form of microfibrils (Huang et al. 2009; Sainz 2009; Kenney and Idaho National Laboratory (INL) 2007).

11.2.2 Biofuels from Enzymes

Aerobic and anaerobic microorganisms both produce enzymes cellulose-degrading enzymes. Bacteria and anaerobic fungi produce cellulosomes. It is a complex of cellulolytic enzymes associated with their cell wall. The secretion of cellulases is either free or cell surface-bound (Binod et al. 2019). With the unraveling of new places and areas, cell wall deconstructing enzymes have been and being isolated and

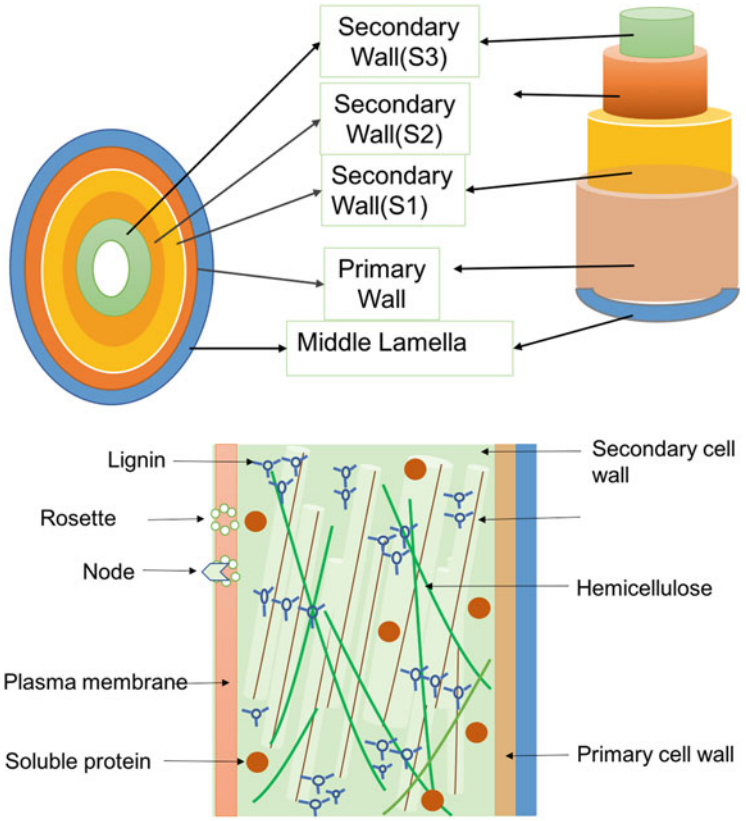

Fig. 11.2 Cell wall primary structure (above) and secondary structure (below) with cellulose microfibrils, hemicellulose, pectin, lignin, and soluble proteins (Sticklen 2008)

characterized from a variety of samples in order to be used in pretreatment investigation. The objective is to achieve higher resistance to the conversion temperatures and a range of pHs. Enzymes cellulases and hemicellulases convert the polysaccharides into fermentable sugars through enzymatic hydrolysis (Himmel et al. 2007).

11.2.2.1 Cellulases

Filamentous fungi produce extracellular cellulases. Due to the ability to produce extracellular cellulose, fungi have been highly researched for cellulase production for a long time. Both fungi and bacteria can degrade biomass; hence the biological method of producing sugars with enzymes is an eco-friendly and accomplishable method (Wilson 2009).

The maximum operating efficiency of the enzymes and their complex composition are always unrepresentative of each other. *Trichoderma viride* and *T. reesei* are amongst the excessively studied microbes (Schuster and Schmoll 2010).

Cellulases are not a single but a group of enzymes composed of endoglucanase and exoglucanases, it also includes cellobiohydrolases and β-glucosidase. Their topologies include β-sheet proteins, β/α-barrels, and α-helical protein. Lignin prevents cell wall hydrolysis by not let enzymes accessing polysaccharides and acting as a barrier. Enzyme production by microbes exposes cellulose to be broken down by cellulases (Schuster and Schmoll 2010). The microbes have the ability to synthesize different functional enzymes effectuating fermentable sugar for biofuels. Cellulases have many applications in industries, and the production of second-generation bioethanol is amongst one of them, it is a low-cost material obtained from lignocellulose. The most abundant renewable biomass; bioethanol production does not create any food insecurity, when lignocellulose is used, hence avoiding any food crops before harvesting. Biological pretreatment concerns with the ligninolytic potential of certain microorganisms that can reduce the recalcitrant nature and cleave it via hydrolytic enzymes. The addition of a molecule with two glucose units into the cultures can increase the cellulase expression. Differential hybridization in many studies showed that gene regulation occurs at transcriptional level (Siqueira et al. 2020; Srivastava et al. 2015).

Abstraction of expression of the glycolytic phosphoglycerate kinase gene, *pgk1,* is the common metabolism observed in cultured media of cellulose. Identification of the functional regions in the promoters of the cellulase genes or the regulatory proteins involved is still a highly debatable topic (Da and Srikrishnan 2012).

11.2.2.2 Factors Affecting the Production from Cellulases

Currently, there are two important factors restricting the production of cellulosic ethanol. The first is the production of strain-specific cellulases, and the other is non-appliance of existing commercial hemicellulases that can increase the output of multiple carbon fermentable sugars. Bioconversion of the complex lignocellulosic material to simple sugars is a complicated process. Genetically modified thermophilic bacteria is the envision for the future as it may lead to enhanced cellulase production through recombinant technology. Isolation of cellulase gene from thermophilic bacteria and its expression in suitable hosts via recombinant technology might enhance cellulase production. It could be done through a classical approach, whole-genome isolation, whole metagenome isolation, or a pre-study with bioinformatics (Verma et al. 2021; Kuhad et al. 2011).

11.2.3 Induction of Cellulase Expression

Plant-based material or cellulose is used in the media to promote high cellulase expression. Expression of cellulose is promoted in cellulose, lactose, and cellobiose that are poor carbon sources rather than in glucose and glycerol (Amore et al. 2013). Cellulase expression is thought to be induced using natural compound sophorose, but the status of cellobiose stays controversial as it needs to be fed in a controlled manner. Cellulase expression might be interfered by the type of nitrogen source that is used. Most of the natural carbon sources used to study cellulase expression offer a competitive growth to other microbes like fungus (Ilmén and Saloheimo 1997).

11.3 Literature Review

11.3.1 Fungus as the Source

When the expression of the cellulases of filamentous fungus Trichoderma reesei QM9414 was studied with genes encoding enzymes cellobiohydrolases and endoglucanases, the steady-state mRNA for cellobiohydrolases was highly expressed. It is also being concluded that cellobiose as a carbon source as an inducer does not show immediate effect and seems to vary depending on the culture conditions, in a study where cellobiose promoted cellulase transcription to a moderate level when compared to that of lactose (Ilmén and Saloheimo 1997). Accumulation of glucose in the culture medium might result in glucose repression of cellulase transcription. The inducing power very much depends on the ratio between carbon source, formation of glucose, and their uptake from the medium. Most of the natural carbon sources used to study cellulase expression offer a competitive growth to other microbes like fungus. Sorbitol and glycerol generally have a neutral effect; it neither promote nor inhibit expression. Glycerol and sorbitol without affecting the fungal growth show a cellulase gene induction in cultures with 1–2 mM sophorose. To understand what is the source of inducing compounds and if they are released from cellulose, the studies have been performed only with glucose and no inducer as a carbon source. If the amount of glucose in a media is subdued, the level of mRNA is found to have a difference in fully induced and repressed states in an actively growing fungus. Without having to add an inducer and still able to cellulase expression after glucose depletion, it can be crucial to keep the fungus alive under starvation conditions (Ilmén and Saloheimo 1997; Margolles-clark et al. 1997).

Aerobic, anaerobic bacteria, and fungi are the models to study cellulolytic enzyme systems. Fungi such as *Trichoderma reese*i, *Penicillium spp*., *Aspergillus niger,* and basidiomycetes secrete extracellular cellulolytic enzymes. Higher fungi also have oxidative systems and can be capable of degrading lignocelluloses through their ligninolytic enzymes.

Fungi are studied for genetic modification reason being their capacity to produce large amounts of extracellular cellulases. Three mainly synergistically acting enzymes in cellulases are cellobiohydrolase/exoglucanase, endo-β-1,4-glucanase, and (c) β-glucosidase. The action of endoglucanase is expedited by lytic polysaccharide monooxygenases. Non-hydrolytic proteins accelerate the action of endoglucanase. Optimization of physical and nutritional parameters can be done by engineering the cellulases, it is also the way to enhance cellulose production. Strain improvement can be approached via random mutagenesis and site-specific mutagenesis in cellulases. One of the most explored fungal strains for commercial cellulase production is *T. reesei* RUT-C30, followed by *Penicillium sp., Aspergillus sp., Myceliophthora,* and *Humicola sp*. (Srivastava et al. 2020).

A study concluded that in a growing fungus, the regulation of cellulase expression depends on glucose repression (Amore et al. 2013). This effect is reversed after the glucose is impoverished and derepression of cellulose occurs with no other inducer present. The use of a mutant can support new ways for biodiesel production; some mutant studies support the theory that separate regulation could exist for different fungal cellulolytic enzymes. This suggests that there is a high possibility that the cellulose enzymes are coordinately expressed. It is always to be mentioned that biologically relevant mRNA levels are not easy to be detected and site-specific proteins might be the ones governing glucose repression or the cellulase expression (Mach and Zeilinger 2003).

11.3.2 Algae as the Source

Photosynthetic algae, both micro and macro, are thought to have the potential to be turned as a possible biofuel resource. Microalgae have the competence to store triacylglycerol and fat that can be turned into biodiesel and ethanol. Due to a high lipid profile, it is believed that crude oil deposits have been created by microalgae over a period of time. Therefore, scientists have a huge interest in understanding and exhilarating the productivity of algae to produce biofuels. What makes microalgae even more interesting is the fact that they are an attractive source of fuel that intake carbon dioxide and keep the environment low on carbon. They grow on marginal land hence are not a competition to terrestrial crops and flourish in waste or saltwater. It means algae does not compete with the resources of food-based crops, which is a problem with lignocellulose-based biofuels. Metabolic pathways of microalgae can significantly be manipulated to produce a greater quantity and a variety of biofuels (Demirbas 2010).

Algae efficiently use photosynthesis to obtain important oils and biomass from carbon dioxide. These oils can be later transformed into feedstocks to produce biofuels, Omega-3 fatty acid oils, and feed for animals feed. Productivity of algae can give an estimate about the approximation of area to fulfill the requirements in a particular country. The protein that comes as a byproduct of fuel production from algae might serve as a very useful food source for protein and other useful products.

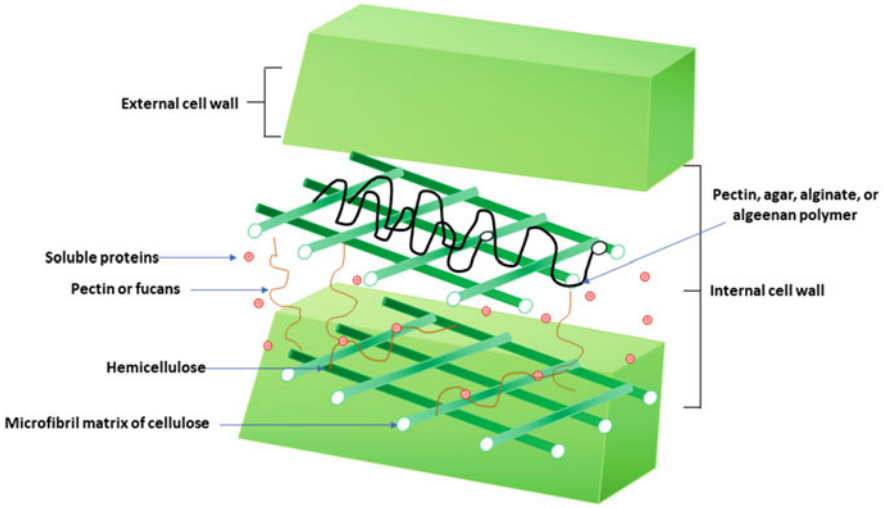

Fig. 11.3 Representation of the structure of microalgae cell wall membrane

The synergistically designed facility takes the edge off liquid fuel hence relaxing the consequences of biofuels production on the land, mitigating global warming, and promoting biofuel production to relieve energy shortage (Demirbas 2011). Microalgae can be aggregated by adjusting the pH; with these adjustment properties of the cell surface, biomass concentration can be modified as per the requirement. After the culture of microalgae, the medium can be recycled, reducing the cost and environmental pollution (Fig. 11.3). The high-pH-induced flocculation method showed an effect on biodiesel production by showing changes in the lipid extraction process and fatty acid profiles of marine microalgae (Castrillo et al. 2013; Liu et al. 2013a).

Nonetheless, and contrary to microalgae, seaweed is a renewable feedstock, and there are some potential concerns and impacts on ecosystems. Polymers in seaweed are mixed sugars, and depolymerization of seaweed polysaccharides is relatively easy, but the conversion of such sugars to biofuels is not an easy task. Excess of seaweed farming can alter natural habitats leading to nutrient depletion and reduction in biodiversity (Kraan 2013).

Economically profitable biofuel production from seaweed is acquired by an efficient conversion of mixed sugars in seaweed hydrolysates. Henceforth, metabolic engineering can be used during the fermentation of sugars. Red marine algae have galactose (up to 23%) as major sugar compound in the hydrolysate Ceylon moss (Wei et al. 2013). Galactose fermentation by engineering *S. cerevisiae* is the eminent way to produce ethanol. The wild-type yeast *S. cerevisiae* is capable of galactose fermentation by controlling the ethanol yield and productivity. However, ethanol production rate and galactose yield are not high when compared to glucose. Also, glucose represses the utilization of galactose by stringent transcriptional repression of GAL genes that are responsible to code enzymes for galactose metabolism.

Hence, excess consumption of glucose and galactose in red seaweed hydrolysates might reduce overall ethanol productivity, which is thought to be improved by metabolic engineering (Johnston et al. 1994).

11.4 Genetic Gateway to Obtain Biofuels

Genetic engineering enables microbe to produce a high number of metabolites. The inherent complexity of the organisms ranges from simple protein structures to folded and globular protein with a variety of medicinal properties. The genetic manipulation requires a preamble of whole-genome sequence to understand and select the desired sites for genetic alterations (Peralta-Yahya et al. 2012). A few examples of such moderation in the gene of the different microbes are mentioned in Table 11.1.

Table 11.1 Improvement of the biofuel production by genetically altering the organisms

Organism	Mutant/gene	Modification	Origin	Reference
T. reesei Qm 6a	RUT-C30	There was an increase by 20 times in cellulase secretion	Fungal	(Peterson and Nevalainen 2012)
Fusarium oxysporum	NTG-19	Cellulolytic activity was 80% more than its parent strain	Fungal	(Kuhad et al. 1994)
Aspergillus nidulans	creAd3	D-glucose metabolism was seen to be improved	Fungal	(Van et al. 1995)
Aspergillus niger DSM 26641	*A. niger* DSM 28712	β-1,4-endoxylanase activity was found to be enhanced by 82%	Fungal	(Ottenheim et al. 2015)
Cellulomonas flavigena	(M4, M9, M11, and M12)	Xylanolytic activities were enhanced	Bacterial	(Mayorga-Reyes and Ponce-Noyola 1998)
Escherichia coli strains	*cydC-D86G, cydC-D86V*	Biofuels and bi-products under ionic liquid stress were higher in the concentration	Bacterial	(Eng et al. 2018)
Chlorella vulgaris	SDEC-3 M	The mutant is supposed to benefit CO_2 biofixation from industrial exhaust gas	Algal	(Qi et al. 2016)
Chlorella minutissima (CM)	CM7	Monounsaturated fatty acids showed an elevation	Algal	(Mehtani et al. 2017)
Saccharomyces cerevisiae	*Saccharomyces cerevisiae* UAF-1	Ethanol production was improved by 12.0% with aeration	Yeast	(Abbas et al. 2017)

11.4.1 Principle

Targeted strain engineering aims at a considerable amount of transformants by homologous integration or deletion of the expression cassette. Nevertheless, introns in the genes and glycosylation convolution cause low-efficiency gene targeting.

Filamentous fungi have the tendency to bring about post-translational modification. The modification includes attachment to a functional group of another molecule by a glycosyl donor and synthesis of organosulfur compounds. They also secrete metabolites that have the ability to expand on a cheaper substrate that makes them suitable for industrial applications. They almost make a good choice to be used as a host for recombinant DNA except for the challenges on non-homologous recombination. By inactivating the double-strand breaks in the DNA pathway this challenge can be resolved (Dellomonaco et al. 2010).

11.4.2 Some Examples from the Previous Studies

Genetic induction or end-product inhibition in microbial cells produces a higher amount of cellulases. Catabolite repression or end-product inhibition in a mutant of *B. pumilus* resulted in a four times higher yield when compared to *Trichoderma reesei* (Kotchoni et al. 2003). RUT-C30 obtained through mutation in *T. reesei* Qm 6a at Rutgers University showed a 20 times increase in cellulase secretion. Most of the available studies state enhancements in cellulase production by mutation without mentioning the changes that might have occurred at genetic level (Peterson and Nevalainen 2012). *Penicillium decumbens* is used in China for the industrial production of lignocellulolytic enzymes. When a comparative genomics analysis by Liu et al. (2013b) was made with the phylogenetically similar species *Penicillium chrysogenum* it was found that the cell wall degradation has advanced with *P. decumbens*. The reason suggested was its strong cellulolytic ability due to more genes involved in cell wall degradation than cellular metabolism, that happens in a medium with cellulose as a carbon source. It has made the lignocellulolytic enzyme system in *P. decumbens* became variegated with hemicellulases and proteins in the cellulose binding site (Liu et al. 2013b). Genetic engineering seems a very efficient method for gene expression by regulating the promoters and can be achieved with minimum changes in the genetic content. Mutagenesis is done to bring the expected changes in the DNA sequences of a gene with specific primers, termed as site directed mutagenesis. The changes are incorporated in a genome by homologous recombination using amino acid sequence primers. However, the sequence in a genome and the site to target are not easy to identify. This process of improving the properties of a protein by alteration in its amino acid sequence increases the secretion of cellulase. Serinine and threonine on the surface of xylanase in *A. niger* BCC14405 were replaced with arginines (Sriprang et al. 2006). The modified enzyme had increased activity than the wild-type strain. As the enzyme activity

also increased half-life of the mutant stability was simultaneously raised. To obtain a hyperthermostability, *Thermotoga maritima* cel5A endoglucanase, when subjected to site directed mutagenesis and CBM engineering, demonstrated 10% higher activity at one site when compared with the native cel5A (Arumugam et al. 2008).

Aspergillus and *T. reesei* are used to express genes from different origins, improving cellulose production. Fungal and bacterial cellulose or carbohydrate binding domains were used from *T. reesei* and *Clostridium stercorarium xylanase* A to be integrated with cel5A. Avicel was used to observe hydrolytic activity in which engineered carbohydrate bonding molecule from both species showed better activity. The activity was linked to binding ability, which was checked via immune gold labeling assay. Mutagenesis of D232A in fungus Macrophomina was used to generate an engineered form for the production of an enzyme with novel substrate requirements. The substrate size of the engineered one was found to be higher than the wild-type 5 b-1,4-endoglucanase but with an equivalent activity on cellohexaose (Druzhinina et al. 2017; Hilden and Johansson 2004). The modified endoglucanase can be used to get complexed carbohydrates by a double decomposition reaction with water present. A study by Liu et al. (2013b) involved *Penicillium oxalicum* mutant JU-A10-T gene with the wild strain 112–2. It has high cellulolytic ability on the processing of decayed organic matter, comparison of whole-genome sequencing, transcriptomes, and secretomes was done. The study revealed that a new lignocellulose-degrading enzyme has emerged (Liu et al. 2013b; Wang and Jones 1997).

11.5 Strategies for Genetic Modification of Microorganisms

Creation, selection, or improvement of strains of desired microorganisms to direct the well-suited output rely on the microbial strains that will be used to catalyze the biosynthesis of the desired compound. Biotechnologies develop befitting strains for fuel by synthetic process.

The imposition of these regulations concerns with the underlying risk and their assessments by the government, taking into consideration proper planning and management. Modification of microorganisms for fuels such as n-butanol, isobutanol, mixtures of alkanes or lipids is evident in number or researches (Keasling et al. 2009; Bhatia et al. 2017).

This is done by overexpression, directed evolution, and codon optimization of key endogenous enzymes to increase the yield of the targeted product (Mythili et al. 2016; Jiménez-Díaz et al. 2017).

The genetic modification deals with the introduction of two or more genes encoding heterologous enzymes to create entirely new biosynthetic pathways or enabling new enzymatic activities of different feedstocks as energy sources. It might also knock out genes encoding enzymes in competing pathways and augment the flow of carbon into a desired pathway. The potential risks are unique to each type of microorganisms, such as in algae; it is possible to have effects on native populations

in order to create or intensify and create a dangerous mutant (Jang et al. 2012; Mary 2011).

To obtain a befitting product, the use of various both prokaryotic and eukaryotic species and strains is common in industrial production. Some of the prevalent and focused microorganisms are yeast fungal and bacterial strains such as *Saccharomyces cerevisiae, Aspergillus* and *Trichoderma, Lactobacillus,* and strains of *Escherichia coli.* The existing strains always have a scope of improvement to have more productivity with genetic engineering for valuable properties (Rubin 2008).

The indication toward the improvisation of carbon fixation enhances pathway in proteins like RuBisCO or alteration in lipid synthesis in algae can help both with the environmental pollution reduction and biodiesel production. To ensure the safety conduct and industrial use of genetically modified organisms, they need to have appropriate risk assessment tools. The methods selected must express, enhance transporter proteins, maximize the carbon flow, and remove toxins and harmful compounds. The change in metabolism should emphasize easier cell lysis and making existing pathways more applicable for commercial purposes. For industrial purposes, fermentation of the microbes must be conducted in a protected environment and must prohibit any exposure or accidental release of the microorganism. Any inherent exposure of the genetically engineered organism that is to be produced commercially. Its exposure into the environment should be assessed for all possible hazards (Jiménez-Díaz et al. 2017; Chen and Dou 2016).

11.6 Regulations for Genetically Modifying Plants and Microbes for Biofuel Production

To fulfill the current demand, the production of renewable fuels or bio-based chemicals is carried out with genetically modified microorganisms. The production includes microalgae, fungus, plants, and cyanobacteria. Researching and finding new biological methods of manufacturing renewable fuels from petrochemical feedstocks foresees a great potential toward more sustainable industrial activities. Without much speculation, genetically modified organisms exhibit certain advantages over other microbiological methods to obtain biofuel. Some of the factors of risk are summarized in Fig. 11.4.

Genetically modified organisms offer remodeled productivity, less operational costs, a variety of feedstocks, and most importantly, substantial carbon footprints. Genetically modified microorganisms (GMM) in most countries require regulatory guidelines before they could enter the market. The government has certain regulations when it comes to genetic engineering of plants and animals. In the late 1980s, risks caused by potential genetically engineered microorganisms to the environment were already getting huge attention. Early scientific reviews laid the foundation for regulatory risk assessments of proposed field tests.

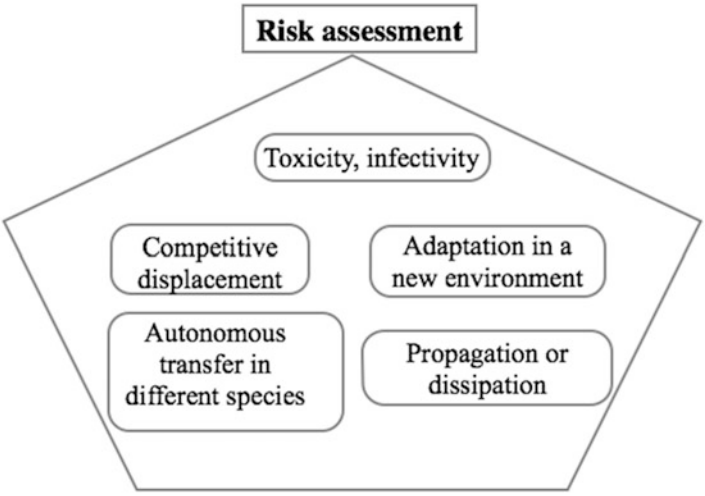

Fig. 11.4 Factors that can influence the risk associated with genetically modifying an organism

EPA and USDA regulations govern the use of modified organisms for the production of fuels or chemicals in the US EPA works under the Toxic Substances Control Act (Glass 2015; Wozniak-Karczewska et al. 2019; Wozniak et al. 2012). The aim of these regulations is to declare it to the agency before merchandising any genetically modified microorganisms.

Ecologists have recommended that engineered strains can perform like non-engineered strains when they are introduced into new environments. This gives a fundamental estimation to predict and monitor the behavior using appropriate risk assessment tools (Viebahn and Chappin 2018). However, we cannot deny the fact that scientific concerns about the potential environmental effects of microorganisms with new traits are reasonable. Tribal Energy Resource Agreements and United States Environmental Protection agency ensure risks are minimized, and it is scalable to establish means of genetic manipulation. The choice of the reactor, its design, and features govern the choice of the starting culture and product.

In the 1990s, there was a standard that was set for the development of biodiesel in order to promote the use of alkyl esters-based biodiesel in pure form or as blends in automotive fuels. The standards vary in the USA and Europe. For the first time in 1999, the American Society for Testing and Materials (ASTM) adopted a provisional specification PS121 for biodiesel. ASTM D6751 was approved in 2002 for middle distillate fuels. ASTM D6751, 2012 onward defines two grades of biodiesel: grade 2-B and grade 1-B. The grades have a strict policy on monoglycerides and cold soak filterability derived from vegetable oils and animal fats. Two automotive standards for biodiesel/diesel fuel are ASTM D975 to allow up to 5% biodiesel to be blended into the fuel, and ASTM D7467, for biodiesel blends from B6 to B20.

In October 2003, the standard for biodiesel EN 14214 was accomplished in Europe for unblended FAME diesel fuel and some biodiesel blends. These standards

set the stepping stone in international standards and became the starting point for biodiesel specifications developed in other countries. Low-level blends are categorized by EN 590. For fatty acid methyl esters fused in diesel engines, EN 14214 makes the regulations.

A category B100 could be used unblended in a diesel engine or blended with diesel fuel to produce a blend conforming EN 590. The changes to cover heating oil applications were inducted by EN14214:2012 to cover blends up to B10. Monoglycerides content was also considered as a separate class. EN 590 covers biodiesel/diesel fuel blends up to B7. The version released in 2004 introduced blending up to 5% of fatty acid methyl ester (FAME) in diesel fuel, which was increased to 7% in 2009. The European biodiesel specification, EN 14214, a European indication that applies only to mono-alkyl esters made with *methanol*, specifies that ester content should be 96.5%, and no additives other than fatty acids can be added. Increased oxidation stability, reducing the sulfated ash limit to 0.005% from 0.02%, and limiting blends to B5 maximum are some of the guidelines for B100 used to make biodiesel/diesel fuel blends used all over the world (ACEA 2009; ASTM 2002; Tasios et al. 2013).

The work carried out in a stepwise and responsible manner can respond to the needs of developing novel sources of energy worldwide. Reducing carbon emissions and avoiding any harmful environmental impacts is the considerable factor that will control, manage, and constrain the genetic engineering of an organism for biofuel production.

Certain uses of GMM could be subjected to FDA regulations because of the production of foods, pharmaceuticals, or other products. However, a standard alternative for companies working with nonpathogenic microorganisms and obtaining ethanol, can be utilization of the excess and waste from the biomass in animal feed.

In the USA, animal feed ingredients are monitored by a non-profit organization that is called Association of American Feed Control Officials. They define if the ingredients are fit for animal feed and pet food.

11.7 Future Outlook

11.7.1 Artificial Intelligence Can Help in Genetic Modification of Biofuels

As we struggle to find out a way to restore diminishing fossil fuel resources, the scientific community is working hard to find alternative sources of fuels; one such form is biofuel, through plantations of certain plants, which takes up to 90 days to grow and be ready processing the biofuel. The goal of the scientific community is to design a plant by a genetic modification that has the ability to produce a large amount of biofuel in less time.

At the molecular level, in a biofuel plant, a gene is responsible for the synthesis of the triglycerides (hydrocarbons). It is necessary to carry out research to identify this gene and isolate it (Rao and Pingali 2008), which might take time for the human eye to get the sequence of the gene. By leveraging Artificial Intelligence, it can be done quicker and with limited sources through AI Machine learning and Deep learning models.

Artificial Intelligence applications in biotechnology include analysis required in modification of genetics, drug screening, predictive modeling. These problems have been solved, which are difficult for humans to solve in a short period of time. AI now exists in every field of study, from recognizing a pattern, forecasting, DNA sequencing of tens of thousands of genes, and plays a crucial role in biotechnology (Klyuchko 2017).

11.7.2 Machine Learning in Action

With the sophisticated machine learning models, we can achieve different clinical trial datasets, enable simulated screening, and analyze vast amounts of data. The basic concept connecting machine learning with artificial intelligence and deep learning is represented in Fig. 11.5. Apart from savings on clinical trial costs, with ML models, we can also gain exclusive insights and feed them back into the process. Machine Learning (ML) concept gives computers the ability to think and helps us to solve many problems. Machine learning is the part of artificial intelligence focused on algorithms which has the ability to learn from experience, and when exposed to new data, its accuracy is measured without explicit programming (Oliveira 2019; Kim et al. 2020).

The ML Algorithms take data as an input, and the output is predicted data or actions. The algorithms improve as they are exposed to more data.

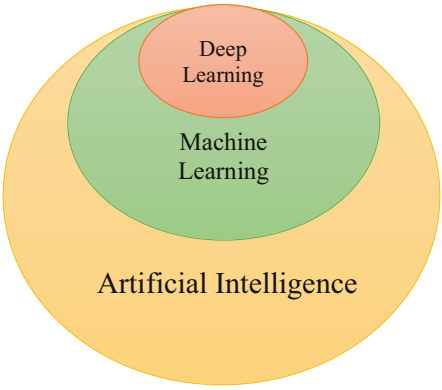

Fig. 11.5 A simple pictorial depiction showing how the artificial intelligence is linked with deep learning and machine learning

As the Dataset grows, the performance of the ML model degrades and may tend to drop its accuracy. Hence, it will be difficult to just work with ML alone. The reasons are

- High dimensions:
  When we have a large number of inputs and outputs, which are nothing but high dimensions, ML is not useful.
- Crucial AI problems:
  Machine learning cannot solve natural language processing, image recognition problems due to their huge data amounts.
- Feature extraction:
  For complex problems, such as object recognition, handwriting recognition, etc., ML will face a big challenge (Jordan and Mitchell 2015).

11.7.3 Deep Learning Methods

Deep learning is an approach of machine learning that has been designed based on the knowledge of the human brain and neurons, a simple depiction in Fig. 11.6. In recent years, deep learning has seen tremendous growth in its popularity and usefulness in the field of biotechnology (Sugomori et al. 2017).

Deep learning is a subset of machine learning methods based on artificial neural networks with multiple layers representation learning in different dimensions, which can be understood with Fig. 11.7.

Machine Learning can be supervised, semi-supervised, or unsupervised. The difference is in supervised learning, we provide labeled data, whereas unsupervised learning machine comes up with its own patterns without labels.

Deep learning algorithms use the concept of multilayer perceptron's. Data is filtered through multiple layers, with each consecutive layer using the output from the previous one to inform its results (Ardabili et al. 2020; Aghbashlo et al. 2021).

Deep learning models will achieve its accuracy level and can provide more data to process. If there is not enough data, then we can apply different deep learning techniques like data augmentation to generate more data for training our model, the more training we provide, algorithm will learn to perform better when unexposed

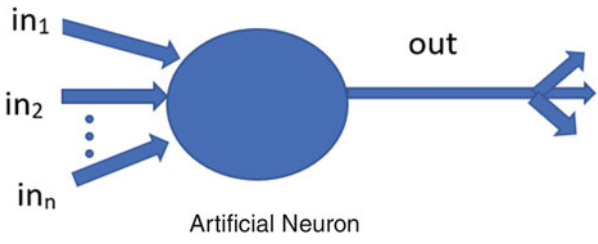

Fig. 11.6 Flow of information in machine learning approach

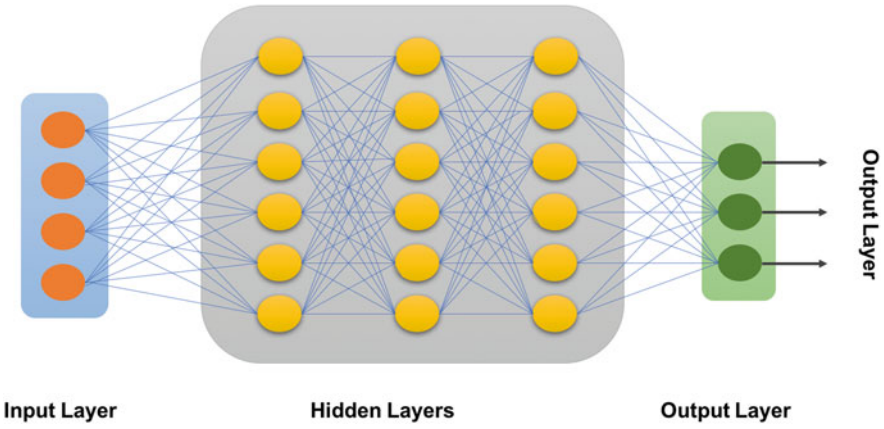

Fig. 11.7 Schematic representation of deep learning methods

data is tested, deep learning models learn from previous results to enhance their capability to make correlations and connections.

- DL algorithms are capable to focus on important features, and this can be achieved through minimal coding.
- DL models are capable to solve high dimensions.
- The main idea behind Deep Learning is to build learning algorithms that mimic biological neurons neural network system.
- Deep Learning is implemented through Neural Network.

Like many other disruptive technologies in Biotechnology, AI is generating much anticipation with Deep Learning's convolutional neural networks, which can work with minimum data as well. Also, a transfer learning model which gained information from a previously trained data can be applied to a new set of data, which can give more accurate results as it has seen certain features of data (Mosavi et al. 2020).

As per American physicist, Kaku and 3M Company (2011), we could be witnessing the next transition from transistors based on silicon to transistors based on atoms. In a decade, Moore's Law will slow down and computer power will level up, and we are going to many such transitions, one of them could be Quantum Computing.

This new field of Quantum Computing is now on the leading edge of computing. World's leading organizations like IBM, Google, etc., are working toward Quantum Supremacy.

As per MIT "One of the goals of quantum computation and quantum information is to develop tools which sharpen our intuition about quantum mechanics and make its predictions more transparent to human minds." The development of Quantum Machine Algorithms has begun for the genetic sample classification.

Unlike classical computing, which is built on either 1 or 0, whereas quantum computing has both the positions at once, that is 1 and 0, and is based on Quantum mechanics (Li et al. 2001; Roe 1998).

There is a future of biotechnology when combined with quantum computing in deriving molecular level properties to modify the genetics of biofuels.

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