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Application of Crispr Technology for the Generation of Biofuels: A Review

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Abstract

The use of crude oil and its derivatives to produced energy have contributed to Global warming and climate changes. Biofuels are produced by conversion of biomass to generate combustible fuels. First generation employ the traditional method to convert food-crops raw materials to generate biofuels in the form of biodiesel, bioethanol and biogas. First generation has so many disadvantages such as food insecurity. Second generation involves production of biofuels using non-food crops with high content of lignocellulose, residues or waste from industry, forestry and agriculture as the feedstocks. Abiotic stress is one of the disadvantages of second-generation biofuels which require enhanced technologies to live up to commercial scale. The third generation of biofuels involves the use of tiny microorganisms such as algae and bacteria. These organisms can be modified using gene editing tools such as CRISPR-Cas system to produced high quantity of biofuels with low GHG emission and low cost.

Keywords: CRISPR; Biofuels; Biomass; Bacteria; Algae

Introduction

The world is facing an increase environmental challenges and energy crisis due to highly dependence on crude oil and it derivatives which lead to intensive usage of petroleum and refined products which in turn causes a serious environmental concern [1,2]. The conventional (old) traditional techniques use to refine crude oil by industries need upgrading in order to reduce the emission of greenhouse gases (GHG) such as methane CH_4, Carbon dioxide CO_2, Nitrous oxide NO_2, Sulfur dioxide SO_2, for sustainable environmental development. Production of biofuels in large scale or commercial scale quantity is still a challenge [3]. According to Intergovernmental panel on climate change (IPCC) report, the world population is on the rise which leads to increase in energy demand per capita and thus result in rapid rise in GHG emission which in turn contribute to global warming, climate change, rise in global sea levels, loss of biodervisity, loss of glaciers etc. [4,5].

Oil and it derivatives are the world primary sources of energy. Both industries and transport sectors rely highly on these sources. Even though some industries have diverted to solar energy. It was estimated that the world consumed approximately 84 million barrels per day and due to reliance on fossil oil, this figure is estimated to increase to 116 million barrels per day by 2030 [2]. The interest for the generation of biofuels from renewable sources is gaining momentum as a result of depletion on non-renewable energy such as fossil fuels and it effect on climate changes and environmental pollution [6]. Green technologies have gained significance attention due to their ability to generate clean and safer fuels [4]. The generation of biofuels from biomass as feedstocks has the potential to substitute petroleum and it derivatives as transport fuels. A statistical market analysis has shown production of biofuels will reach 1900 million barrels by 2020 which will contribute to 6% of global fuel production [7].

The proposition of the termed "Biorefinery" emerges as the only reliable solution to counter these challenges. Bioenergy has the potential to end global energy crisis [8]. The word "Biorefinery" is defined by International Energy Agency (IEA) as a process of sustainable production by subjecting biomass as a raw material to generates different bio-based products and bioenergy. Biorefinery rely on both chemical and biological techniques or processes for the conversion of renewable feedstocks as the raw material such as biomass, agricultural and food waste to produced biofuels and other refined chemical [8]. Both plant and animal waste and by-products can be employed to generate energy. Nevertheless, biofuels can be generated from both macro and microorganisms such as algae and different species of bacteria [9]. The main difference between conventional or traditional refinery and Biorefinery is traditional refining approach subject non-renewable raw materials to convert fossil crude oil into refine product. This process is characterised by anaerobic or low oxygen content which result in higher carbon emission which in turn lead to serious environmental issues [8].

Scientists have been using CRISPR as a genome editing tool to make a significant and positive alteration of genes in bacteria, fungi, plant and human cells [10]. The technology has shown diverse application in producing genetically modified organism (GMO) that can be use in industries and pharmaceutical companies to produce high amount of product such as yogurts, wine, alcohol and drugs. CRISPR stand as the best candidate that can be employed by scientist to solve energy crisis in the world [9].

This paper reviews the use of non-renewable and renewable sources of energy, the conventional use of traditional approach for refining crude oil and Biorefinery. Firstly, an overview of the effect of fossils oils and global warming is provided. The application of CRISPR technology in agriculture and environment are highlighted. Lastly, the use of CRISPR to increase bioenergy is described.

Bioenergy

Bioenergy is termed as the use of natural resources to generate renewable energy. Natural resources use includes plants, animals and their by-products or waste products as shown in Table 1 [9].

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Biomass	Types	Example
Plant-Based	Agricultural Biomass	Straws from wheats and rice, grasses, seeds, crop residues, stalks, husks, food by-products etc.
	Forestry resources	Leaves, wood, loggings etc
Non- Plant Based	Livestock resources	Waste from butcheries (i.e. abattoirs), fishery waste (or industrial fishery by-products) etc.
	Industrial biomass	Sewage sludge
	Household biomass	Garbage waste

Table 1: The Classification of Biomass.

Biomass

Biomass is any form of renewable energy that is generated from organic matter such as human and animal by-products, organic and industrial waste, wood, straw, by-products of food, forestry and other agricultural processes (organic crop waste) as shown in Figure 1, [2,8,9]. The generation of energy from biomass is categorised into three major categories [2].

Direct burning: This is the production of heat by directly combustion (i.e. burning) of solid biomass. This process has been used for decades to generate heat which in turn generate steam that is use by engines to generate electricity [9].

Biogas: The fermentation of wet biomass results in the production of widely used methane gas. The anaerobic process of exposing dry biomass to higher temperatures result in the formation of syngas [9].

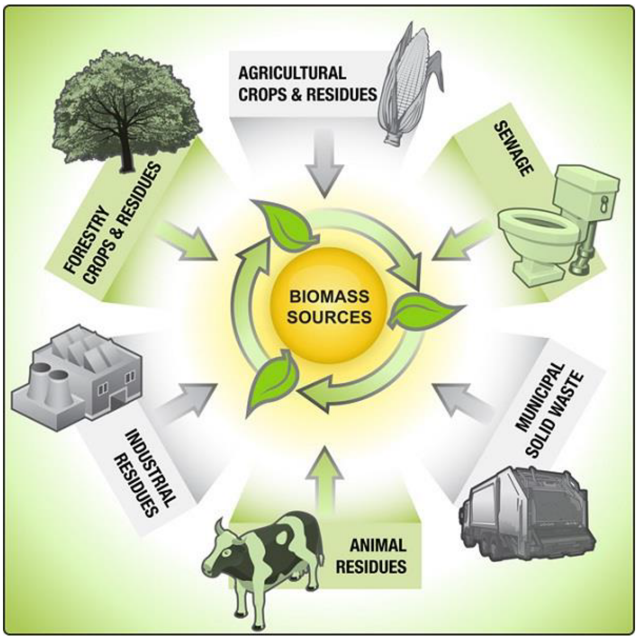

Figure 1: Sources of Biomass [11].

Biofuels

Biofuels are termed as the use of biomass to generate combustible fuels. They are sustainable, renewable, environmentally friendly and less threat to ecosystem [4]. Biofuels are classified into three generations. First generation is the production of biofuels using food and feed (such as corn, sugar cane etc) or the used of unprocessed organic matter such as wood pallets and chips that are mostly used for cooking to generate electricity. The usage of this raw material compete with their usage as food and pose a serious, ethical, political and environmental concern. In order to divert away from subjecting food as raw materials, second generation of biofuels are utilize which involve using waste, residues and crops that are not edible which results in the liquefaction processes of ethanol and biodiesel to generate biofuels [2].

Production of biofuels: Biofuels are mostly produced via two processes; chemical reaction and fermentation of biomass. The chemical reaction between alcohol and greases result in the production of biodiesel. The fermentation processes of plant sugars result in the production of ethanol [4].

First generation (1G)

First generation employ the conventional or traditional method to convert raw materials such as starch, animal fat, sugars and vegetable oils obtained from grains (corn, wheat, seed etc.) to generate biofuels in the form of biodiesel, bioethanol and biogas derived from starch and biomethanol from vegetable oils as illustrated in Figure 2 [2,4].

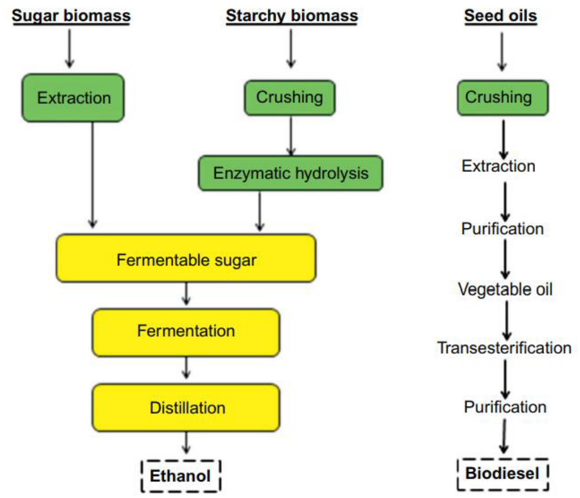

Figure 2: Flow chart of 1G biofuels production [12].

Bioethanol: Bioethanol is the most utilized biofuel which is produced by conversion of feedstocks biomass as starchy crops (from wheat and corn), sugar cane (*Saccharum officinarum* L) and sugar beet [11,12]. It was estimated in 2006 that total global generation of bioethanol has hit 51.3 billion litters with USA solely produced approximately 20 billion litters per the year using corn as the primary biomass. After USA, Brazil is the second largest producer of bioethanol with approximately 18 billion litters per the year using sugar cane as the main feedstocks followed by European Union (EU) with approximately 3.5 billion litters per the year using both starch and sugar beet [13].

Biodiesel: Biodiesel is generated from conversion of feedstocks obtained from oils such as soybeans, sunflower, rapeseed, palm oil and other oil derived from edible agricultural waste. As shown in Table 2, the total production of biodiesel was estimated to reach 6 billion as of 2006. Germany is the world leading producers with 2.5 billion per the year using sunflower and rapeseed as main feedstocks. USA is the second largest producers with approximately 1 billion litters. A higher increase in generation is reported in Italy, Austria and France [2,14].

Biogas: Biogas is generated from the anaerobic conversion of feedstocks such as grasses, starch-derived corn, manure and organic waste (which fall into second generation of biofuels due to the fact that wastes are not viable competition with feed and foodstuffs) as shown in Figure 3. Generation of biogas is common in most European countries (such as Sweden and Germany) which utilise biogas for the generation of electricity. Biogas is further converted or upgraded to biomethane to serve as transportation biofuels. As of 2006, Sweden is the world leading producer of biomethane making up to 45% of the countries fuel consumptions [2].

Advantage of first generation: First generation of biofuels has so many advantages, they possess high sugar and oil content and they are easily converted to biofuels. Researchers have employed Life Cycle Assessment (LCA) technique to analyse many biofuel production stages in order to evaluate their environmental performances (score). As shown in Figure 4, LCA result has shown that when biofuels (in the form of biodiesel and bioethanol) are utilize instead of conventional gasoline, petroleum and diesel as transportation biofuels, a net reduction of GHG is observed which contribute to global warming and consumption of fossil fuels (non-renewable source of energy) [15,16]. To evaluate life cycle effect under difference environmental conditions such as ozone depletion, local air pollution, eutrophication, acidification etc, LCA is employed and the result demonstrated that using biofuels is the only current and reliable alternative to reduce emission of toxic gases that contribute to global warming and cause of several diseases in both plant and animal [2,16].

S/No	Countries	Biodiesel (Billion litters)
1	USA	5.5
2	Brazil	3.8
3	Germany	3
4	Indonesia	3
5	Argentina	3
6	France	1.5
7	Thailand	1.4
8	Spain	1.1
9	Belgium	0.5
10	Columbia	0.5
11	Canada	0.4
12	China	0.3

Table 2: Top 12 countries with the highest production of biodiesel in 2016 [14].

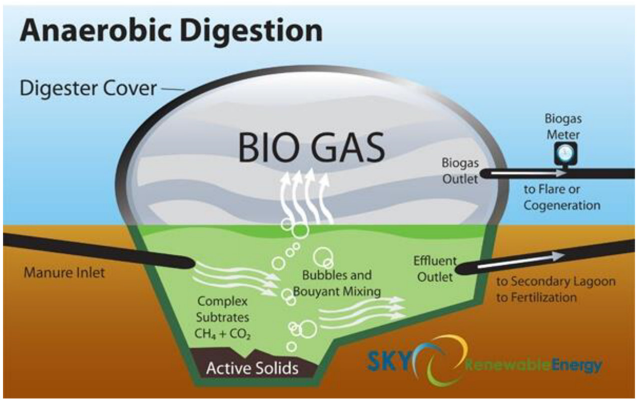

Figure 3: Generation of Biogas using Cow dung: Jordan Green [15].

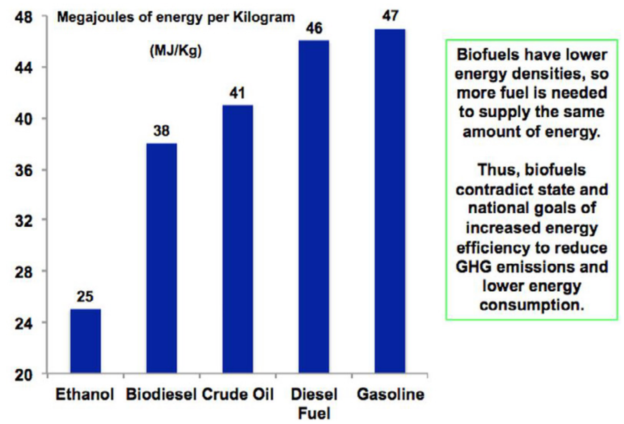

Figure 4: Biofuels Have Lower Energy Densities and thus Less Energy Efficient [17].

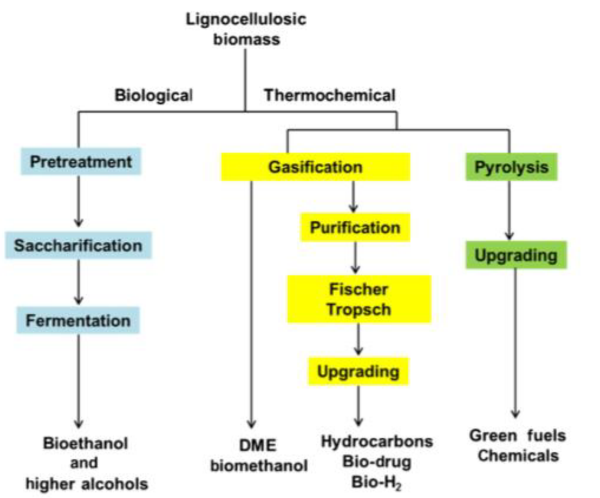

Figure 5: Flow chart of 2G biofuels production [12].

Challenges of first generation: First generation of biofuels from raw materials such as starches, sugars and vegetable oils poses a serious concern due to their contribution to food insecurity and competition for use as foodstuff, feedstocks, raw materials for industrial production of products such as chemicals, alcohol and wine and agricultural land, water and fertilizer [7]. First generation of biofuels required large hectare of land and high energy input to cultivate the main raw materials. Farmers prefer to sell these crops to industries that convert these products to biofuels, processed food and drinks which may lead to food shortage around the world [16].

Second generation

Second generation involves production of biofuels using non-food crops as the feedstocks mostly in the form of lignocellulosic materials such as non-food crops with high content of lignocellulose, residues or waste from industry, forestry and agriculture [5,17,18]. As shown in Figure 5, lignocellulosic materials are converted to biofuels in the form of synthetic liquid biofuels and biogas via processes such as enzymatic technique, thermochemical and flash pyrolysis etc. [2,19].

Pros and cons of using second generation: The generation of biofuels using second generation is highly promising as it paved a way to divert from the usage of foodstuffs crops and reduced food insecurity [7]. These approaches according to LCA studies offer solution to the challenges face by first generation of biofuels in terms of environmental performance, efficiency of land use. The utilization of waste residue from both agricultural and industrial sectors help minimise water pollution that is toxic to aquatic organisms and land pollution that poses serious health and environmental concerns [16]. The readily availability, widespread and relatively cheap of these lignocellulosic materials make them an ideal feedstocks for the production of biofuels. These materials can be supplemented as feedstocks with non-food crops such as perennial grasses [19].

These residues can be converted to yield or produced valuable biofuels for generation of electricity, production of chemical compound and heat or steam for use as energy for industrial engines. The second generation of biofuels from Biorefinery can lead to a safer environment, better energy, economic development and net reduction of GHG emission. Most of the technologies for production of second generation of biofuels are still under development to live up to commercial scale production and required dedicated investors to help transform the sector [2].

Application of Genetic Engineering in Bioenergy

For decades scientist have been trying to modify both prokaryotic and eukaryotic cells in order to improve production of by-products and to cure genetic diseases. The discovery of Recombinant DNA technologies in 1970 has opened the window for scientist to edit cells and develop new biological functions [20]. The discovery of Zinc Finger Nucleases (ZFN) in 2005 and Transcription activator-like effector nucleases (TALENS) in 2010 as genetic tools have lit hope but these technologies have limitations and are very expensive [21].

The discovery of CRISPR-Cas system which is an adaptive immune response or mechanism use by bacteria against invasion by viruses [22]. This technique is harnessed by scientist to precisely modify gene by simply inducing a single or double strand break. Genetic engineers took advantage of this break to insert a homologous sequence with either point; base or nucleotide mutation and cells repair the break using Homologous Directed Repair (HDR) [23-27]. This approach is used by scientist to turn off (knock out) or turn in (knock in) gene of interest at any point in the genome. CRISPR-Cas system has become the most widely and adopted gene editing tool due to its simplicity, affordability, (cheap) and reliable method compare to ZFN and TALENS [20].

Lignocellulosic feedstocks with desired traits are highly required for the transition from the use of non-renewable energy (i.e fossil fuels) to renewable source of energy (i.e biofuels). Due to the diversity and structural complexity of plant cell walls, scientist has been trying to enhance these structures such as lignin, cellulose and hemicellulose for efficient utilization of plant as biomass or feedstocks as shown in Figure 6. Lignin is one of plant secondary metabolite that is employed by scientist to generate biofuels. Genetic engineers have adopted different genetic techniques to modify plant cell composition and architecture to enhance production of biofuels [24].

The third generation of biofuels involves the use of tiny macro and microorganisms such as algae and bacteria. Microalgae biomass which is found in abundance in nature in both fresh and salty water, they have lower emission of GHG and can be easily reproduced [25]. The comparison between 1G, 2G and 3G of biofuels is presented in Table 3.

Generation of biofuels from genetically modified algae

Since 1970s, scientists have been researching and working to optimise the production of high amount of biodiesel and bioethanol from phototrophic algae. Algae are very slimy and tiny organisms, like phototrophic organisms (i.e. plants) algae also need energy from sunlight, Carbon dioxide and other essential nutrients (such as Phosphorus and Nitrogen. Microalgae contain variety of biocomponent such as protein, lipid, carbohydrate, antioxidants and pigments. Due

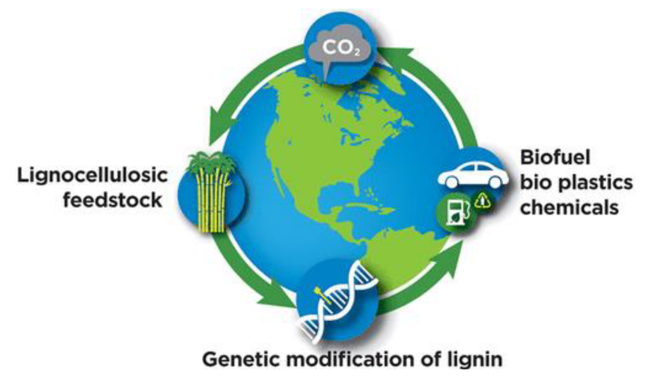

Figure 6: Genetic modification of Lignin of plant cell wall for the production of biofuels [24].

(G)	Raw materials (Biomass)	Biofuels (Products)	Advantages	Disadvantages
1G	Food based resources starch, animal fat, sugars and vegetable oils obtained from grains (corn, wheat, seed etc.)	biodiesel, bioethanol and biogas	High sugar and oil content and they are easily converted to biofuels. Net reduction of GHG	food insecurity due to competition for use as foodstuff, required large hectare of land and high energy input to cultivate
2G	non-food crops as the feedstock's mostly in the form of lignocellulosic materials, residues or waste from industry, forestry and agriculture	synthetic liquid biofuels and biogas	Reduced food insecurity. Minimise water pollution that is toxic to aquatic organisms and land pollution. Readily available, widespread and relatively cheap. Can be supplemented as feedstock's with non-food crops. Safer environment, better energy, economic development and net reduction of GHG emission.	Most of the technologies are still under development to live up to commercial scale. Required dedicated investors to help transform the sector
3G	microorganisms such as algae and bacteria	Hydrogen gas, bioethanol and biodiesel	Abundance in nature. lower emission of GHG and same as 2G	Required the use of Genetic engineering which need intensive research, tools and knowledge

Table 3: Comparison between 1G, 2G and 3G Biofuels.

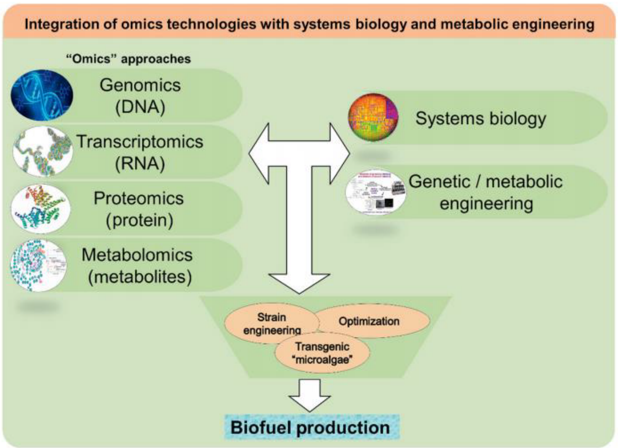

Figure 7: Research among Omics Approaches, Systems Biology, and the Genetic Engineering Approach for Optimization of Microalgae for Biofuel Production [7].

to biochemical component, microalgae can be used to produced Hydrogen gas, bioethanol and biodiesel. Algae overtime became sluggish and stop growing or multiplying. In this dormant stage, their cellular mechanism starts synthesizing fatty lipids [25,26].

The primary advantages of using algae to generate biofuels is it does not require much energy input, use of fertilizer (as the case of 1G), employing microalgae for the production bioethanol and biodiesel will counter the challenges of food insecurity, microalgal biomass can also be integrated with waste water treatment (2G) for higher production of biofuels. Some of the challenges of using microalgae are "Abiotic stress" conditions such as salinity, nutrient depletion and repletion, heat stress, phytochromes, UV radiation, light etc [7]. "Algomics" which refer to the studies microalgal cell metabolism using different omics such as (genomics, transcriptomics, proteomics and metabolomics) have contribute immensely to the use of microalgae for biofuel production [26]

Several microalgal species, such as *Chlorella sp. NC64A, Micromonas pusilla, Phaeodactylum tricornutum, Ostreococcus tauri, Nannochloropsis oceanica, Chlamydomonas reinhardtii, Volvox carteri, Ostreococcus, Coccomyxa sp.* C-169, *Aureococcus anophagefferens, Dunaliella salina, Botryococcus braunii* UTEX 572 etc have been screen and sequenced by scientist and database of the genes are available for gene edition to enhance microalgal lipid metabolism for the generation of biofuels as shown in Figure 7. However, different microalgal organelles such as plastid genomes and mitochondria have also been sequenced [7,25]. In order to solve the challenges of abiotic stress, scientist employed transgenic microalgae which are modified by targeting responsible genes using forward or reverse approaches and screening for random knockout libraries [25]. The 20 factors affecting transcription which regulate lipid production in algae are studied by scientist. 18 of the transcription factors are knocked out by employing CRISPR-Cas system which led the algae to double its lipid production [9].

A group of scientist from the University of California have used CRISPR to tweak specific genes which result in algae doubling their lipid production. A collaboration research by both synthetic genomics and Exxon Mobil companies aim to advance and enhance the production of biofuels. The resulting research has the potential to generate estimated amount of 10,000 barrels of biofuels-derived algae by 2025 [9].

It has been shown that triggering triacylglycerol (TAG) accumulation in microalgae can enhanced the production of biofuel. Kaye et al. [28] employed Nannochloropsis oceanica a microalgae with high potential for production of biofuels, the group enhanced the biosynthesis of polyunsaturated fatty acids (PUFA) (i.e. TAG) by overexpressing endogenous Δ12 desaturase (NoD12). Chien et al. [29] genetically modified Chlorella sp. by codon-optimization of some genes. The expression of genes encoding for enzymes that catalysed the TAG pathway caused an increased levels of TAG by up to (20-46 wt%) and total lipid storage (35-60 wt%) compared to the wild-type. Xue et al. [30] overexpressed the gene encoding malic enzyme (which play a critical role in both carbon fixation and pyruvate metabolism in microalgae) in Phaeodactylum tricornutum. The overexpression of these genes resulted in malic enzyme activity which considerably increased total lipid content by 2.5-fold.

Kamennaya et al. [31] modified *Cyanobacterium synechocystis* sp using gene editing technologies to increase the number of copies of the endogenous bicarbonate transporter BicA which are needed for more efficient use of Carbon dioxide. These genetically modified strains under carbon dioxide pressure successfully produce more BicA, resulted in a biomass and growth rate twice compare to the wild-type.

Generation of biofuels from genetically modified yeast

Yeast is one of the most widely organism used in industries for the production of alcohol and bread. During bioproduction recycle process, yeast undergoes stress as a result of generating excessive metabolite or protein which is very harmful to yeast. Pre-treatment of chemical is one of the process of bioproduction which is utilize to increase the speed of cellulose breakdown process into sugars, these chemicals are very toxic to the yeast [9].

Yeasts are employed for the fermentation of sugars into biofuels. Scientist have employed CRISPR-Cas system to protect yeast from harmful and toxicity effect of chemicals during biofuel production. Scientist use CRISPR to make two alteration to the single gene responsible and thus make yeast resistant (i.e. tolerant) to chemicals used in pre-treatments.

Generation of biofuels from genetically modified bacteria

Acetogenic microbes such as Clostridium autoethanogenum are utilised for the fermentation of biogas. Industries use these organisms to produce ethanol in commercial scale production. Before the discovery CRISPR as a gene editing tool, the exploitation of this microbe for commercial use is highly challenging due to limited understanding of biochemical mechanism of Acetogenic microbe and the availability of gene editing technique to study some specific genes responsible for generation of bioethanol. Recently, CRISPR has improved efficiency of gene knock-out in these organisms.

Conclusion

In order to divert from non-renewable source of energy such as fossil fuels and the use of crude oil and it derivatives which contribute to Environmental pollution and global warming. A sustainable, renewable and environmentally friendly source of energy is highly required. Green technologies which employ the use of renewable energy and biomass to generate biofuel remain the only potential alternative to solve world climate crisis.

First Generation (1G) of biofuels rely on edible raw materials (food-crops) such as corn, wheat, vegetable oils, animal fats etc. to generate bioethanol, biodiesel and biogas but the usage of this raw materials contribute to food insecurity due to competition with food resources and land use for cultivation. Second generation (2G) provide solution to the challenges of 1G as it employ the use of non-edible raw material (non-food crops) with high content of lignocellulose such as agricultural waste, industrial residues and household waste but 2G of biofuel is still under development to live up to commercial scale.

Third generation (3G) use organisms such as algae and bacterial biomass which are found in abundance. 3G offer to solve the challenges of both 1G and 2G. The use of CRISPR/Cas system to modify these organisms to produce high quantity and overcome the toxicity of chemicals and abiotic stress (for commercial production) added flavor and a greater dimension to 3G. Thus, 3G of biofuels has the potential to solve environmental challenges, reduce emission of Greenhouse gases and prevent global warming.

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