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Current situation of biofuel production and its enhancement by CRISPR/Cas9-mediated genome engineering of microbial cells

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ABSTRACT

Geopolitical and economic factors have motivated the scientific community to utilize renewable energy resources. In addition to the modifications in major steps and processes of biofuel production, manipulation of microbial genome engineering tools is essential in order to find sustainable solution of continuous depletion of fossil-fuels. Recently, the CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated nuclease 9), a prokaryotic molecular immunity system, has emerged as a novel technology for targeted genomic engineering. This genetic machinery seems to be a groundbreaking discovery to engineer the microbial genomes for desired traits such as enhancing the biofuel tolerance, inhibitor tolerance and thermotolerance as well as modifying the cellulases and hemicelluloses enzymes. In this review, a summary of different generations of biofuels, integrated processes of bioconversion of raw materials into biofuels and role of microbes in biofuel production has been presented. However, the ultimate focus of the review is on major discoveries of CRISPR/Cas9-mediated genome editing in microorganisms and exploitation of these discoveries for enhanced biofuel production.

1. Introduction

There is a huge demand of fuel due to its consumption in transportation, energy generation and industrial purposes. Recently, the demand for petroleum-based fuel has resulted in a number of economic and environmental concerns and attentive efforts are needed to encourage the emerging alternative fuels (Gowen and Fong, 2011). Biofuels, produced from biomass, render an environmentally clement and cost-efficient solution for fossil fuel impoverishment. These substitutive and inexhaustible sources of fuel as biodiesel and bioethanol have captivated increasing attention from industry, decision-makers, and scientists because of their valuable advantages (Xing et al., 2012). The narrative about the biofuels as a prospective alternative to petroleum-based transportation fuels have been growing during the past few years (Gowen and Fong, 2011; Stephanopoulos, 2007). Specifically, the production of ethanol and butanol are mainly based on sugar or starch feedstock fermentation, whereas the biodiesel is being yielded by transesterification of lipids obtained from soya beans, canola seeds and other crops (Munasinghe and Khanal, 2010). The cost-effective and inexhaustible raw materials such as lignocellulosic feedstock derived from agricultural wastes (such as sugar cane bagass, sugar beet or corn stalks) and energy crops (such as popular or switchgrass) are used to manufacture biofuels adding an advantage of not severely affecting food supplies (Bajpai, 2017; Kovačić et al., 2017). However, various investigative and technology related obstacles are involved in the deployment of lignocellulosic biomass as a raw material for the biofuel industry.

Many microbial strains are well-known for fermentation potential yielding biofuels. Among yeasts, *Saccharomyces cerevisiae* is one of the most widely utilized microbes for fermentation of monomeric sugars for ethanol production on industrial scale. The bacterial species employed for fermentation purpose include *Zymomonas mobilis, Clostridium thermosaccharolyticum*, *C. thermohydrosulfuricum*, *Thermoanaerobacter mathranii*, *T. brockii* and *T. ethanolicus*. The site-specific genome editing is a leading domain of genomics that seems to be helpful in the improvement of microbial strains for biofuel production. The genetic

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engineering is a widely used method for site-specific modifications in the genome for the manipulation of a particular feature in the indigenous microorganisms including knocking-down, knocking-out, and knocking-in the genes. This contradicts the conventional genetic engineering where the gene to be manipulated is first singled out and then manipulated *in vitro* and added back to the host, or a heterologous gene is inserted by genetic transformation methods to change a particular feature of the organism (Garneau et al., 2010; Wiedenheft et al., 2012). The RNA-guided endonuclease-mediated (REM) and modified endonuclease-mediated (MEM) site-specific genome editing methods have been recently employed for strain improvement. CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated nuclease 9), a natural bacterial defense system, is a characteristic example of REM based genetic engineering method and multifaceted tool for genetic engineering that employ a guide RNA (gRNA) to point Cas9 to a particular sequence. This facile RNA-guided genome-engineering technology has been regarded as a groundbreaking tool in biology and has multiple inventive uses in biofuel manufacturing (Xu et al., 2016).

The CRISPR/Cas9 technology has proven to be a novel and smart tool in industrial research and have been employed to engineer the genomes of various microorganisms such as bacteria, yeast, filamentous fungi, and algae (DiCarlo et al., 2013; Fang et al., 2017; Jiang et al., 2013; Wang et al., 2016). The instigators of CRISPR/Cas9 have modified this tool into an adaptable and robust approach for genetic engineering. This review summarizes generations of biofuels, processes and microbes involved in biofuel production with special emphasis on discoveries of CRISPR/Cas9-mediated genome editing in microorganisms in order to link these discoveries for improvement of biofuels production.

2. Biofuel sources and its generations

Biofuels are liquefied fuels that are generated from the different biological materials such as animal matter and waste plants (Azad et al., 2015; Rodionova et al., 2017). These are mainly categorized into two types like primary and secondary biofuels. The primary biofuels generally used in their unrefined form for cooking, heating, and production of electricity. Some of the examples of primary biofuels are fuel-wood, pellets, wood chips, crop remains, and landfill gas (Rodionova et al., 2017). Secondary biofuels are refined form of primary biofuels, which are generated either in the form of solids (e.g. charcoal), liquids (e.g. biodiesel, bioethanol, and bio-oil), or gases (e.g. biogas, and hydrogen). The secondary biofuels that are used in vehicles and numerous industrial processes include biodiesel, bioethanol, and biogas. These are manufactured by the biological processing of biomass (Doshi et al., 2016). Based on the raw materials and biological processes that are employed for their manufacturing, secondary biofuels are further classified into four generations as shown in Table 1 (Azad et al., 2015).

2.1. First generation biofuels

Starch (from potato, barley, wheat and corn) or sugars (from sugarbeet and sugarcane) are usually fermented to manufacture first-generation biofuels such as bioethanol and butanol. Where, bioethanol is regarded as the most eminent biofuel of the first generation which is generated by fermenting carbohydrates distilled from crop plants (Naik et al., 2010). Crop plants with a high concentration of carbohydrates are fermented with the help of enzymes synthesized by *S. cerevisiae* to get bioethanol. The *S. cerevisiae* work on six-carbon sugars (usually glucose) to produce bioethanol (Nigam and Singh, 2011). Another highly efficient first generation biofuel is biodiesel which is manufactured by trans-esterification or cracking of straight vegetable oils of sebaceous plants (e.g. sunflower, palm, rapeseed, soybeans and coconut etc.), commonly used for cooking purposes (Rodionova et al., 2017). They offer multiple advantages especially to underdeveloped countries by considering the fact that lower production costs of biofuels enable low-paid societies to have access to inexhaustible forms of transport fuels (Demirbas, 2011). Substantial economic and environmental constraints are also associated with the first-generation biofuel production systems apart from their various advantages. For example, as the production efficiency expands, their competition with agriculture for cultivable land also increases. This is the most frequent problem associated with the production and use of first-generation biofuels in the last few years (de Vries et al., 2010).

2.2. Second generation biofuels

Novel starch, sugar and fatty crops like jatropha, cassava or miscanthus are used to manufacture second-generation bioethanol and biodiesel with the help of traditional technologies. Biobutanol and syndiesel® are some other popular second generation biofuels which are manufactured from lignocellulosic materials (e.g. straw, wood, and grass) (Rodionova et al., 2017). The use of total above-ground biomass for the manufacture of second-generation biofuel provides a better land use efficiency in comparison to first-generation ones. Moreover, reduced cost of raw material, as well as utilization of inedible lignocellulosic biomass (the woody part of plants), which does not compete with food, lend an advantage to second-generation biofuels (Havlík et al., 2011; Schenk et al., 2008). Non-edible components of corn or sugarcane, forest harvesting residues, agricultural residues, as well as wood processing waste such as leaves, straw or wood chips are used as sources of lignocellulosic material. Nevertheless, the technology which is used to transform lignocellulosic materials into sugars is costly and entails the use of special enzymes. This simply means that manufacturing second-generation biofuels on a commercial scale are not feasible currently (Brennan and Owende, 2010).

2.3. Third generation biofuels

Microalgal biomass is used to generate third generation biofuels. Microalgae are autotrophic life forms of aquatic environment e.g. cyanobacteria (Brennan and Owende, 2010; Lee and Lavoie, 2013). The growth yield of the microalgal biomass in comparison to traditional lignocellulosic biomass is very peculiar (Scott et al., 2010). In contrast to conventional crops, micro-algal biomass gives 15–300 times more oil for biodiesel production. The high energy index, minor cost, eco-friendly and completely inexhaustible feedstock are few of the prodigious benefits that make microalgae an exceptional source of biomass. It mainly reduces the stress on already decreasing water and land resources because of its ability to grow on undeveloped land and water which is not appropriate for food production. The most promising feature which enables algae to be used as third generation biofuel is their excessive oil content. Among all the species of green algae, *Chlorella vulgaris*, *Chamydomonas reinhardtii*, *Dunaliella salina* are the most utilized species for obtaining biofuels owning to their high oil content (around 60–70%) (Azad et al., 2014). With multiple advantages, it also has its pitfalls as the third generation biofuel technology is in its infancy. The major drawback is its high estimated cost and reliance on fossil fuels in production steps elevating the environmental concerns (Liang et al., 2009; Scott et al., 2010).

2.4. Fourth generation biofuels

The fourth-generation biofuels are manufactured by making use of modern techniques such as geo-synthesis or low pressure, advanced biochemistry, petroleum-hydro-processing and low-temperature electrochemical processes. These techniques use the captured carbon from the environment to produce fourth generation biofuels (Azad et al., 2015). The different authors have defined the fourth-generation biofuels in diverse ways. For example, Lü et al. (2011) has utilized metabolically engineered forms of microalgae to produce fourth generation

Table 1
Classification of biofuels on the basis of their feedstock along with their benefits and limitations.

Generation Type	Biofuel Type	Feedstock	Benefits	Limitations	References
Primary biofuels					
First generation	Fuel-wood, Landfill gas, Wood chips, Organic material etc.	Forest residues, Slash, Pellets, Wood chips, Crop residues etc.	Domestic energy source, Higher reliability, Better waste utilization, Reduce local pollution etc.	Technology cost, Production of value added co-products, Required storage facilities etc.	(Doshi et al., 2016; Nigam and Singh, 2011; Rodionova et al., 2017)
Secondary biofuels					
First generation	Bio-ethanol, Vegetable oils, Bio-diesel, Bio-syngas Bio-gas etc.	Rice, Wheat, Sugar, Edible vegetable oils etc.	Reliable, Locally distributed, Price stability, Less expensive, Nontoxic etc.	Tax credits on production, Most criticized, Emits CO_2, Less fertile soil etc.	(Azad et al., 2015; Hirani et al., 2018; Naik et al., 2010)
Second generation	Bio-diesel, Bio-alcohols Bio-oil, Bio-DMF, Bio-hydrogen, Bio Fischer-Tropsch diesel, Wood diesel etc.	Non-food crops, Non-edible vegetable oil, Waste cooking oil, Animal fact, Wheat straw, Corn, Wood waste, Solid waste, Energy crops etc.	Reduce CO_2 emission, Biodegradable, Better waste utilization, Reliable than first generation, Employment generation etc.	Expensive, Complex process, Increase NOX gases, Lower energy content, Stability concerns, Food-fuel competition etc.	(Doshi et al., 2016; Hirani et al., 2018; Scott et al., 2010)
Third generation	Bio-diesel, Vegetable oil, Bio-gas etc.	Non-food crops/algal starch	Relatively cheap, High biomass productivity, Low fouling, Easy maintenance, Less prone to contamination.	Scalability problem, Need maximum light exposure, Periodic cleaning, Temperature maintenance, High initial investment cost, High energy usage etc.	(Azad et al., 2015; Demirbas, 2011; Dragone et al., 2010)
Fourth generation	Bio-hydrogen, Bio-methanol, Bio-electric current etc.	Algae, Solar energy	Reliable, Environment friendly, Relatively cheap.	Land requirement for cultivation, High initial cost for solar panel, Power outputs can be variable some areas so large no. of battery banks must be required, Require good solar exposure etc.	(Azad et al., 2015; Cheng et al., 2011; Rizwan et al., 2016)

biofuel. This concept has been applied to manufacture inexhaustible fourth generation biofuels by using chemical processes (Cheng et al., 2011). While Demirbas (2009) defines fourth generation biofuel as the changeover of biodiesel and vegetable oil into biogasoline by utilizing the advanced technologies. Unlike fourth generation biofuels, the second and third generation biomass feedstocks take up CO_2 while cultivating and transforming it to fuel. Two processes have been cited in the literature for the manufacturing of fourth generation biofuels. In the first method, the carbon that is available by industry emitted CO_2, is captured into the water with the electrochemical process and is used to manufacture liquid methanol. Electricity and heat energy can be manufactured by using the inexhaustible energy sources like wind, solar, geothermal, hydro etc. (Azad et al., 2015). A sequence of electrolytic cracking and catalytic production is performed in these processes which guides to a low pressure and temperature electrochemical manufacturing process. Liquid inexhaustible methanol is a cost-efficient, eco-friendly fuel which can be mixed with biodiesel and aviation spirit. In the second method, old oil and gas fields or saline aquifers are used to geosequester the captured CO_2. This carbon captured in fourth generation biofuel manufacturing locks more carbon than it releases making it carbon negative rather than neutral. It has been reported that this method also decreases CO_2 emissions by replacing fossil fuels by capturing the CO_2 emissions from the environment.

3. Integrated processes for the bioconversion of raw material into biofuels

The bioconversion of feedstocks into biofuels is accomplished in three steps *viz.* pretreatment, hydrolysis, and fermentation. The most important, complicated and costly step in the conversion of biomass into biofuel is the pretreatment. The four types of pretreatment processes namely physical, physiochemical, solvent and biological are employed most commonly for the disintegration of cell walls to expose the cellulose and hemicellulose for downstream processing. Generally, all pretreatment strategies are applied in a synergistic manner for better efficiency (Alvira et al., 2010; Mood et al., 2013). The pretreatment is followed by acidic or enzymatic hydrolysis of the feedstock. The hydrolysis process is used to convert the polysaccharides present in the raw material into fermentable sugars (Sun and Cheng, 2002). Finally, microbial fermentation is employed to convert monomeric sugars like glucose, galactose, and mannose into ethanol or other alcohols (Liu et al., 2015a, b).

For the production of biofuels; four process configurations have been developed (Fig. 1) namely, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP) (Devarapalli and Atiyeh, 2015).

3.1. The separate hydrolysis and fermentation

This technique involves two stages such as saccharification (hydrolysis) and fermentation of hydrolyzed substrate. In separate hydrolysis and fermentation (SHF), enzymatic hydrolysis and fermentation are performed separately under the optimal conditions. This approach has many features like each step is carried out under optimized environment and the interaction between saccharification and fermentation is negligible (Saini et al., 2015). The SHF results in the complete breakdown of cellulose into monomeric sugars. The optimal temperature for cellulases mediated hydrolysis and fermentation is 50 °C and 35 °C, respectively (Zhao et al., 2011).

The disadvantages related to this approach are end-product inhibition of enzyme's activity and contamination (das Neves et al., 2007; Galbe and Zacchi, 2002; Sarkar et al., 2012). The continuous addition of hydrolytic enzymes may serve as one of the solutions to cope with enzyme inhibition but the process will no longer remain cost efficient if the expensive enzymes are used (Zhao et al., 2011). These drawbacks of SHF process opened the way for the establishment of a second integrated technique called SSF.

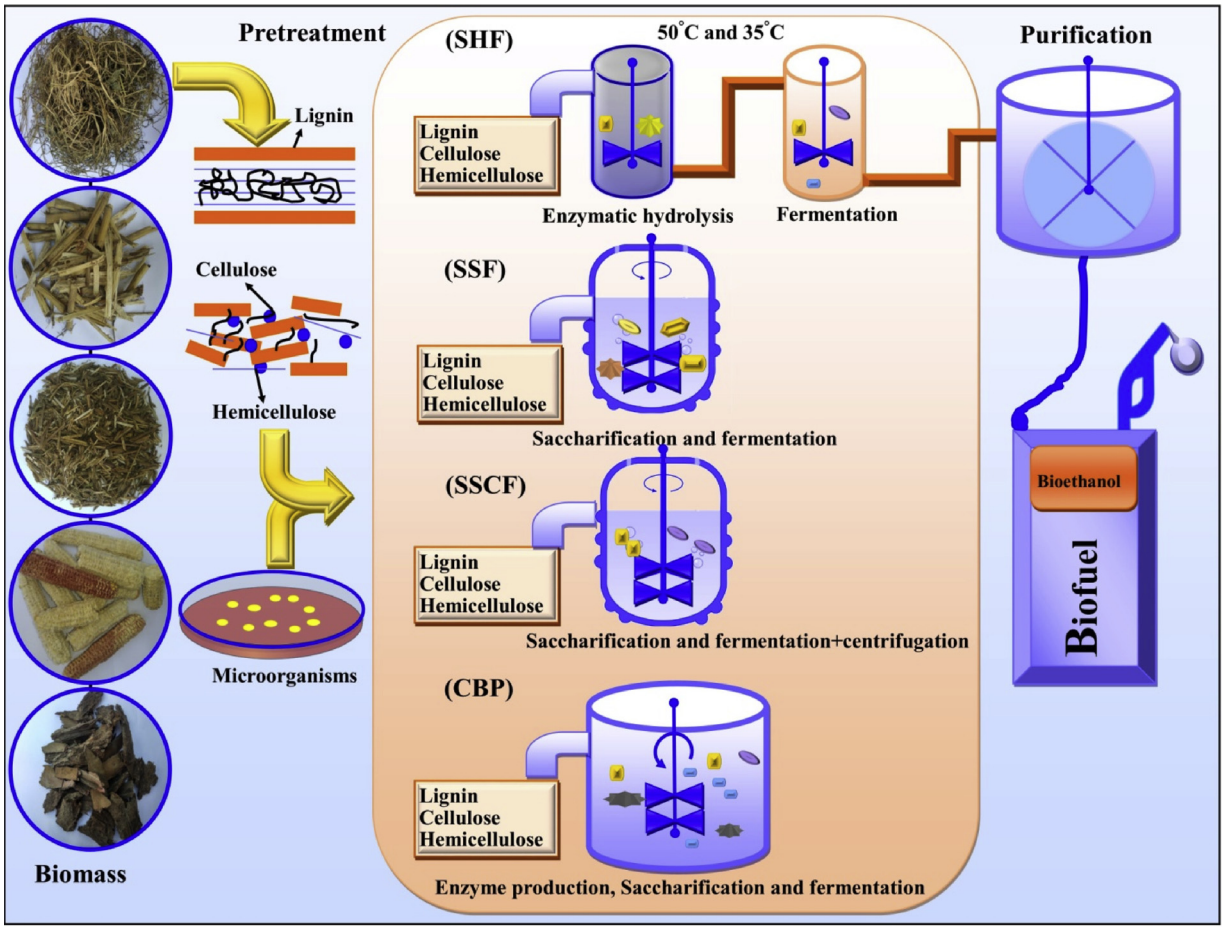

Fig. 1. Schematic representation of integrated processes for bioconversion of raw material into biofuels.
Where, SHF stands for separate hydrolysis and fermentation; SSF for simultaneous saccharification and fermentation; SSCF for simultaneous saccharification and co-fermentation; CBP for consolidated bioprocessing.

3.2. The simultaneous saccharification and fermentation

In this technique, both saccharification and fermentation are performed synergistically in a single vessel. In simultaneous saccharification and fermentation (SSF), monomeric sugars, resulted from enzymatic hydrolysis process, are immediately converted into ethanol through fermentation and therefore avoids the associated complications including sugar accumulation, enzyme activity retardation and contamination (Hahn-Hägerdal et al., 2006). Furthermore, SSF is cost-effective than SHF due to the requirement of a single reactor for both stages (Kádár et al., 2004). In SSF, the hydrolysis of starch-containing raw material is carried out by the treatment with an endoenzyme (i.e. amylase) at 90–110 °C followed by the treatment with an exoenzyme (i.e. glucoamylase) at 62 °C for 30 min. The amylase hydrolyzes starch into dextrins, while glucoamylase converts dextrins into glucose. Then, the fermentation of hexose sugars is carried out at a lower temperature (30–32 °C) for biofuel production. This technique has been extensively employed for bioethanol production (Öhgren et al., 2007).

The requirement of different optimum temperatures for hydrolysis and fermentation is the major limitation of SSF (Kádár et al., 2004). This SSF limitation can be overcomed by using high-temperature tolerant microorganisms e.g., *Kluyveromyces marxianus* during saccharification process (Bjerre et al., 1996). Another drawback related to this approach is that glucose fermenting microbes cannot convert the hexoses present in the hydrolysate into biofuel (Lin and Tanaka, 2006).

3.3. The simultaneous saccharification and co-fermentation

In simultaneous saccharification and co-fermentation (SSCF) technique, five and six carbon sugars can be fermented simultaneously, however, the primary requirement in this approach is the utilization of compatible co-fermenting microbes that can work efficiently on operating temperature and pH (das Neves et al., 2007). It is difficult to find a single potential microbe having the ability to ferment hexose as well as pentose sugar. Moreover, another limitation of this process is the dearth of ideal co-fermenting microbial strains for biofuel production on a commercial scale (Talebnia et al., 2010).

The mixed-culture approach utilizing the mixture of both C6-fermenting and C5-fermenting microbes is proved as an effective strategy for the complete conversion of monomeric sugars (produced from feedstock hydrolysis) into biofuel. It has been reported that the efficient microbes for SSCF process are *S. cerevisiae* and *Candida shehatae* known for synergistic action (das Neves et al., 2007).

3.4. The consolidated bioprocessing

Another technique of process configuration is consolidated bioprocessing (CBP) in which the saccharification and fermentation processes are accomplished by a single microbe (Lynd et al., 2008). All stages of bioconversion (i.e. enzymatic hydrolysis and fermentation) are performed in a single reactor in a sequential manner. The CBP process requires low capital investment making it cost efficient technique (Kang et al., 2014; Zhao et al., 2011). Bacterial species such as *C. thermocellum* and several fungal species such as *Fusarium oxysporum, Neurospora*

crassa, and *Paecilomyces* sp. have been reported to show such activities (Sethi and Scharf, 2013).

The CBP technique has limitations of poor biofuel yields and the long-time requirement for the completion of fermentation reactions (e.g. 3–12 days), which makes the process inefficient (Szczodrak and Fiedurek, 1996).

4. Role of microbes in biofuel production

The microorganisms of various groups such as bacteria, yeast, and filamentous fungi play a pivotal role in the production of biofuels. All major stages of bioconversion including pretreatment, hydrolysis, and fermentation involve potential microbial strains for efficient biofuel production.

4.1. Role in the pretreatment

Microbe-mediated pretreatment of feedstock results in the disintegration of structural components and expose the chemical constituents (i.e. celluloses and hemicellulases) for downstream processing (Chandel et al., 2013; Zabed et al., 2016). For this purpose, **white** rot (such as *Pleurotus florida*, *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*) and soft rot fungi (like *Trichoderma reesei)* (Zabed et al., 2016) are most commonly used because they are equipped with extensive enzyme system for feedstock disintegration. The enzyme system includes lignin peroxidases, laccases and manganese peroxidases (Zhao et al., 2011).

4.2. Role in the hydrolysis

Generally, the hydrolysis of biomass into their monomeric sugars is carried out through acidic or enzymatic hydrolysis. The latter, due to low energy requirement is preferred over chemical process (Ferreira et al., 2009). In enzymatic hydrolysis of various microbe mediated enzymes (such as cellulases, hemicellulases and many more) are utilized for hydrolysate production for fermentation process (Sun and Cheng, 2002). Various bacterial and fungal genera have been reported to produce these hydrolytic enzymes. Several potential bacterial species of *Clostridium*, *Bacillus*, *Cellulomonas*, *Ruminococcus*, *Bacteroides*, *Erwinia*, *Thermomonospora*, *Acetovibrio*, *Streptomyces* and *Microbispora* genera have been reported for hydrolytic enzyme production. On the other hand, potential fungal genera reported for hydrolytic enzyme production include *Trichoderma*, *Schizophyllum, Sclerotium, Aspergillus, Fusarium, Humicola, Schizophillum* and *Penicillium* (Balat, 2011; Gupta and Verma, 2015).

4.3. Role in the fermentation

Similarly, various microorganisms including bacteria, yeast, and filamentous fungi have the potential to convert monomeric sugars into ethanol under anaerobic environment. It has been reported that different microorganisms have the varied potential for ethanol production and only a few microbial strains with high ethanol production potential are utilized on a commercial scale. The yeast *S. cerevisiae* is most commonly employed for ethanol production on an industrial scale due to its robust and suited nature (Galbe and Zacchi, 2002; Sarris and Papanikolaou, 2016). Various bacterial species have also been investigated for ethanol fermentation on a commercial level including a gram-negative bacterium *Z. mobilis* (Sulfahri et al., 2016) and numerous species of anaerobic thermophilic bacteria like *C. thermohydrosulfuricum*, *C. thermosaccharolyticum*, *T. mathranii*, *T. ethanolicus* and *Thermoanaerobium brockii* (Balat, 2011). Filamentous fungi are the poor producer of ethanol and cannot survive under high ethanol concentrations (Zabed et al., 2016).

5. Genome modification: the new revolution in genomics

Genome modification is an efficient tool to introduce desirable characters into a single organism. This process alters the native genome in a very precise manner to change the physiological characters of an individual microbe (Singhania et al., 2017; Ulaganathan et al., 2017) for the enhanced production of a particular metabolite (Singhania et al., 2017). By using this approach, a gene can either be introduced, deleted and up or down regulated at a specific site within an organism. In contrast to conventional genetic engineering, this technique did not include conventional gene isolation, *in vitro* engineering and then retransfer to host cell to change the physiological properties of that individual (Ulaganathan et al., 2017). Genome engineering can be performed in two ways: (i) REM engineering (ii) MEM engineering. The CRISPR)/CRISPR associated protein 9 (Cas9) system mediated genome engineering belongs to REM (Horvath and Barrangou, 2010; Wiedenheft et al., 2012), whereas MEM technique includes transcription activator-like effector nucleases (TALENs) system (Miller et al., 2011), zinc finger nucleases (ZFNs) system (Bibikova et al., 2003; Kim et al., 2011) and meganucleases system (Smith et al., 2006) of genome engineering.

All these genome engineering techniques revolutionized the biological investigations and related research fields. The ZFNs and TALENs are limited due to the unavailability of efficient delivery systems, off-targeted effects, toxicity and low efficiency, whereas, CRISPR/Cas9 system has emerged as a promising tool to address the limitations of ZFNs and TALENs (Gaj et al., 2013).

6. CRISPR/Cas9 system – an overview

The CRISPR/Cas9 system which is originally a prokaryotic defense system against phage attack has been employed as an influential instrument for site-directed mutagenesis (Zaidi et al., 2017). The CRISPR contains small repeated sequences of DNA flanked by small segments of spacer DNA. Spacer DNA is of a bacteriophage or plasmid origin and is integrated into bacterial genome because of its encounter with a bacterial virus or plasmid. The Cas are the CRISPR associated genes which translate into nuclease or helicase proteins with the task of cutting or unwinding DNA (Rodriguez, 2016; Schaeffer and Nakata, 2015). In prokaryotes, CRISPR system operates by integrating phage or plasmid DNA sequences into its own genome and whenever it encounters that phage again, the system identifies it using the transcribed RNA sequences and directs a Cas enzyme for cleaving the DNA at a targeted position. The Cas9 is a cutting enzyme which has the ability of cleaving the DNA at two active cutting sites on each strand of the DNA double helix. It was initially isolated from bacterium *Streptococcus pyogenes* (Louwen et al., 2014).

The two scientists Doudna and Charpentier have done the major work in discovering the mechanism of CRISPR/Cas9 system. They reported that bacteria counter the phage attack by transcribing spacer sequences and palindromic repeats into a long RNA molecule which is cleaved into pieces (called crRNAs) with the help of trans-activating RNA (tracrRNA) and protein Cas9 (Niewoehner et al., 2014). Subsequently, it was reported that a single guide RNA (sgRNA) can be established with the combination of tracrRNA and crRNA which can ultimately be organized into a powerful tool for targeting and cleaving specific DNA sequences with the help of Cas9 nuclease. Consequently, it provides the ability of gene editing because crRNA carries a sequence which pairs with tracrRNA and develops a hairpin loop-like structure enabling the Cas9 enzyme to cleave DNA sequence by recognizing crRNA as a guide. The cleaved DNA is repaired either by Non-homologous end-joining (NHEJ) or homology-directed repair (HDR). For this purpose, a DNA repair template is used which inserts a specific DNA sequence for the desired outcome (Louwen et al., 2014; Wright et al., 2016). Usually, a plasmid or a virus-based vector is applied to transfect target cells with CRISPR/Cas9 system. The sgRNA is programmed in

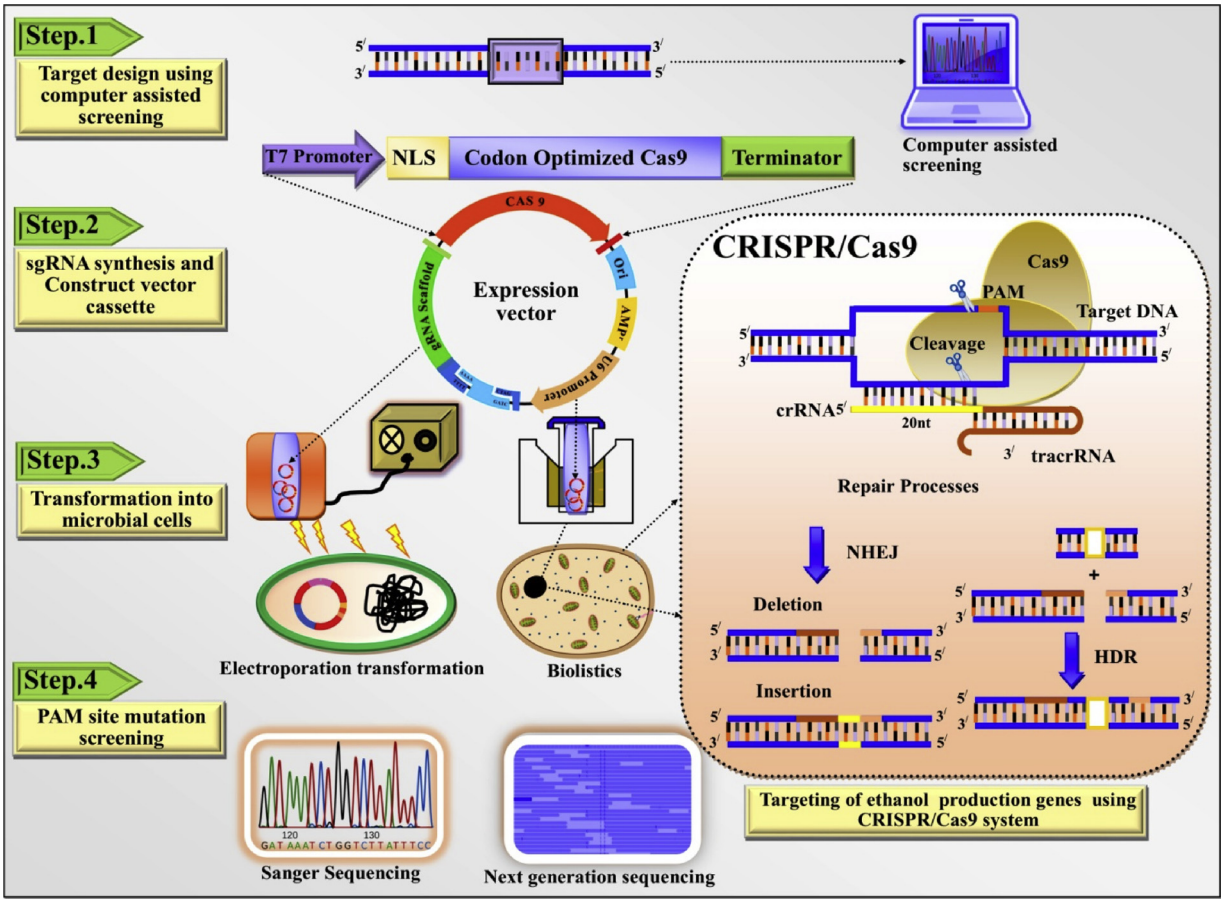

Fig. 2. Schematic representation of the CRISPR/Cas9 system of genome editing in microbial cells for enhanced biofuel production.
The Cas9 and the targeted genomic sequence guided by the sgRNA are delivered into the microbial cells either by electroporation, biolistic gene gun or heat shock transformation. The cassette expressing Cas9 is driven by the T7 promoter, while gRNA scaffold is driven by the U6 promoter. Cas9 stimulates DNA strand separation and allows a sgRNA to hybridize with a specific sequence in the targeted gene for targeted DNA cleavage. The CRISPR/Cas9 system positions the target DNA into the active site of Cas9 in proper orientation in relation to a PAM binding site and allows separate nuclease domains of Cas9 to independently cleave each strand of the target DNA sequence 3 bp upstream of the PAM site. The double-stranded DNA disintegrates and then undergoes either error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR) DNA repair. Approximately one in three deletions or insertions that restore the proper reading frame in the gene's coding region can restore gene activity. Real time PCR, sanger sequencing and next generation sequencing can be used to determine the expression profile and nature of mutations within the targeted gene.

such a way that Cas9 cleaves the genome only at a desired location. The DNA repair template is also developed in a way to overlap with the sequences on either side of the cleaved position and code for the desired function (Fig. 2). Up till now this innovative tool of site-directed mutagenesis has been applied in a wide range of basic and applied research. It can be harnessed as a revolutionary methodology for genome editing of microbial cells for enhanced biofuel production. Various successful studies have been conducted to engineer microbes using CRISPR/Cas9 technology for the improved characteristics like b-cyclodextrin glycosyltransferase production and resistance to spore formation in *Bacillus subtilis*. Similarly, *S. cerevisiae*'s genome was altered to increase mevalonate or (R-R)-2,3-butanediol production and xylose utilization. With each passing day, improved CRISPR/Cas9 protocols are being published and we expect this technique to be progressively more ingrained in daily laboratory practices (Vervoort et al., 2017). Table 2 summarizes the CRISPR/Cas9 mediated genome editing in diverse microbial groups.

7. CRISPR/Cas9-mediated genome engineering of microbial cells for enhanced biofuel production

Researchers are optimistic in harnessing the full potential of microbial cells for enhanced biofuel production with the help of new groundbreaking technologies in next 5–10 years (Stephanopoulos, 2007; Ulaganathan et al., 2017). The metabolic performance of the microbial cells needs to be improved by CRISPR/Cas9-mediated site-directed mutagenesis to accomplish these goals. Several reports have emerged recently on the application of CRISPR/Cas9-mediated genome engineering of microbial cells for enhanced production of biofuels (Gao et al., 2015; Nymark et al., 2016; Voloshin et al., 2016) and have been summarized here (Table 3).

7.1. Genome engineering for enhancing the biofuel tolerance

In many microorganisms, metabolic pathways can be affected by the end products as many biofuel products have antimicrobial activities. Therefore, microorganisms require genome editing to possess enhanced tolerance to the end products (Dunlop, 2011). Many *S. cerevisiae*, *Z. mobilis,* and *Escherichia coli* strains exhibit disparity in their tolerance to biofuels. At the same time the ethanol tolerance is a complicated feature which requires several pathways and networks to be understood. Genes corresponding to ethanol tolerance have been identified in *S. cerevisiae* by performing several functional, comparative, transcript profiling and genomic analyses (Lewis et al., 2010; Ma et al., 2010). Targeted as well as random mutagenesis has pinpointed various genes in which the mutation resulted in either increased or decreased ethanol

Table 2
A brief summary of CRISPR/Cas9-based genome editing in diverse microorganisms.

Microorganism(s)	Targeted Gene(s)	Promoter(s) for Cas9	Promoter(s) for sgRNA	Transformation method	Results obtained	References
Prokaryotes						
Escherichia coli	gfp-mut2	inducible promoter	constitutive promoter	Electroporation mediated transformation	The use of CRISPR arrays (generating multiple crRNA guides) affect many genes at the same time and will allow genetic network organization.	(Bikard et al., 2013)
Staphylococcus aureus	egfp-pest reporter	T7 promoter	U6 promoter	Electroporation mediated transformation	By modifying the PAM recognition site, SaCas9 targeting range can be increased by two to four folds.	(Kleinstiver et al., 2015a)
Clostridium beijerinckii	spo0A	spoIIE promoter	spo0A promoter	Electroporation mediated transformation	Highly efficient markerless gene deletion from the bacterial chromosome can be achieved through a single-step transformation.	(Gao et al., 2015)
Streptomyces coelicolor	actIORF1 (SCO5087) and actVB (SCO5092)	tipA promoter	ermE* promoter	calcium chloride transformation Conjugation	Highly efficient CRISPR-Cas9 system can be used to delete genes or gene clusters, implement precise gene replacements and reversibly control gene expressions.	(Tong et al., 2015)
Streptococcus pneumoniae and *E. coli*	cas1, cas2 and csn2	—	—	Electroporation mediated transformation	The approach relies on dual RNA: Cas9-directed cleavage at the targeted genomic site to kill un-mutated cells.	(Jiang et al., 2015)
C. beijerinckii	spo0A	spoIIE promoter	Srna promoter	Electroporation mediated transformation	Combining inducible expression of Cas9 and plasmid-borne editing templates; efficient gene deletions and integrations can be achieved.	(Wang et al., 2016)
E. coli	polB, dinB, and umuD	T7 promoter	U6 promoter	Electroporation mediated transformation	Stability of microbial host genomes through shuffling between a mobile genetic element-free strain and the target cells using CRISPR-Cas assisted MAGE has been achieved.	(Umenhoffer et al., 2017)
Eukaryotes						
Saccharomyces cerevisiae	LYP1 and CAN1	Gal-L promoter and TEF1 promoter	SNR52 promoter	Electroporation mediated transformation	Targeted double-strand breaks can increase homologous recombination rates of single- and double stranded oligonucleotide donors by 5-fold and 130-fold.	(DiCarlo et al., 2013)
S. cerevisiae	ACS1, ACS2	TEF1 promoter	SNR52 promoter	Electroporation mediated transformation	Multiple genetic modifications in a single transformation step with high efficiency can be achieved.	(Mans et al., 2015)
Phaeodactylum tricornutum	CpSRP54	LHCF2 promoter	U6 promoter	Biolistic-mediated transformation	Genome editing based on CRISPR-Cas9 and mutant selection based on high resolution melting PCR assays have been developed for diatoms.	(Nymark et al., 2016)
Chlamydomonas reinhardtii	MAA7, CpSRP43 and ChlM	beta2-tub promoter	PsaD promoter	Electroporation mediated transformation	Cas9-RNPs help to avoid cytotoxicity and off-target effects in induced knockout and knock-in mutations within microalgae.	(Voloshin et al., 2016)
Phytophthora sojae	GFP	Ham34 promoter	RPL41 promoter	DNA-PEG-calcium transformation	Single guide RNA (sgRNA) based gene replacement and mutant-screening strategies have been established in filamentous-fungus like oomycetes.	(Fang et al., 2017)

Table 3
Gene/protein targets for enhancing biofuel tolerance, inhibitor tolerance, thermotolerance and outcomes of cellulase / hemicellulase specificities by genome engineering.

Microorganisms	Target genes/proteins	Modifications	References
Escherichia coli	cAMP Receptor Protein	Enhanced biofuel tolerance	(Basak and Jiang, 2012)
E. coli	cAMP Receptor Protein	Oxidative stress tolerance	(Chong et al., 2013)
Zymomonas mobilis	NADH dehydrogenase	Increase biofuel tolerance and production	(Kalnenieks et al., 2008)
Saccharomyces cerevisiae Tn 4	RMD6	Enhanced ethanol tolerance	(Cho et al., 2011)
S. cerevisiae	MSN2	Enhanced ethanol tolerance	(Cho et al., 2011)
S. cerevisiae Tn5	CAR1	Enhanced ethanol tolerance	(Shima et al., 2003)
S. cerevisiae CEN.PK113–7D	ASG1, GIS4, and SAC6	Improved constitutive acetic acid tolerance	(González-Ramos et al., 2016)
Z. mobilis	RpoD	Enhanced furfural tolerance and should improve cell growth	(Sahu and Prasad, 2015)
S. cerevisiae	SSK2	Enhanced furfural tolerance	(Park et al., 2012)
Z. mobilis	NADH dehydrogenase, Cytochrome bd type ubiquinol oxidase and ubiquinol peroxidase	Increased thermotolerance and biofuel production	(Notarnicola et al., 2012)
S. cerevisiae Tn2	SSK2/Mapkinase kinase and PAM1	Enhanced biofuels production	(Cho et al., 2011)
S. cerevisiae	CDC19/pyruvate kinase	Increased pyruvate kinase activity	(Benjaphokee et al., 2012)
S. cerevisiae	C-5 sterol desaturase	Conversion of ergosterol to biofuel	(Caspeta et al., 2014)
Acidothermus cellulolyticus	Cel5A	Altered product specificity and reduced product inhibition	(Chong et al., 2013)
Clostridium cellulovorans	Endoglucanase (EngB)	Improved thermostability of the gene	(Murashima and Kosugi, 2002)
Bacillus sp. strain KSM-64	Endo-1,4-β-glucanase	Improved thermostability of the ezyme	(Ozawa et al., 2001)
Trichoderma reesei	Cel 7A	Decreased product inhibition of enzyme	(Cho et al., 2011)
Thermobifida fusca	Cel 6B	Enhanced resistance to cellobiose	(Zhang et al., 2000)
Streptomyces sp. *strain* S9	XynAS9	Improved thermostability of the gene	(Xing et al., 2014)
T. reesei	BD-11 (GH11 xylanase)	Improved thermostability of the gene	(Hokanson et al., 2011)
Aspergillus usamii	AuXyn11A	Improved thermostability of enzyme	(Couturier et al., 2013)
A. niger	Xyn10 A	Improved thermostability of enzyme	(Guo et al., 2015)

tolerance e.g. The NADH dehydrogenase enzyme is located in the mitochondrial membrane of *Z. mobilis* and it functions in catalyzing the transfer of electrons from NADH towards coenzyme Q (CoQ). A frameshift mutation in this gene (generating a stop codon at codon 41) has resulted in the enhanced ethanol tolerance and its production (Ulaganathan et al., 2017). The shift of the NADH flux from respiration towards ethanol production was identified to be associated with mutation stimulated aerobic growth (Kalnenieks et al., 2008). In *C. thermocellum*, the ethanol tolerance was increased by the point mutations which resulted in the replacement of specific amino acids at particular sites of alcohol dehydrogenase enzyme. This increase in the tolerance is attributed to the change in co-factor requisition by alcohol dehydrogenase enzymes (Brown et al., 2011).

Additionally, several methods have been devised to minimize the antimicrobial toxicity of biofuels. Activation of stress responsive genes, the heterologous expression of efflux pumps or heat shock proteins and the modification of membrane proteins are few of them (Dunlop, 2011; Mukhopadhyay, 2015). Synthesis, transport, and the correct folding of proteins are the major functions of the heat shock proteins. Up-regulation of heat shock proteins plays a major part in averting protein aggregation under stress response. According to Rutherford and coworkers, mutated genes (*rpoH, dnaJ, htpG,* and *ibpAB*) related to heat shock and protein misfolding were identified during the transcriptional profiling of *E. coli* cells, when exposed to butanol (Rutherford et al., 2010). Underexposure to butanol, variations in their cell wall composition of microorganisms were observed that prevented the butanol entry by using cis-trans isomerase, the enzyme which enables microorganisms to convert their cis fatty acids into trans fatty acids. Modifications in the ratio of trans to cis fatty acids can alter the fluidness of the membrane, and thus influence its accessibility and solvent tolerance (Bhatia et al., 2017). The above mentioned genome modifications can be carried out with greater efficiency and flexibility by using CRISPR/Cas9 genome editing technique.

7.2. Genome engineering for enhancing the inhibitor tolerance

The main target of genome editing is to increase the tolerance of microorganisms to highly toxic compounds like fermentation process inhibitors and specific growth inhibitors (Tkalec et al., 2014). A considerable amount of cellulolytic enzymes and the microbial growth inhibitors are formed during fermentation due to pre-treatment process of lignocellulosic biomass with physio-chemical methods, ultimately resulting in low ethanol yields (Jönsson et al., 2013). These inhibitors can be classified into three different types: (a) organic acids/weak acids, (b) furan derivatives and (c) phenolic compounds (Pandey, 2011). The increase in the bioethanol production is dependent mainly on the increase in the tolerance of *S. cerevisiae* and other potential ethanogenic microorganisms to the inhibitors. For example, the constitutive acetic acid tolerance of *S. cerevisiae* was increased by single amino acid replacement in four different genes at various locations e.g. ADH3 (G416 T, T966 G, T201 A), *SKS1* (G821 T, C617 A), *GIS4* (G1322C, G295 A) and *ASG1* (G1248 A, G1248 T, A1979 G, G2881C) (González-Ramos et al., 2016). Likewise, the furfural tolerance of *Z. mobilis* was increased by single amino acid replacements at various sites including P195 T, V355 M, K219E, V522 G, Q649 L, G14C, L18 P, E145 G, L361 F, P511 T, L606 in RpoD protein. Moreover, it was observed that the furfural tolerance of *S. cerevisiae* can be increased by the disruption of *SSK2* gene with transposon insertion (Ulaganathan et al., 2017). Hence, the CRISPR/Cas9 can be a viable tool for the genome editing of microbial enzymes in order to improve the inhibitor tolerance for enhanced biofuel production.

7.3. Genome engineering for developing thermotolerance

Several constraints limit the use of thermophilic microorganisms in industrial lignocellulosic bioethanol production despite their availability in a considerable number. The biotransformation of LCB into bioethanol carried out smoothly with greater efficiency and flexibility by using thermophilic microorganisms due to variety of features (such

as reduced production cost, structural changes in cellulose at elevated temperature facilitating easy breakdown and increased stability and specific activity of enzymes). The genome modifications of themostable microbes for aforementioned traits are highly laborious in contrast to editing microbial strains that are already applied in bioethanol production for improving thermostability (Ulaganathan et al., 2017). Thermotolerance is a complicated feature that involves multiple genes. The bioethanol production usually involves high temperature which in turn results in the growth reduction of ethanol-producing microorganisms. Accumulation of trehalose and heat shock proteins have been found to be associated with increased thermotolerance (Ulaganathan et al., 2017). Improved thermotolerance and increased ethanol production in *Z. mobilis* and *S. cerevisiae* have been achieved due to single amino acid modifications in the pyruvate kinase and NADH dehydrogenase (Benjaphokee et al., 2012; Caspeta et al., 2014). Similarly, better thermostability was observed in *S. cerevisiae* when the gene of Dfg5 glycosyl phosphatidylinositol-anchored membrane protein was deleted (Nasution et al., 2015). The modification of proteins involved in thermo-resistance can also be performed through CRISPR/Cas9 by employing the potential laboratory and environment microorganisms.

7.4. Genome engineering for modifying cellulases and hemicellulases

Specificities of cellulases can be changed by specific alterations of amino acids using genome editing tools. Previously, these modifications were difficult to be carried out because of the problems linked to protein engineering methods. Additionally, such modifications were carried out in laboratory strains and it was an onerous job to reproduce them in bioethanol producing industrial strains. Recombination-based genome engineering can facilitate the precise modifications in the genetic material of microorganisms. These methods can be applied for bioethanol production to modify the specificity of cellulases (Ulaganathan et al., 2017). A modification in one or more amino acids of cellulases increased the heat tolerance in microorganisms such as *C. cellulovorans* (E116D and V192 A), *Bacillus* sp. strain KSM-64 (N179 K and D194 K), *Humicola insolens* (C313S), *C. phytofermentans* (N144I, N291 K, E158 V andV245 G), *C. thermocellum* (S329 G) and *Melanocarpus albomyces* (S290 T, G4C/M70C/S290 T) (Chokhawala et al., 2015; Ozawa et al., 2001).

Hemicellulases which also take part in cell wall composition can be utilized for ethanol production since they contain a considerable amount of pentose sugars (Dumon et al., 2012; Subramaniyan and Prema, 2002). The most familiar enzyme among hemicellulases is Endo-1, 4-β-xylanase which carries out the random breakdown of the β-1, 4-xylosidic links in xylan producing xylo-oligosaccharides. Earlier reports of protein engineering have demonstrated that amino acid modifications (e.g. Y111 T; N148D; S15 L; A10 V; V224 A; S27 T; G133D; M93 V) at particular locations can change the specificity of xylanase enzymes (Díaz et al., 2004; Gallardo et al., 2010). Amino acid substitutions in microbial xylanases like from *Aspergillus usamii*, *B. subtilis*, *A. niger* and *Yarrowia lipolytica* have increased their ability of thermotolerance. Similarly, specific amino acid modifications have been associated with enhanced xylanases activity from *Thermomyces lanuginosus* and *Geobacillus stearothermophilus* (Ulaganathan et al., 2017). The alteration in hydrolytic enzymes for desired characteristics can be done by using CRISPR/cas9 based genome editing techniques in more effective and better way.

8. Current status of research and future perspectives

Effective strategies are needed to cope with the current issue of global energy demand (Voloshin et al., 2016). Currently, researches are directed towards finding an optimal solution to enhance the efficiency of lignocellulosic-based biofuels with the minimal energy requirements and water consumption. Future studies must consider the optimization of each step involved in the production of biofuels including pretreatment, hydrolysis, fermentation, and purification. Pretreatment is the costliest step and accounts for about 33% of the total cost. The production of genetically modified microbes with the desired fermentative and cellulolytic properties and the use of enhanced co-cultures are required to increase the product yield under stressful conditions (Jiang et al., 2018).

A great deal of research efforts has been made during the past few years for the development of efficient bioconversion strategies for biofuel production. Various process conformations such as SHF, SSF, SSCF, and CBP have been developed for the commercial production of biofuels (Saini et al., 2015) which are considered to be economical and have the potential to reduce the end product inhibition and operation numbers. Major stages of all these strategies are derived from potential microbial species (Zabed et al., 2016). There are certain limitations associated with every technique affecting the biofuel production. These limitations need to be overcome with optimal efficacy for increasing the yield and quality of biofuels (Saini et al., 2015). Such limitations have also drawn the attention towards the use of genetically modified microorganisms to drive the bioconversion of huge quantities of low-cost raw materials into biofuels (döEspaux et al., 2015). Various reports on microbial genome modifications for enhanced biofuel production have been published (d'Espaux et al., 2015; De Bhowmick et al., 2015; Gonçalves and Simões, 2017; Ulaganathan et al., 2017).

Genome engineering of microorganisms may serve as one of the solutions to cope with the limitations of bioconversion approaches. Out of various genome engineering techniques such as ZFNs and TALENs, CRISPR/Cas9 has proved to be the most efficient in recent years for the manipulation of genes (Shin et al., 2016). The CRISPR/Cas9 has also emerged as the most efficient tool for genome editing in microbial cells for enhanced biofuel production and characteristics improvement (Bikard et al., 2013; Gao et al., 2015; Hsu et al., 2014; Kleinstiver et al., 2015b). Though, development of microbes with improved characteristics is required for efficient biofuel production, nevertheless, the development of an efficient process configuration is another major challenge that impedes the biofuel production on commercial scale. Hence, extensive research is required in future for the improvement of an optimized integrated process for the commercialization of biofuels. Moreover, the manipulation of CRISPR/cas9 system for enhanced biofuel tolerance, inhibitor tolerance and thermotolerance as well as modification in cellulose and hemicellulase should be key subjects of future research in terms of enhanced biofuel production.

9. Conclusion

Limited fossil fuel resources and increasing environmental concerns have influenced the scientific community to establish reliable systems for economical and sustainable production of an alternative source of fuels. The main focus of this review was to highlight the recent progress in CRISPR/Cas9-mediated genome engineering of microbial cells for possible boost in the efficiency of microbial cells for biofuel production. The most important goals of CRISPR/Cas9-mediated genome engineering may be redesigning of microbes for higher product concentration, enhanced inhibitor tolerance, modifying cellulases and hemicellulases, improved product yield, and product tolerance. It can be concluded that these combined techniques will result in the improvement of microbial cells capable of producing cost-efficient biofuels on industrial scale.

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