Minireview Article

Exploration of various microbial systems for the biofuel production

Abstract

Fuels on a broad scale are divided into two categories based on their renewability, i.e., renewable and non-renewable. The increasing scarcity, prices of fossil fuels, and the constantly increasing level of CO2 in the environment have led to an immediate need to investigate alternative fuels. Microorganisms are extensively being used for the generation of biofuels. The qualities of *E. coli* to be accessible to culture and maintenance have made it stand out among the microbes. Their ability to metabolize pentoses further draws it near to being used for biofuel production. Fermentative bacteria, particularly the class clostridia (obligate anaerobe), have been successful hydrogen producers. Dark fermentation employed by clostridia has dual advantages, i.e., production of biohydrogen and waste reduction since clostridia can utilize a broad range of substrates, including organic wastes. Moreover, the application of metabolic engineering can provide additional processes to clostridia required for the efficient production of biobutanol. The review further explores the two yeast systems, conventional and non-conventional systems. Synthetic biology tools have explored the traditional system, which comprises Saccharomyces cerevisiae for commercial purposes. The non-conventional yeast system has several advantages over the conventional ones, like ethanol tolerance, thermotolerance, inhibitor tolerance, and genetic diversity. However, the application of synthetic biology tools is still being explored in microbes like *E. coli*, Clostridia species, *Saccharomyces cerevisiae, and Yarrowia lipolytica*. The review also incorporates excellent commercial strain features like economical fuel tolerance, inhibitor tolerance, and thermotolerance control on redox balance and yield increases. The main focus is to bridge the gap between lab-scale production and commercialization.

Keywords: Biofuel, Clostridia, *E. coli*, Metabolic Engineering, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*

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1.0 Introduction

Human civilization has always been keen to explore and extract the most efficient outcome from a source. This propagated on shifting energy sources from manual to being dependent on the burning of fossil fuels. The Industrial revolution further triggered the dependence on fossil fuels.

On a global scale, 87% of total energy is derived from fossil fuels, further bifurcating it, 28% is derived from coal, 21% comes from natural gas, and 38% from oil. The ignition of fossil fuels poses a threat to the environment by releasing harmful gases such as CO, SO2, CO, CO2, and NOx. The most detrimental is the CO2, which traps solar heat in the atmosphere called the Green House Effect. The need to look for an alternative energy source even becomes more alarming as the world has limited fossil fuel energy resources. Even UN IPCC (United Nations Intergovernmental Panel on Climate Change) estimated the atmospheric temperature to rise between 1.1oC to 6.4oC in the next 100 years if no reformatory steps are taken into consideration [1,2].

Biofuel has become the need of the hour to combat environmental concerns. Biofuel is the liquid or gaseous fuel that is predominantly obtained from biomass. The liquid biofuel includes biodiesel, ethanol, Fischer-Tropsch diesel, and methanol. The gaseous biofuel comprises hydrogen and methane. Apart from being non-pollutant, biofuel is a sustainable, locally available, sustainable and reliable fuel [3]. The biofuel has low emissions as the biofuel's carbon content in question is from the carbon concealed in the growing biomass and thus released into the environment [4]. Due to public awareness regarding climate change and the policies that encourage to generation and utilize biofuel to reduce greenhouse emissions, there is a steadily increasing demand for biofuel globally, as depicted in figure 1 [5,6]. The rate of biofuel production is also on the rise at present (as shown in figure 2); however, the biofuel generation rate is not adequate to fulfill global biofuel demand. Thus, extensive research is being carried out to increase biofuel productivity and decrease biofuel costs. Synthetic biology and genetic engineering techniques are utilized to construct recombinant strains to synthesize biofuels with higher efficiency by using cost-effective and readily available substrates such as agricultural and industrial wastes [6,7,8].

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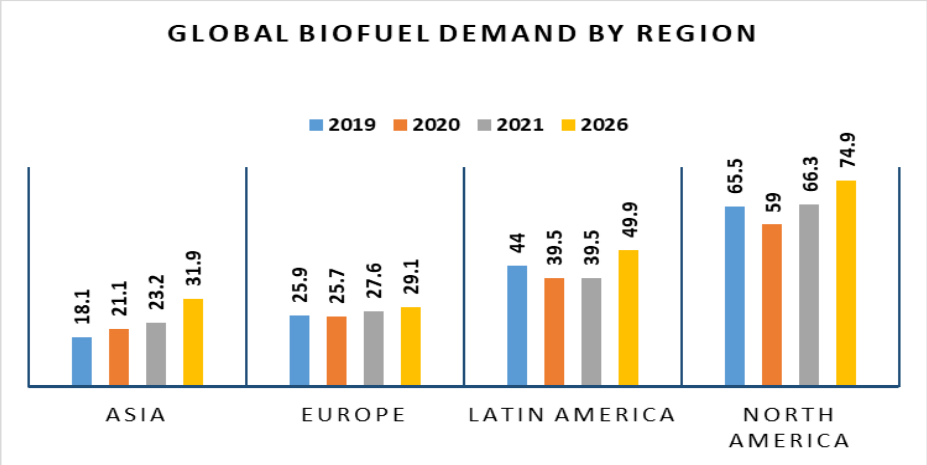

Figure 1: Global biofuel demand by region from 2019 to 2026 (expected)

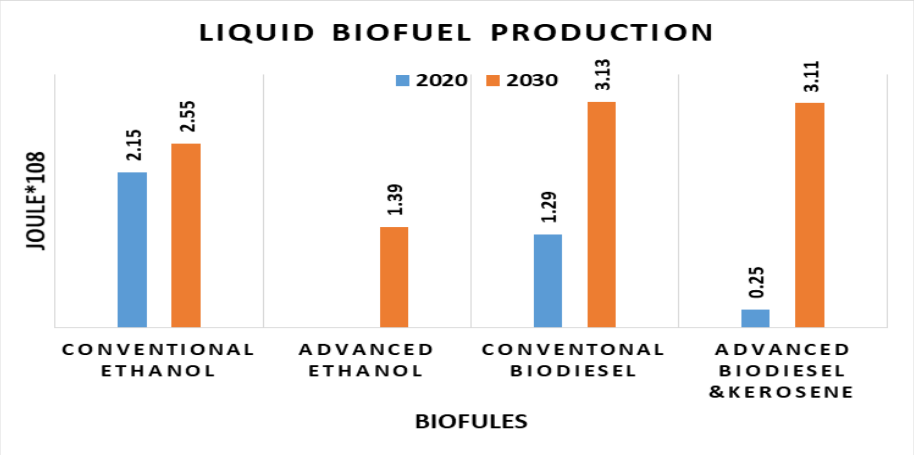

Figure 2: Liquid biofuel production in 2020 and 2030 (expected)

Synthetic biology is the combination of various biological components narrowed down from the enormous data derived from metabolomics, proteomics, transcriptomics, and fluxomics [9]. The spotlight of synthetic biology is the construction of biological systems, assembling novel biological regulon that governs gene expression in response to a specific input. This aspect is being explored in the industry to create cell factories that cause the efficient production of fuels and chemicals [10].

Synthetic biology progresses to write new genetic information, creating designed non-natural genes, proteins, and biological processes in the organism [11]. Synthetic biology cannot work in isolation. It

needs to be coupled with metabolic engineering and systems biology. After designing the synthetic pathway, the metabolite flux is maintained to allow an economically feasible process. The genotype-phenotype interaction also needs to be looked upon [10]. The ideologies remain a bit obstructed because of the constraints due to the pressure of a higher number of introns and the large size of some genes. Advances in DNA sequencing technologies can overcome it all [12].

The present review attempts to give a comprehensive account of the alternatives to non-renewable energy sources, mainly because of the growing demand coupled with the scarcity of fossil fuels. Furthermore, it provides an overview analysis of the different microbes that carry great significance for creating novel renewable fuel systems.

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2.0 Different forms of biofuel and their production pathways

Acetone, butanol, and ethanol (ABE) mixture can be synthesized from various starches and sugars through the process known as ABE fermentation. ABE fermentation is biphasic, i.e., it contains two different phases. Initially, acetic acid and butyric acid are generated in the acidogenesis phase, followed by the re-assimilation of these acids to synthesize solvents such as acetone, butanol, and ethanol in the ratio of 3:6:1. The ABE fermentation is usually carried out in the batch mode of fermentation as this mode of operation features effortless operation and lower cost and contamination risks [13,14]. ABE fermentation is primarily utilized to produce biobutanol from Clostridium, including *C. acetobutylicum and C. beijerinckii*. Previously, conventional substrates such as molasses, starch, and glucose were used to make biobutanol. As the cost of the substrate influences the biofuel price, the option to utilize cost-effective and readily available non-conventional substrates such as wastes and agricultural residues is being explored [14]. Likewise, the fermentative process that converts the substrate to biohydrogen is called dark fermentation. A diverse array of biochemical reactions

involving a wide range of bacteria represents this complex process. Fermentation is a metabolic activity in an anaerobic environment for ATP regeneration. During the dark fermentation process, obligate anaerobes such as Clostridia convert organic molecules (which contain mostly sugar) and yield ATP, NADH, and pyruvate by glycolysis. Mediated by pyruvate synthase, pyruvate is eventually converted to acetyl CoA and CO2, giving out reduced ferredoxin (Fd). In certain conditions, NADH created may be oxidized then the ferredoxin is reduced by ferredoxin reductase. Electron-bifurcating or Fd-dependent [FeFe] hydrogenase now utilizes this reduced ferredoxin to reduce protons that, in turn, emit hydrogen. The acetyl CoA, in the end, is changed into acetate or butyrate with the aid of various metabolic pathways [15,16].

Butanol-acetone (*C. acetobutylicum*): Hexoses → butanol + acetate + acetone + ethanol + CO_2 + H_2

Some of the major substrates utilized for biofuel generation and their production strategies are provided in table 1.

S. No	Biofuel	Form	Substrates	Production strategy	References
1	Biogas	Gaseous	Lignocellulosic Biomass	Acid-base hydrolysis, Biological degradation, Anaerobic Digestion	[17,18]
2	Bio-hydrogen	Gaseous	Agricultural waste, Food waste, wastewater	Microbial decomposition, Dark-fermentation, Photo-fermentation(Continuous mode)	[19]
3	Biodiesel	Liquid	Vegetable oils (edible & non-edible), waste frying oil, waste animal fat, algal and microbial oil.	Acidogenic fermentation, Transesterification	[20,21]
4	Bioethanol	Liquid	Molasses, sweet sorghum, lignocellulose, Agricultural waste	Alcoholic fermentation	[22,23]

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5	Bio-methanol	Liquid	Biomass feedstock, Non-biogenic waste streams, flue gases.	Gasification, Pyrolysis	[24,25]
6	Biobutanol	Liquid	Lignocellulosic biomass, Algal biomass, Molasses, Wasted vegetables.	Acidogenesis, Solventogenesis, ABE fermentation(Batch mode)	[19,26]

Table 1: Biofuel and primary substrates utilized for their biofuel generation along with production strategies.

3.0 Microbial systems for biofuel production

3.1 *E. coli*

As the demand for energy increases daily, metabolic engineers introduce new approaches to manipulate the biobutanol production pathway in butanol-producing strains to overcome these limitations. *E. coli* strain MG1655 (DE3) is metabolically engineered to produce biobutanol from glycerol by introducing a butanol synthesis pathway of *C. acetobutylicum*. *E. coli* is the most suitable microorganism for genetic engineering as more information is present about its genetic and physiological characteristics, enabling modification using different genetic tools. The natural tolerance towards butanol is absent. However, it is still preferred for large-scale production over other strains because of its ability to ferment pentose sugars. Glycerol is present more abundantly and is a cheap carbon source and also a by-product during biofuel synthesis. *E.* coli has the ability to metabolize glycerol which is present more abundantly and is a cheap carbon source. Glycerol is often a by-product during biofuel synthesis, which directly affects the production rate of biobutanol by making it cost-effective [27].

Nevertheless, scientists recently engineered the GlpF gene (channel protein gene), which amplifies the transport of extracellular glycerol into the cytoplasm during biobutanol production. *E. coli* have further been engineered to combat carbon catabolite repression, a particular reference to metabolization of glucose among the pool of various sugars available. The work of action is the introduction of the point mutation in a transcriptional activator for catabolic operons that helps facilitate the catabolic activation independent of the catabolite repression control [4,27].

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Similarly, *E. coli* is a well-characterized microbe and has become one of the most significant microbes for enhanced hydrogen production due to its ease of manipulation [28]. It has been discovered that glycerol can be fermented by *E. coli* generating hydrogen as the end-product. Since hydrogen synthesized by *E. coli* has a negative impact on its cellular growth and glycerol fermentation, the recombinant *E. coli* strains are constructed utilizing engineering strategies such as metabolic and protein engineering to improve biohydrogen yield [29]. It is feasible to induce the generation of DHAP and inactivate ethanol and acetic, lactic acid production by redirecting metabolic pathways in *E. coli.* Thus, the resulting engineered *E. coli* strains can synthesize large formate but a lower quantity of acetate and ethanol compared to wild type during glycerol fermentation, leading to enhanced biohydrogen production. These recombinant strains also exhibited rapid growth on glycerol and achieved a significant anaerobic growth rate. Moreover, in vitro synthetic enzymatic pathway is used as a basis to develop a contemporary model for metabolic engineering that ensures increased biohydrogen yield from biomass in a relatively inexpensive manner. The resulting metabolically engineered *E. coli* strains demonstrated a 67-fold increase in biohydrogen production. This approach seemed to be practical for effective biohydrogen generation from glycerol [30].

3.2 *Clostridia* species

A versatile class of gram-positive bacteria, clostridia are the anaerobes that can utilize varieties of carbon sources, including glucose, fructose, xylose, glycerol, starch, arabinose, and cellobiose. This flexibility of clostridia to use a wide range of substrates becomes quite useful when essential for developing new and efficient procedures to generate biofuel [31]. *Clostridia sp.* are very likely candidates for biofuel production such as biohydrogen due to their relatively high efficiency. Apart from hydrogen, these obligate anaerobes also put together various other substances of industrial interest like bioethanol, biobutanol, lactate, acetate, and butyrate. Recently, Clostridia has attracted the immense attention of researchers for its use in hydrogen production. The microbes of this class generate hydrogen throughout the exponential growth phase, but when the bacterial population gets to the stationary growth phase, the process of metabolism transfers from the hydrogen production stage to a solvent production stage [1].

Clostridia sp. oxidizes organic molecules mainly containing sugar and yields ATP, NADH, and pyruvate via glycolysis during the dark fermentation process [16,32]. Some of the well-known

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and widespread species of class clostridia used for studies and biohydrogen generation include *C. butyricum*, *C. acetobutylicum*, *C. pasteurianum*, *C. cellulolyticum*, *C. tyrobutyricum*, *C. lentocellum*, *C. propionicum*, *C. saccharoperbutylacetonicum*, *C. thermocellum*, and *C. bifermentans* also have been reported to produce biohydrogen [33,34,35]. *C. butyricum*, *C. Pasteurianum*, and *C. beijerinkii* yield a high amount of biohydrogen, whereas *C. propionicum* is a poor producer of biohydrogen [36]. *C. thermocellum* and other thermophilic *Clostridium* species can use cellulose as a carbon source and generate biohydrogen from cellulosic substances like delignified wood fibres [37].

Similarly, Bioutanol has attracted the immense interest of researchers from industrial and academic areas as a potential renewable and clean energy source. Biobutanol is an excellent biofuel with superior compatibility to existing internal combustion engines and petroleum infrastructures since it has high energy density but lower volatility, water miscibility, flammability, and corrosiveness [38,39,40]. The solvent-producing clostridia, predominantly the type strain *C. acetobutylicum* ATCC 824, *C. beijerinckii*, and *C. tyrobutyricum*, have been modified utilizing metabolic engineering techniques to increase the production titer and outcome of the overall biological processes for the biobutanol generation [41]. The introduction of the acetate kinase gene (ack) featuring a deletion mutation in *C. tyrobutyricum* resulted in acetate pathway inhibition which doubled the biohydrogen output when glucose-based media was used. Similarly, an increase in the hydrogen yield was observed in *C. tyrobutyricum* and *C. paraputrificum* when homologous overexpression of the FeFe-H_2ase encoding gene was induced within the microbes. Another strategy to enhance hydrogen generation is the application of antisense methods. Utilizing this technique, the downregulation of hydrogenase uptake expression in *C. saccharoperbutylacetonicum* hydrogenase can ultimately enhance hydrogen production and make the whole process efficient [15].

Moreover, essential genes from other microorganisms can be included to construct synthetic pathways in clostridia. The incorporation of a new gene promotes metabolic pathway reconfiguration resulting in simplified bioprocessing and higher biobutanol yield [42,43]. The expansion of the substrate spectrum can increase the hydrogen production from clostridia. With regards to this course of action, *Thermoanaerobacter ethanolicus* contain specific genes that can utilize xylose. This particular gene can be then cloned and expressed in *C. thermocellum* which

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gives rise to a recombinant strain possessing the ability to use hemicellulose and cellulose as the carbon source. This modification allowed the engineered organism to produce two-fold the amount of hydrogen employing multiple substrates in contrast to single substrate utilization [44].

3.3 *Saccharomyces cerevisiae*

The yeast system tends to be more appealing than bacteria due to its capacity to perform a variety of post-translation modifications, ability to compartmentalize reactions in organelles, high secretion capacity, and no susceptibility to infectious agents [45]. *Saccharomyces cerevisiae* belongs to the conventional yeast system. It belongs to Kingdom Fungi, Divison Ascomycetes, Family Saccharomycetaceae, and Class Saccharomycetes. The abundance and dominance of *S. cerevisiae* in spontaneous fermentation caused them to be the choice for most fermentation processes [46]. *S. cerevisiae* exhibits temperature tolerance up to 35°C, provides advantages like rapid metabolic activity, high fermentation rate and output, the decline in the solubility of the gas, increasing temperature causes the viscosity of media to drop down, energy requirements reduced and lowered the risk of the combination of the by-products [47].

Metabolic engineering has expanded doors for biobutanol production; the approach combines the biosynthesis of valine and its breakdown along with the relocation of the overall degradation pathway into mitochondria or cytosol. It follows the Homologous end-joining pathway preventing ectopic integration of targeted constructs. Thus, eliminating competitive pathways for reducing carbon outflow into unproductive pathways is essential for producing target chemicals by *S. cerevisiae* [48].

The possibility of uninterrupted bioethanol generation is dependent on renewable and inexpensive raw materials, including lignocellulose, which is a major by-product of food and agriculture-based industries [49]. The concurrent bioprocessing of bioethanol fermentation and saccharification via lignocellulose requires S. cerevisiae to tolerate higher temperatures. Additionally, thermotolerance in the strains increases bioethanol yield and decreases the cost of production. One of the primary obstacles to bioethanol synthesis from *S. cerevisiae* is the

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development of the strains capable of sustaining growth under various inhibitory environments involved during the production process, including high temperatures (≥40°C) [50]. It was observed that the regulatory protein IrrE in *Deinococcus radiodurans* regulated transcription levels of numerous genes after exposure to radiation, which includes genes responsible for DNA replication and repair. This indicated that IrrE provided *D. radiodurans* with the radiation resistance capability through the regulation of pathways involved in DNA replication and repair. The modified or wild-type IrrE was recently discovered to enhance heterologous hosts' tolerances to stress. Researchers have engineered the IrrE from *D. radiodurans* to confer *S. cerevisiae* with the enhanced tolerance to elevated temperatures and inhibitors in lignocellulose hydrolysates. Wild and mutant IrrE were incorporated and expressed in *S. cerevisiae* strains using genetic engineering tools. It was found that the strains expressing wild or mutant IrrE demonstrated increased bioethanol yield as well as higher glucose consumption and specific growth rates in elevated temperatures [51].

3.4 *Yarrowia lipolytica*

Yarrowia lipolytica (Y. lipolytica) can accumulate lipids at a level of more than 50% of dry cell weight, the reason being the complexity and diversity of the multigene families present in the genome. These genes even facilitate the utility of a wide range of hydrophobic substrates (HS). Therefore *Y. lipolytica* can be considered oleaginous yeast. The cell surface invagination facilitates HS uptake from the medium. Storage molecules get deposited in a specialized compartment known as the lipid body (LB). These also play a role in synthesizing triacylglycerol (TAG) and sterol esters. A new lipid-binding protein was identified, regulating lipid trafficking between the cytoplasm and LB [52].

Lipids accumulation in *Y. lipolytica* is from the different pathway (a) de novo synthesis, where lipids are formed from precursors like acetyl and malonyl CoA. (b) ex novo accumulation pathway where the uptake of fatty acids, oils, and TAG occurs from the culture media. It further involves hydrolysis of HS. Lipid accumulation depends on the physiology of microorganisms, nutrient limitation, and environmental conditions of secondary metabolites like citrate and bioethanol [53]. The unique ability of *Y. lipolytica* to utilize and degrade HS paved its path to be used in bioremediation processes and fermentation to produce value-added oils, enzymes, and

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intermediate metabolites. It is further expanding its domain to wastewater purification in fat separating processes. The ability to break down HS has made it suitable for decreasing chemical oxygen demand (COD) in oil mill wastewater [54].

The capacity to accumulate lipid and its composition depend on the growth and culture condition of the *Y. lipolytica*. It provides a range of spectrum for the choice of substrate/final product combination. For example, replacing the carbon source from glucose with oleic acid increased the capacity to accumulate lipid, lipid particle size, and modified lipids and protein. Thus, it can be concluded that the composition of the substrate being used and yeast selectively remove or assimilate FA from the substrate, producing fats with predetermined composition [55].

Heat stress is a significant element in microbial engineering that regulate metabolic flux distribution, target product generation and cellular growth rate. Commercial manufacturing of biological products is primarily restricted by the cost of the energy involved for cool purposes during large-scale fermentation, which considerably increases the overall operational cost [56,57]. To assemble and select thermotolerant *Y. lipolytica* carries significant importance since it helps to lower the cooling cost and unravel the mechanism of heat tolerance at the molecular level. Through the transcriptome analysis, the genes involved in the thiamine metabolism pathway that confers thermotolerance to the phenotypes can be identified in the thermotolerant *lipolytica* strain. It is now possible to transfer the optimal gene combination utilizing molecular manipulation systems to thermotolerant phenotype to the wild-type *lipolytica* optimum to enhance lipid production in higher temperatures when compared to optimal cultivation temperature [57].

Genetic Engineering approaches are also being explored to increase lipid accumulation. Redirecting the carbon flux towards TAG assembly, which is progressed by deleting assembly, which is advanced by deleting the glycerol-3-phosphate dehydrogenase gene (GUT 2), increases the lipid accumulation three times when compared with the optimal gene combination was transferred. *Y. lipolytica* has the tremendous property of forming single-cell oils (SCO). SCO is the edible oil obtained from a microorganism that shares similarities with the one obtained from plants and animals. SCO is rich in PUFA, essential for human nutrition and development [58]. Genetic manipulations can enable *Y. lipolytica* to function as sustainable SCO production

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platforms by efficiently utilizing xylose as a cheap and readily available substrate. Xylose is the main component of lignocellulosic by-products from agriculture, food, forestry and paper-based industries. Studies have revealed *Y. lipolytica* contains a dormant pentose pathway. It was recently reported that the recombinant strain with three genes (derived from dormant pentose pathway) overexpressed in a Po1d genetic background exhibited growth on xylose [59].

4.0 Conclusion

Interest in finding an alternative to fossil fuel has increased due to increased fuel prices. It shifted the approach to biofuel generation by manipulating microbial; cellular metabolism. Analyzing complex metabolic pathways is a challenge in synthetic and metabolic engineering concepts. *E. coli* has widely been known as it is easy to cultivate. The advantage it provides for the choice in the biofuel industry is its ability to metabolize pentose sugars and is easily modified using available genetic tools. *Clostridium*, the genus of strictly anaerobic bacteria, has exhibited the potential to produce biohydrogen with relatively higher efficiency. To maximize the biohydrogen yield, it is feasible to recover the energy by combining the biohydrogen generation process from clostridia with other techniques that utilize the by-products of overall processes. The metabolic biosynthesis pathway of clostridia is required to be well analyzed, characterized, and favourably engineered to develop cost-effective and sustainable procedures for producing biofuel-like biobutanol through biological processes. The analysis of advanced biological processes, metabolic engineering of clostridia, and renewable bio-based resource utilization will definitely play a key role in improving operating conditions crucial for increasing biobutanol productivity.

S. cerevisiae has established synthetic and microbial engineering approaches. The aspects are still being explored in the case of the non-conventional yeast system. Improved genome editing methods are already helping researchers to overcome some of the challenges associated with Non-homologous end-joining dominated DNA repair pathways in these organisms. *Yarrowia lipolytica* has its metabolism inclined towards the accumulation of lipids, and its ability to use hydrophobic substrates efficiently makes it a suitable candidate for the production of biofuels. The metabolism study in *Y. lipolytica* has revealed the substrate transport processes and genes concerned with regulation processes. Nitrogen limitation is the primary type of limitation

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studied, and the C/N ratio is the crucial factor governing lipid accumulation. The approach combines lipidomic, metabolomic, and genetic approaches, and using fed-batch culture will unleash tremendous information about the regulation of lipid metabolism.

The future proposition can be to engineer non-conventional strains, imparting them the ability to digest lignocellulosic biomass. Lignocellulosic biomass is the most readily available substrate, cutting down the biofuel production cost. The genome-scale metabolic model has been employed to evaluate the cellular metabolic constitution of the microbes. CRISPR-Cas gene-editing technology has been successfully put to use in the non-conventional yeast system enabling rapid gene manipulation that allows modification of metabolic pathways for enhanced biofuel production. CRISPR-Cas platform has the ability to allow the establishment of transcriptional regulation, genetic circuits, and other metabolic networks in non-conventional yeast systems that would pave the way for the development of efficient biological approaches for biofuel generation.

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Authors' Contributions

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