

Exploration of various microbial systems for the biofuel production

Abstract

Fuels on a broad scale are divided into two categories based on their renewability as 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 easy to culture and maintain have made it stand out among the microbes. Their ability to be able to metabolize pentoses further draws it near to being used for biofuel production. Fermentative bacteria, particularly the class clostridia (obligate anaerobe), have been recognized as 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 n-butanol. The review further explores the two yeast systems, conventional and non-conventional systems. The traditional system which comprises *Saccharomyces cerevisiae* has been explored by the tools of synthetic biology 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 being 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*

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 CO, SO₂, CO, CO₂, NO_x, and HC. 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 1.1°C to 6.4°C in the next 100 years if no reformatory steps are taken into consideration [1,2].

Biofuel becomes 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].

Synthetic biology is the combination of various biological components which have been narrowed down from the enormous data derived from metabolomics, proteomics, transcriptomics, and fluxomics [5]. 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 [6].

Synthetic biology progresses to write new genetic information, creating designed non-natural genes, proteins, and biological processes in the organism [7]. 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 [6]. The

ideologies remain a bit obstructed due to the constraint due to the pressure of a large number of introns, and a larger size of some genes involved. Advances in DNA sequencing technologies can overcome it all [8].

The present review is an attempt 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 can be taken advantage of, for the creation of novel renewable fuel systems.

2.0 Microbial systems for biofuel production

2.1 *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 [9].

Nevertheless, scientists engineered the GlpF gene (channel protein gene), which amplifies the transport of extracellular glycerol into the cytoplasm during butanol 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 [4,9].

2.2 *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 [10]. *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 ethanol, butanol, 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. Mediated by pyruvate synthase, pyruvate is eventually converted to acetyl CoA and CO₂, giving out reduced ferredoxin (Fd). In certain conditions, NADH so 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 [12,13]. Some of the well-known and widespread species of class clostridia used for studies and biohydrogen generation include *C. butyricum*, *C. acetobutylicum*, *C. beijerinckii*, *C. pasteurianum*, *C. cellulolyticum*, *C. tyrobutyricum*, *C. lentocellum*, *C. propionicum*, *C. thermocellum*, and *C. bifermentans* also have been reported to produce biohydrogen [14,15,16]. *C. butyricum*, *C. Pasteurianum*, and *C. beijerinckii* yield a high amount of hydrogen whereas, *C. propionicum* is a poor producer of hydrogen [17]. *C. thermocellum* and other thermophilic *Clostridium* species can use cellulose as a carbon source and generate hydrogen from cellulosic substances like delignified wood fibers [18].

Similarly, n-Butanol has attracted the immense interest of researchers from industrial and academic areas as a potential renewable and clean energy source. n-Butanol 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 [19,20,21]. 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 n-butanol generation [22]. 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 n-butanol yield [23,24].

2.3 *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 [25].

Saccharomyces cerevisiae belongs to the conventional yeast system. It belongs to Kingdom Fungi, Division 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 [26]. *S. cerevisiae* provides 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. The thermotolerance in the strains increases ethanol yield and decreases the cost of production [27]. Metabolic engineering has expanded doors for butanol 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* [28].

2.4 *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 cytoplasm and LB [29].

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 ethanol [30]. 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 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 [31].

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 to selectively remove or assimilate FA from the substrate produce fats with predetermined composition [32].

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 wild-type strains. *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 [33].

3.0 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 favorably engineered to develop cost-effective and sustainable procedures for producing biofuel-like n-butanol 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 to improve operating conditions crucial for increasing n-butanol 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 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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