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Review Article

Advances in microbial biofuel production by metabolic and enzyme engineering, synthetic biology, metagenomics, and genome editing applications

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Microorganisms are the primary source of genetic diversity on earth due to their unparalleled metabolic and functional variability. With the depletion of fossil fuels, a sustainable alternative approach is the use of biofuels, where plant biomass as feedstock is essentially degraded to sugars with the aid of microbe-derived enzymes, followed by the conversion of those sugars to biofuels. Several cellulolytic and non-cellulolytic enzymes are involved in biofuel synthesis. Molecular cloning, along with the advancements in genetic and metabolic engineering in microbial cells, plays a significant contribution to biofuel overproduction. Advanced molecular technologies such as metagenomics and synthetic biology approaches are also being used to construct effective microorganisms for biofuel manufacturing. Obtaining novel enzymes from undiscovered microbial consortia and functional gene analysis is possible through a metagenomics approach. While synthetic biology provides engineered biological systems to generate required biofuel productivity, the CRISPR-Cas genome editing tool is another revolutionary tool being utilized for efficient biofuel production. This article provides a brief overview of different methods of biofuel production using microorganisms.

Introduction

The increasing energy demand worldwide and the rapid depletion of fossil fuels have prompted the development of biofuels and bioproducts [1]. Furthermore, the particulate matter produced by burning fossil fuels is directly linked to carbon dioxide emissions and climate shifts, posing serious harm to the environment where biofuels are a viable alternative [2]. The rising trend of fossil fuel usage resulting in transportation and aviation emissions also contributes to global warming through the greenhouse effect [3]. A multitude of ecological and economic benefits can be achieved through biofuels, particularly reduced fossil fuel consumption, enabling the use of inexpensive and renewable raw materials, and the production of an ideal substrate for a wide range of microorganisms [4]. Currently, 10% of the world's primary energy supply comes from bioenergy. Bioenergy, primarily biofuels, is estimated to have the capacity to provide anywhere from 10% to over 60% of the world's primary energy supply [5]. The proportion of bioenergy in the global energy matrix is projected to range from 7.5% to 37% by 2050 [6].

At present, diverse gaseous and liquid biofuels, including biodiesel, bioethanol, biohydrogen, bio-oil, and Fischer–Tropsch H_2, are being derived from renewable resources as biomasses [7–11]. Based on the source of feedstock, biofuels are categorized into four groups. Plant biomass has a complex polymeric composition that makes it inherently recalcitrant. The first generation of biofuels is produced mostly from plant sugars and starches found in food crops [12]. Second-generation biofuels are derived from non-food crops often referred to as lignocellulosic biomass. Lignocellulose, a complex carbohydrate polymer of the secondary cell wall, comprises about 40–50% cellulose $(C_6H_{10}O_5)_n$, which provides strength and flexibility; 20–40% hemicellulose $(C_5H_8O_4)_n$, which links lignin and cellulose molecules; 18–25% lignin $(C_9H_{10}O_3(OCH_3)0.9–1.7)_n$, a naturally renewable aromatic polymer which provides hydrophobicity and structural stability; and other natural extractable compounds [13,14]. Substantial

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cellulosic biomass degradation occurs owing to the synergistic activity of hydrolytic enzymes produced by cellulolytic microorganisms such as bacteria, algae, and fungi [15]. Microalgae are used as an alternate feedstock for the third generation of biofuels because of their rapid growth, volume, and substantial lipid and carbohydrate ratios [16]. Fourth-generation biofuels are synthesized through genetic engineering of desired microbes, particularly algae and cyanobacteria [17,18]. Plant biomass reduced to simple sugars with the use of microbial enzymes, particularly genetically engineered, and then converted to biofuels by respective microorganisms is a sustainable method for biofuel production [19].

In recent times, the use of microorganisms in metabolic engineering has advanced through molecular cloning, allowing for increased biofuel production [20]. Microbes, predominantly bacteria, fungi, yeast, and microalgae may accumulate intracellular lipids, mainly triacylglycerol, into a significant portion of their biomass [21]. Compared with rapeseed plants and biomass, fast-growing microorganisms can convert a larger amount of feedstock into significantly higher yields. Likewise, developments are being made in genetic engineering and biotechnology to transform extensively explored microorganisms such as *Saccharomyces cerevisiae* and *Escherichia coli* into bioenergy cell plants [22]. Mining enzymes from microbial communities and modifying the proposed metabolic route for biofuel production are also becoming increasingly feasible with the revolutionary advances and understanding of techniques such as synthetic biology and metagenomic sequencing [23,24]. Moreover, innovative molecular engineering tools such as CRISPR-Cas systems are also being utilized to induce enhanced biofuel production while exploring the potential of modified microorganisms and the biomass utilized [25]. This review highlights biofuel production in relation to molecular tools using microorganisms.

Utilization of microorganisms in biofuel production

Due to the diverse metabolic profiles, microorganisms can produce biofuel by fermenting a range of substrates obtained from food crops (sugars, starch, rapeseeds), non-food crops such as lignocellulosic biomass (bagasse, corn stalks, cottonwood), and agricultural and inorganic waste materials [26]. Although there is now an extensive understanding of the diversity of microbial life on earth, the majority of environmental microbes are as yet unexplored and unidentified in relation to biofuel production. Therefore, in-depth studies are currently underway globally to exploit unexplored microbial communities [27]. It is vital to introduce the fuel synthesis pathway into native and heterologous microbial hosts in order to obtain the requisite biofuel yield. Several parameters, including the primary source of carbon, the toxicity level of the synthesized fuel or substrate, and the operational conditions required to convert the substrate into the fuel, usually determine the choice of microbial host. Additionally, the inherent genetic variation within a species or genus may be utilized to the benefit of the already recognized biofuel-producing organisms [28,29].

Biofuel production approaches in microorganisms

The production of biofuels from renewable resources, such as lignocellulose and cellulosic biomass, offers an alternative to petroleum-based fuels for transportation and energy sectors in the form of liquid fuel [30]. This involves the processing of simple sugars from cellulosic biomass which are then converted into biofuels. Although particular microbes produce biofuels natively, their yields are frequently lower and are less efficient. Therefore, microorganisms are being engineered to convert sugars into biofuels [31]. Cellulose, the most prevalent biopolymer in nature, is utilized as a potential source of sugars for the synthesis of biofuels [32]. Cellobiose and glucose, the biomass saccharides obtained as a result of cellulosic conversion, are efficiently hydrolyzed by microbial enzymes to produce a variety of biofuels [33,34].

Conventional biofuel production in microorganisms involves four main metabolic pathways: (1) the isoprenoid pathway, (2) the keto-acid pathway, (3) the fatty acid synthesis pathway, and (4) the CoA-dependent β-oxidation pathway. Pyruvate is the precursor compound for the isoprenoid and keto-acid pathways, while acetyl-CoA is the precursor for the fatty acid synthesis and CoA-dependent β-oxidation pathways. In order to achieve the high yield and productivity of biofuels, pathways are often further altered [35]. For example, carboxylic acid reductase, obtained from *Mycobacterium marinum*, was found to readily transform aliphatic fatty acids (C_6–C_{18}) into fuels. However, combining the respective metabolic pathway with an exogenous lipase formed a broad range of C–C bonds that were further utilized to produce fuels and other specific chemical products [36]. Nevertheless, a significant disadvantage includes the high expense of extraction from the fermentation substrate, which affects the output yields [37]. For

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this reason, several integrated biological and chemical methods have been established for the production of biofuels, in addition to modifying metabolic pathways. Recently, *Clostridium acetobutylicum* has gained a lot of interest as the nucleophilic α-carbon of acetone, a by-product of acetone-n-butanol-ethanol (ABE) fermentation, and the alcohols' electrophilic α-carbon, formed during the solventogenic phase, facilitate in the production of C–C bonds [38]. These bonds are then able to form high molecular weight hydrocarbons similar to jet fuels. Additionally, transportation fuel reserves comprising C15< are frequently produced from cellulosic biomass by the processing of ABE fermentation products into ketones using a metal-catalyzed alkylation process [39]. Feedstocks for bio-derived oils are also being utilized through integrated processes similar to ABE fermentation. Alcohols and fatty aldehydes are formed from the fatty acids obtained through integrated mechanisms employed to form bio-oil feeds [40]. Despite being an elaborate process, producing biofuel through ABE fermentation presents a number of challenges; in particular, this procedure results in the formation of additional by-products including hydrogen, ethanol, acetone, and other compounds, which generate high purification costs during downstream processing [41,42].

For biofuel production, microorganisms have the ability to metabolize sugars and lipids from raw feedstock, resulting in increased carbon chain length and conversion of the metabolites into fuel compounds. They can selectively hydrogenate, decarboxylate, and deoxygenate feedstock compounds through a variety of biochemical and heterogeneous [37]. Additionally, different molecular engineering strategies such as metabolic engineering, synthetic biology, and genome editing are used to develop and optimize biofuel production in microorganisms [43]. Essentially, recent advancements in metabolic engineering strategies, including the engineering of enzymes, exploration of novel metabolic routes, and optimization of metabolic and fermentation processes, have been employed to augment biofuel synthesis [44]. Non-native hosts are also currently being engineered to achieve sustainable biofuel production [42]. Figure 1 gives a brief overview of the three biofuel production pathways.

Cellulolytic enzymes involved in biofuel production

Enzymatic cellulose degradation of lignocellulose is predominantly dependent on glycoside hydrolases (GHs) and oxidative enzymes for sustainable fuel generation [45]. Cellulase enzymes are crucial to the synthesis of biofuels because they convert the complex sugar chains of cellulose into simpler sugars like glucose [46]. Cellulolytic microbes produce enzymes that work in tandem with other microorganisms or autonomously as cellulases in fungi and bacterial species. A cellulosome is a multi-enzyme network of glycosyl hydrolases in bacteria that is bound onto a non-catalytic scaffolding. The cellulolytic unit is made up of three primary groups of enzymes: β-glucosidases, endoglucanases, and exoglucanases [47]. Endoglucanase, often referred to as carboxymethyl cellulase (CMCase), is responsible for breaking down internal β-glycosidic linkages, revealing the reducing and non-reducing ends of cellulosic polysaccharide chains. Exoglucanase then acts on these open chains, releasing glucose and cellobiose. β-glucosidases then transform cellobiose formed by exoglucanase into glucose [48].

Hemicellulase enzymes comprise β-endoxylanases, β-xylosidase, arabinofuranosidase esterases, glucuronidase, glucanase, mannanase, xyloglucan hydrolase, and monooxygenases [49]. The most common hemicellulose in hardwoods is xylan, which is known to safeguard cellulose against cellulolytic enzymes' hydrolytic activity [50]. Treatment with xylanase increases tissue fibre dilation and permeability of biomass, which increases cellulose attraction to hydrolysis [51]. Xylanases also increase the saccharification of hemi-celluloses (xylan), which results in more sugar being available for fermentation, therefore, enhancing the productivity of the biofuel [52]. The enzymatic digestion of lignocellulosic biomass requires complete hydrolysis of the polysaccharides in order to produce cellulosic liquid biofuels. This can be accomplished through the pre-treatment of biomass [46]. Cellulolytic enzymes are subsequently added after this treatment, which causes the cellulose in the pre-treated biomass to degrade. Fermenting microbes exploit the liquid and solid fractions from the hydrolysis and pretreatment processes in order to produce biofuels [53].

The production and characteristics of thermostable cellulolytic enzymes have been enhanced for commercial purposes by genetic engineering, which has also led to the production of various biofuels. The production of biofuel is frequently achieved by using fungal strains such as *Kluyveromyces* sp., *S. cerevisiae*, and *Trichoderma* sp. [47], as well as thermophilic anaerobic bacteria such as *Clostridium* spp., *Caldanaerobacter,* and *Thermoanaerobacter* [54]. Additionally, commercial microbial hydrolases that act on polysaccharides are now readily accessible. For example, CellicCTec2 (Novozymes) is a blended mixture of

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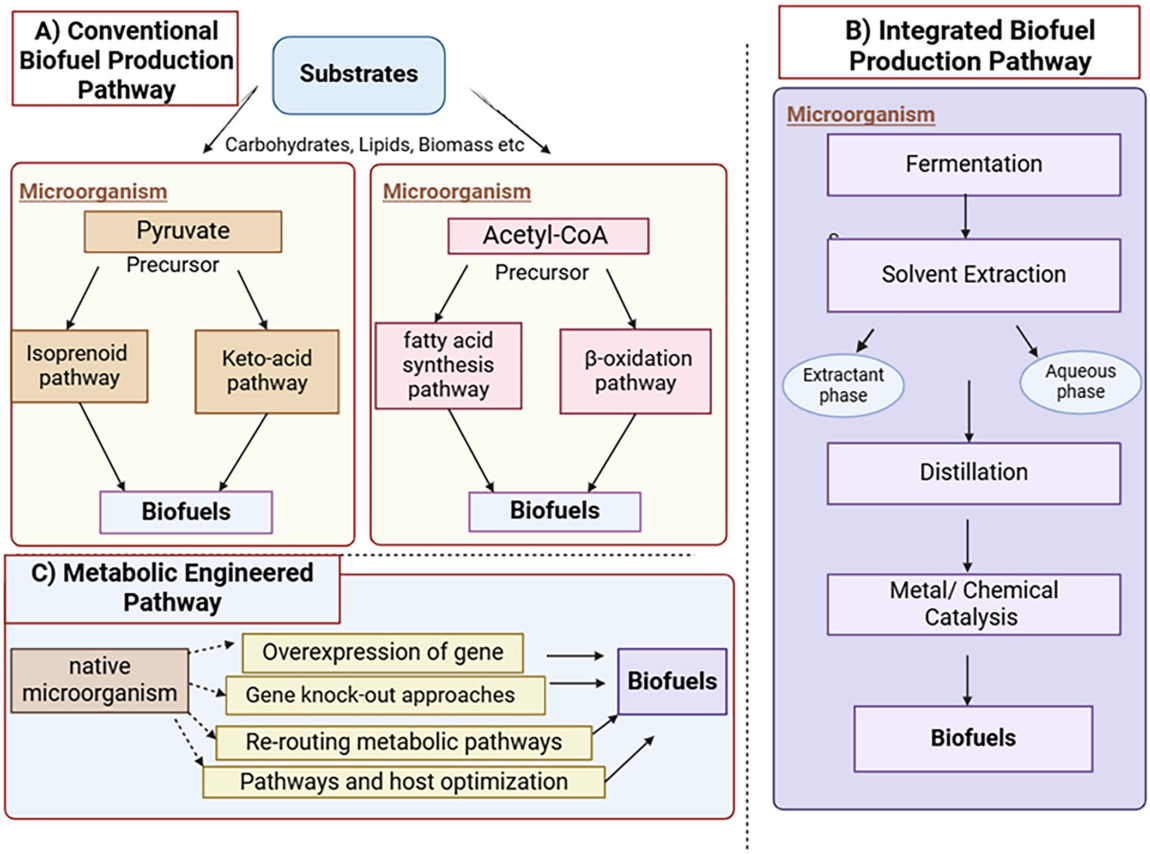

Figure 1: The three different approaches in microorganisms for biofuel production; (A) conventional biofuel production pathway; (B) integrated biofuel production pathway; and (C) metabolically engineered pathway.

cellulases, β-glucosidases, and hemicellulases [12]. In addition, Celluclast, Carezyme, and cellulases from *Aspergillus* sp. and *Trichoderma* sp. are also being commercially produced [47].

Non-cellulolytic enzymes involved in biofuel production

For biofuel production, pre-treatment is an effective way to remove lignin and improve the enzymatic digestibility of biomass polysaccharides [55,56]. The primary enzymes involved in the breakdown of lignin are peroxidases and phenol oxidases. Phenol oxidases are further classified into two groups: laccases and polyphenol oxidases [57]. Laccase, lignin peroxidase, manganese peroxidase, and versatile peroxidase are examples of ligninolytic enzymes that facilitate in the polymerization and de-polymerization of various substrates, which leads to the breakdown of lignin and the creation of biotechnological by-products like biofuels [58]. The financial viability of biofuel-based biorefineries can be balanced by developing alternatives for the lignin or lignin-rich residue valorization by employing microorganisms [59]. Furthermore, amylases and inulinases are involved in the breakdown of polysaccharide molecules in raw starches which are then further hydrolyzed to yield a variety of first-generation biofuels [60,61]. Third- and fourth-generation biofuels also rely heavily on enzymatic hydrolysis and are created from starch and cellulose-rich algal biomass [61]. Other non-cellulolytic enzymes essential for biofuel production at industrial scale are lipases and phospholipases [62]. For biofuel synthesis, microbial non-cellulolytic enzymes are responsible for the trans-esterification of triglycerides [63].

Metabolic and evolutionary engineering for microbial biofuel production

Microorganisms have historically been exploited as a source of biofuel generation either directly or indirectly, either by photosynthetic microorganisms processing inorganic compounds or by microbial

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transformation of organic compounds at a commercial scale [33]. However, by employing potential microbes that were previously unable to produce fuels from feedstock, researchers are now engineering them to reduce the numerous steps involved in pretreating raw materials for fuel conversion into a single microbial process [64]. In recent times, genetic and metabolic engineering have been used for enhanced biofuel production. To create highly productive microbial cell factories, metabolic engineering is an effective strategy [65,66]. It is also an essential tool that is frequently utilized to re-route the biosynthetic pathway in a variety of native and modified hosts in order to manufacture desired end products. These tools alter the surface expression of targeted proteins in engineered cells and the associated intracellular metabolic pathways, enabling precise modifications for microbes to convert respective feedstocks to biofuels [64]. The principle of metabolic engineering, in conjunction with recombinant DNA technology, has improved the synthesis of desired products through integrating the biosynthetic routes, biochemical pathways, and the regulating functions of the cell [13,67]. Nevertheless, metabolic engineering entails an in-depth understanding of the organism's biochemical response system, often putting a constraint on metabolic activity, which in turn lowers the survival and proliferation rate of the modified strains [68,69].

Mutagenesis, along with irrational design approaches, has previously been applied to enhance microbial strains for industrial purposes [34,49]. For this purpose, evolutionary engineering, also referred to as adaptive laboratory evolution, has been broadly adopted as an alternative bottom-up strategy [70]. Microbial strains are exposed to selective stressors such that they evolve in a manner that favours advantageous mutations and the desired phenotypes [69]. The fundamental point of an evolutionary engineering method is to generate genetic variety by imitating evolution [70,71]. Evolutionary engineering employs the organism's innate capacity to adapt to particular stress conditions rather than establishing alternative metabolic pathways [72]. The result is optimized strains capable of enhanced production of biofuels like ethanol, butanol, and other renewable chemicals [73]. Furthermore, elevated yields and viability obtained through adaptive evolution are essential for producing effective microbial strains harnessing ecological tolerance [69]. This process can be carried out with or without using physical or chemical mutagenesis [74,75]. Evolutionary engineering studies can also be integrated with targeted directed evolution strategies to efficiently generate specific mutant libraries in less time and to rapidly analyze genomic variations using high-throughput sequencing [76,77].

Microbial strains have a significant effect on biofuel production. Using molecular biology techniques such as gene editing further optimizes their expression and efficacy to produce biofuels [78]. Furthermore, advances in biofuel characterization methods have vastly enhanced our grasp of their features and capacities, spurring innovation and optimized production [79]. According to several metabolic engineering studies, *S. cerevisiae* and *E. coli* have been used as modified strains for biofuel generation [80,81]. For the fermentation of sugar into a variety of short-chain alcohols, genetically modified *E. coli* has been regarded as a viable biocatalyst for the production of biofuels [82]. For second-generation biofuel production, microbes such as *Bacillus sp*. AY8 [83], *Caulobacter crescentus* [84], *Neurospora crassa* [85], *Rhodotorula glutinis* [86], *Talaromyces amestolkiae* [87], and *Thermoascus aurantiacus* [88] are known to employ cellulosic feedstock as substrate. Microalgae lipids are a substantial feedstock for biofuel production, which are often overexpressed through providing varying growth conditions [89,90]. For the production of biofuels from marine algal biomass, *E. coli* and *S. cerevisiae* have been reported to act as producers for algae such as *Kappaphycus alvarezii* [91] and *Laminaria japonica* [92]. *S. cerevisiae* is also being used as a genetically engineered host for the production of fourth-generation biofuels [93]. Moreover, several reports are available where enzymes including endo-glucanase are responsible for the fourth-generation biofuel production utilizing algal biomass [94]. The halophile *Haloferax volcanii* releases very stable enzymes, such as peroxidase, which might be helpful in the oxidation of complex phenolic molecules required for biomass degradation [95]. Another method for biofuel synthesis is the utilization of endogenously produced ethanol-rendering yeasts as an alternative host medium for fourth-generation biofuels [96,97]. Several other microorganisms including fungi [98,99], bacteria [100–103], yeasts, and algae are found to have potential biofuel-producing genes. As previously stated, metabolic engineering combined with evolutionary research offers a viable platform for the production of next-generation biofuels [104]. In the fungus *Rhodospridium toruloides*, increased xylose consumption rate through adaptive engineering in combination with better microbial oil yields by overexpression of lipid-synthesizing genes resulted in production of biodiesel fuel [105]. Similarly, in *E. coli,* efficient biomanufacturing of fuels was achieved by disruption in the pentose phosphate pathway through insertion of methanol-utilizing genes followed by adaptive evolution by providing methanol as a sole source [106]. Table 1 shows different microbes expressed and characterized through molecular techniques for microbiological biofuel generation.

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Table 1.: Molecular biology tools used for microbial biofuel production.

Sr. no.	Source	Gene	Enzyme	Molecular method for biofuel production	Substrate	Biofuel generation	Reference
Bacteria							
1	*Bacillus* sp. AY8	Cellulose microfibril swelling (Cms)	Cms enzyme	Molecular cloning, in-silico analysis, characterization	Cellulose	2nd	[83]
2	*Caldicellulosiruptor bescii*	Lactate dehydrogenase (Idh)	-	Gene knockout	Cellobiose, maltose	1st, 2nd	[101]
3	*Caulobacter crescentus*	celA	Cellulose	Molecular cloning, expression	Lignocellulose	2nd	[84]
4	*Corallococcus* sp. EGB	AmyC	Alpha-amylase	Molecular cloning, characterization	Maltotriose	1st	[103]
5	*Escherichia coli* KO11	-	Endo-glucanase	Characterization, enzyme treatment	Algae biomass (*Laminaria japonica*)	3rd	[92]
6	*Haloferax volcanii*	katG (hvo_1778)	Peroxidase	Overexpression, characterization	Lignocellulose	2nd	[95]
7	*Klebsiella* sp. NBRC100048	orf2991	Novel alkane synthase	Molecular cloning	Aldehydes	4th	[102]
8	*Thermococcus litoralis*	WP_004069094.1	β-Glycosidase	Expression, synthetic biology	Vegetable oils	1st	[100]
Fungi							
9	*Myceliophthora thermophila*	MtglaA, Mtamy1	Glucoamylase, alpha-amylase	Enzyme expression-components deletion, overexpression	Starch	1st	[99]
10	*Neurospora crassa*	GH5-1	Endoglucanase	Expression, characterization	Azo-carboxymethylcellulose, Cellulose	2nd	[85]
11	*Penicillium oxalicum* GXU20	PoGA15A	Glucoamylase	Expression, characterization	Raw corn, cassava flours	1st	[98]
12	*Talaromyces amestolkiae*	bgl1	β-Glucosidase	Expression, characterization	p-Nitrophenyl glucopyranoside, Cellooligosaccharides	2nd	[87]
13	*Thermoascus aurantiacus*	TaBgl1	β-Glucosidase	Molecular cloning, characterization of recombinant strains	Lignocellulose	2nd	[88]
Yeast							
14	*Rhodotorula glutinis*	Cellobiohydrolase (cbhI)	Cellulose	Molecular cloning	Cellulosic substrates	2nd	[86]
15	*Saccharomyces cerevisiae*	ARO10	Decarboxylase	Cloning, overexpression	Aldehydes	4th	[93]
16	*Yarrowia lipolytica*	PDC1, ADH1	Pyruvate decarboxylase, alcohol dehydrogenase	Chromosome-based co-overexpression	*S. cerevisiae* encoding ethanol	4th	[97]
Microalgae							
17	*Nannochloropsis* sp.	Violaxanthin–chlorophyll a protein (VCP)	-	Homologous recombination, transformation	Carbon dioxide	3rd	[89]
18	*Phaeodactylum tricornutum*	PtPEPCase1	Phosphoenolpyruvate carboxylase (PEPCase)	Transformation, overexpression	Dissolved inorganic carbon	4th	[94]

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Enzyme engineering for microbial biofuel production

Another parameter utilized for efficient biofuel production involves engineering of enzyme machinery. The biofuel produced includes bioethanol (produced through fermentation by yeast or other microorganisms), biohydrogen, or even butanol through different types of fermentation processes. The two main approaches of engineering involve chemical and genetic modifications [107]. Thermophilic filamentous fungi, including *Thermoascus aurantiacus* [108], *Hypocrea jecorina* [109], *Rhizomucor miehei* [110], *Gloeophyllum trabeum* [111], and *Rhizopus chinensis* [112], are being enzymatically engineered for efficient biomass degradation and improved thermophilic activity. Furthermore, for increased substrate saccharification, fungal strains such as *Bipolaris sorokiniana* [113] and *Penicillium funiculosum* [114] were also identified. The strain *Bipolaris sorokiniana* was found to have increased catalytic and specific activity on using a rational design approach where conserved and mutant residues on the protein's active site were utilized for fuel productivity [113]. Likewise, overexpression of genes encoding specific enzymes was recognized for overproduction of final product yield as reported in the bacterium *Nannochloropsis oceanica* [115] or mutant gene overexpression as developed in *Myceliophthora thermophile* [116]. Enzyme engineering through site-directed mutagenesis of microorganisms such as *Anoxybacillus* sp. [117], *Bacillus thermocatenulatus* [118], *Thermomyces lanuginosus* [119], etc., has also resulted in improved specific activity of proteins for biofuel productivity, increased thermo-stability profiles, and enhanced yield. Thermophilic bacteria from the genus *Caldicellulosiruptor* effectively degrades plant biomass to fermentable sugars [120]. For enhanced activity, the six glycoside hydrolase genes located in the glucan degradation locus (GDL) of *Caldicellulosiruptor bescii* were knocked out, resulting in increased breakdown of microcrystalline cellulosic substrates [121]. By engineering the enzyme that catalyzes the first phase in the *n*-butanol pathway (i.e., Thl) and modifying the cofactor specificity of Hbd and Ter, *n*-butanol yield was doubled [122]. An amino acid shift in β-glucosidase-forming enzyme accounts for the enhanced cellulase expression [123]. Bacteria such as *Halothermotherix orenii* [124] and *Ruminiclostridium thermocellum* [125] have been engineered for β-glucosidase activity for effective cellulose degradation. Table 2 shows the various enzyme engineering approaches adapted for efficient biomass hydrolysis and biofuel production.

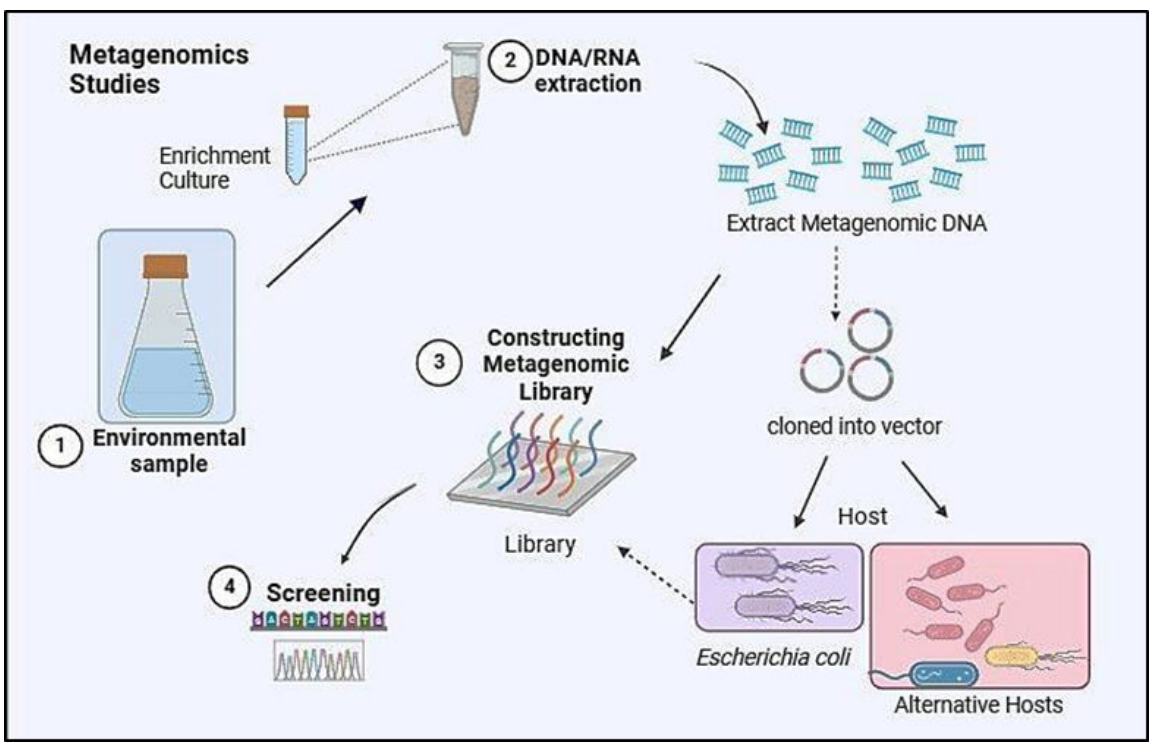

Figure 2: An overview of the functional metagenomics approach for novel biocatalyst identification.

(1) Collection of enrichment culture (2) DNA/RNA extraction (3) Constructing genomic libraries (4) Screening analysis of potential biofuel-producing microorganisms.

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Table 2.: Enzyme engineering approaches for efficient biofuel production.

Sr. no.	Source	Enzyme	Enzyme engineering	Result	References
1	*Anoxybacillus* sp.	α-amylase	Site-directed mutagenesis at Ala-161 in the conserved sequence region with an aspartic acid	Enhanced specific amylolytic activity	[117]
2	*Bacillus thermocatenulatus*	*Bacillus thermocatenulatus* lipase 2 (BTL2)	Site-directed mutagenesis, modulating surface hydrophobicity	Enhanced catalytic activity	[118]
3	*Bipolaris sorokiniana*	Endoglucanase (BsGH7-3)	Conserved residues in the active site	Increased catalytic efficiency and saccharification	[113]
4	*Caldicellulosiruptor bescii*	Glycoside hydrolase CelA	Gene deletion	Increased degradation of microcrystalline cellulosic substrates	[121]
5	*Clostridium thermocellum*	Thl enzyme	Homologous mutations, cofactor engineering	High biofuel yield and thermostability	[122]
6	*Gloeophyllum trabeum CBS*	GH5 cellulase GtCel5	Site-directed mutagenesis	Improved enzyme-substrate interactions	[111]
7	*Halothermothrix orenii*	β-glucosidase	Active site pocket engineering	Enhanced substrate accessibility	[124]
8	*Hypocrea jecorina*	Cellobiohydrolase I (cbh1or Cel7A)	Recombinant enzyme expression	High specific activity	[109]
9	*Myceliophthora thermophile*	Cellobiohydrolase (rMtCel6A)	Codon optimization, recombinant enzyme expression	Enhanced saccharification of cellulose-containing substrates	[116]
10	*Myceliophthora thermophila*	Glucoamylase	Enzyme expression-components deletion, mutant overexpression	Enhanced amylolytic activity	[99]
11	*Nannochloropsis oceanica*	Diacylglycerol acyltransferase 5	Overexpression of DGTT5 gene	Overproduction systems for triacylglycerols	[115]
12	*Penicillium funiculosum*	GH5 xylanase (*Pf*Xyn5)	Recombinant enzyme expression, overexpression	Enhanced saccharification of cellulose-containing substrates	[114]
13	*Rhizomucor miehei*	Lipase	Site-directed mutagenesis at the N-linked glycosylation site	Improved hydrolytic activity and methanol tolerance	[110]
14	*Rhizopus chinensis*	Lipase r27RCL	Site-directed mutagenesis, expression	Enhanced thermostability and expression	[112]
15	*Ruminiclostridium thermocellum*	β-glucosidase	Up-regulation of β-glucosidase gene (bglx)	Enhanced saccharification of cellulose-containing substrates	[125]
15	*Thermoascus aurantiacus*	Xylanase	Integration of transcriptional xylanase regulator *xlnr*	Improved enzyme thermostability	[108]
17	*Thermomyces lanuginosus*	*Thermomyces lanuginosus* lipase (TLL)	Site-directed mutagenesis, expression	Stable α-helix, thermostability, increased yield, enhanced transesterification	[119]
18	*Trichoderma reesei*	β-glucosidase II (Cel1a)	Single-nucleotide mutation (SNP)	Enhanced cellulase expression	[123]

Molecular biology tools for biofuel production

The search for biofuel genes by metagenomics

Since most environmental microorganisms have not yet been cultured, research into engineering these new species is crucial for comprehending the roles that microbiomes play in the generation of biofuels [125]. The increasing quantity of sequence data on metagenomic and meta-transcriptomic processes offers further potential for expanding microbial resource utilization [27]. Applying the metagenomics approach involves growing inaccessible microbial resources, identifying novel genome structures, finding physiologically active small compounds or biocatalysts, and establishing novel metabolic pathways [32,126]. To obtain the novel enzymes using metagenomic strategies for biofuel genes, developing a metagenomic library, extraction of genes encoding novel enzymes from the library, pre-conditioning of feedstock with native or engineered microorganisms, and determination of proper host and vector for gene cloning and protein expression are assessed [127,128]. For the determination of biofuel genetic elements, the metagenomic DNA obtained from the environmental samples is selected for library construction.

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The library is introduced in the genetically tractable host for heterologous expression. The selective host can allow a secondary metabolic process through its biosynthetic and regulatory mechanism. A selection pressure, such as compounds at inhibitory quantities or resistant substrates, is applied to the whole library of potential functional genetic elements, allowing the survival of hosts having active genetic elements solely. Secondary metabolic routes are excellent sources for the exploration of new enzymes because, in contrast with major metabolic pathways, their enzymes frequently catalyze specialized reactions with a variety of substrates [129,130] (Figure 2).

For constructing libraries of large DNA segments, extracted DNA fragments are inserted into appropriate vectors such as cosmids or fosmids (>40 kb insert size), bacterial artificial chromosomes (BACs), or yeast artificial chromosomes (YACs) (<40 kb insert size). For small DNA segments (≤15 kb insert size), plasmids are utilized. The ligated product is then transformed into a selective host, usually *E. coli,* to assess the target enzyme's expression [19,23,131]. Alternative hosts for characterization and expression of biofuel genes include *S. cerevisiae* [114], *Bacillus subtilis* [132], and *Trichoderma reesei* [133]. The microbial biocatalysts for biofuel production explored through metagenomic libraries include psychro-halophilic endo-1,4-xylanase [134], cellulases including GH5 family endoglucanases [32,135], lipases [23], and amylases [136].

Synthetic biology for biofuels production

To create and assemble unique biological systems to achieve scientifically specified objectives, the area of synthetic biology is an emerging field that effectively integrates science and engineering [137]. Sustainable assembly and alteration of DNA sequences based on homologous recombination, elimination, or ligation of sequences in a chosen host system can be used to create synthetic metabolic pathways [138,139]. Construction of heterologous expression hosts, design and construction of enzymes and pathways, data mining and analysis constitute the steps in biofuel production through synthetic biology [140,141]. A number of pathways in *Kluyveromyces marxianus* and *S. cerevisiae* have been reassembled. By using yeast artificial chromosomes (YACs) as hosts for *in vitro* recombination, substantial biofuel yields have been achieved [142]. Synthetic biology tools, rendered through DNA integration techniques and genetic modification, include a) syntrophic interactions, which create codependent channels in microorganisms; b) exogenous molecules, which regulate particular microbial attributes; and c) intercellular signaling, which establishes channels for interaction between microbes [143,144]. Synthetic biology tools are applied to microbes for the bioproduction of fuels, substrate consumption, and the creation of functional biomaterials [145,146]. *S. cerevisiae* [147], *E. coli* [148,149], *Corynebacterium glutamicum* [150], and *Bacillus subtilis* [151,152] are among the microbes that enhance the advancements in molecular biology tools and engineer novel biological systems through synthetic biology. Additionally, commercial synthesis of fourth-generation biofuels through microalgal strains modified by synthetic biology is being observed. Oleaginous microorganisms such as *Chlamydomonas reinhardtii and Microchloropsis gaditana* are being modified through this principle such that their ability of high-lipid output results in a considerable biofuel yield [153,154]. This strategy not only aids in the utilization of non-edible biomass for commercial production of biofuels but also in improved yield gains [155]. A brief overview of the synthetic biology process is shown in Figure 3.

Genome editing technologies (CRISPR-Cas) for microbial biofuel production

Clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins (CRISPR-Cas) technology is a prokaryotic molecular defense system that has emerged as an innovative technology for targeted microbial genomic engineering including site-directed mutagenesis for desired traits such as enhanced biofuel tolerance, inhibitor tolerance, and thermo-tolerance as well as modified cellulolytic enzymes [156,157]. CRISPR-Cas9 genome engineering entails a number of mechanisms; however, a few of the prominent strategies for improved biofuel production include:

1. Inhibition of competitive pathways as metabolic pathways can be affected by the end products as many biofuel products have antimicrobial activities in addition to being inhibitory predominantly due to their hydrocarbon-based composition [29,158]. For biofuel production, CRISPR-mediated repression was

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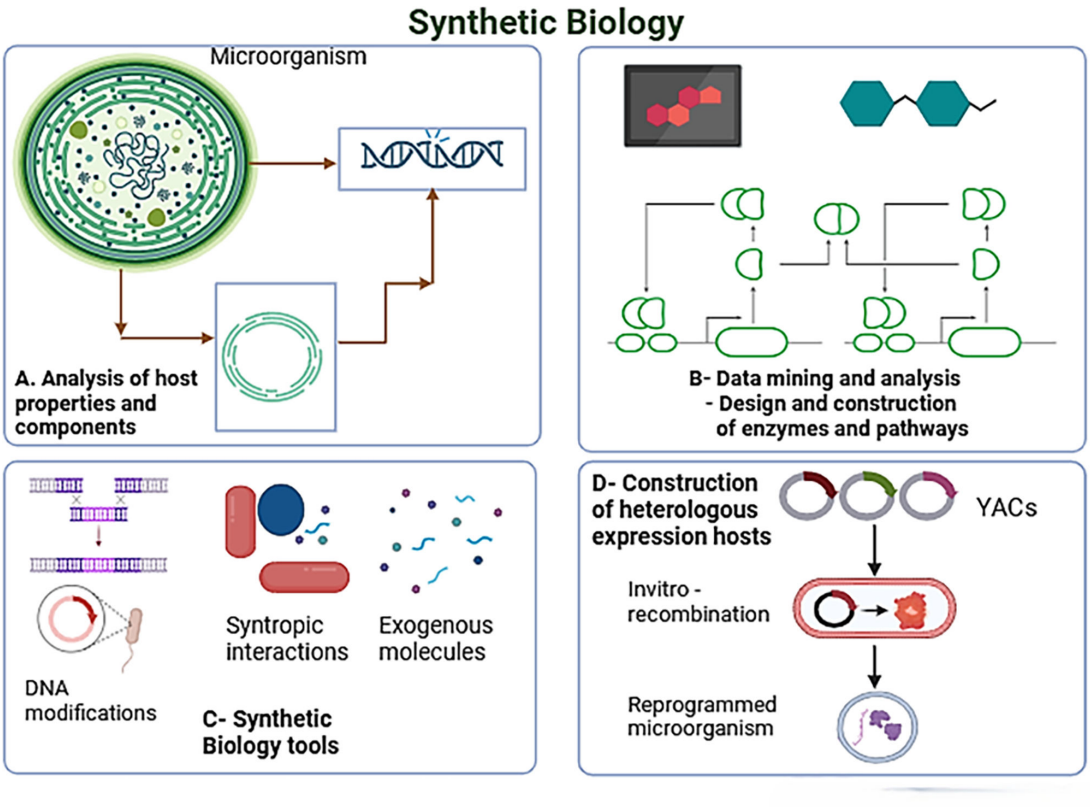

Figure 3: The synthetic biology approach for designing and engineering hosts for the efficient bioproduction of fuels.

used in *E. coli* by knocking down endogenous genes in competing pathways resulting in increased biofuel titer by 18–24%, and up to 98% enhancement in production was also observed [159].

2. Redirection of carbon flux towards improved biofuel synthesis is another essential CRISPR-mediated parameter. By repressing the citrate synthase expression in an *E. coli* strain, the biofuel production was enhanced, demonstrating that redistributing carbon flux through CRISPR is an efficient way of improving yield [160]. Likewise, in another study on CRISPR, through repression of competing pathway genes and redirecting the metabolic flux in *E. coli,* biofuel yield and productivity increased up to 5.4-fold [161].
3. Enhancement of substrate utilization capability using CRISPR is a viable strategy to develop microbial strains for biofuel production. By developing *Chlamydomonas* mutant strains using CRISPR-Cas9 technology, total lipid accumulation by dried biomass utilization was up to 28%, and an up to 27.2% increase in oleic acid production was seen which is an essential component in biofuel production [162].
4. Exploiting endogenous CRISPR-Cas9 to elevate the host specificity for biofuel production results in high transformation efficiency [156]. This system has been utilized for increased biofuel production with a productivity rise as reported up to 26.2 g/l in *Clostridium tyrobutyricum* using native CRISPR-Cas systems [163].
5. CRISPR/Cas9-engineered strains have also been utilized prolifically in microbes for enhanced biofuel yield, including *S. cerevisiae* yielding up to 74.7% as compared with the native strain [164]. CRISPR-Cas9 modifications such as gene knockout and competing gene repression in microbes have also demonstrated improved total lipid content, a prerequisite for biofuel production [165]. *Chlamydomonas reinhardtii* has shown an increase in lipid yield up to 64.25% through a Cas9-engineered strain [166]. Increased biofuel production through site-specific gene editing of a thermotolerance gene in extremophilic microorganisms has also been observed [165].

Although CRISPR has a precise mechanism of function that is cost-effective and has minimal side effects, research on optimizing genome-editing efficiency with fewer or no off-targets is still in progress for

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biofuel production [165–167]. Additionally, the development of a reliable process configuration is another major challenge that impedes biofuel production on a commercial scale [157].

Expanding the role of microbes in biofuel production: addressing energy and sustainability gaps

While significant progress has been made in developing molecular tools for microbial biofuel production, several challenges remain in achieving large-scale sustainability [168]. These challenges include barriers to feedstock utilization, limitations of biofuels like bioethanol, and the urgent need for sustainable alternatives to aviation fuel [169,170]. Overcoming these challenges will require continued advancements in metabolic engineering, synthetic biology, and CRISPR-Cas technology, as highlighted in this review.

Despite the potential of microbial biofuels in treating cellulosic biomass for biofuel production, key obstacles remain. Although lignocellulosic bioethanol has reached commercial-scale production (e.g., Raízen and GranBio bioethanol plants in Brazil), its limitations such as low energy density, water absorption, and the impact on land use and food security necessitate the development of alternative microbial biofuels [171,172]. Furthermore, lignocellulosic biomass requires costly pretreatment, and microbial fermentation often faces challenges in inhibitor tolerance and complete sugar utilization [173,174]. To integrate microbial biofuels into existing energy systems, improvements in fuel compatibility and production scalability are essential [175,176]. However, microbial-derived biofuels offer promising solutions due to their higher energy density, better fuel compatibility, and reduced carbon footprint [177]. To address the issues of transportation fuel and climate change, research is also focused on alternative fuel sources [178]. Bioethanol is unsuitable for aviation due to its oxygenated nature and low energy content. Therefore, hydrocarbon-based biofuels such as synthetic paraffinic kerosene (SPK), hydro-processed esters and fatty acids (HEFA), biodiesel, bio-oil, and Fischer–Tropsch H_2 are necessary [170,179]. Microbial engineering and synthetic biology approaches can optimize metabolic pathways to produce long-chain hydrocarbons, isoprenoids, and branched alkanes, which can serve as drop-in replacements for conventional jet fuel [29,180]. Ongoing research in process efficiency and strain optimization aims to ensure a sustainable and scalable alternative to fossil fuels [176].

Conclusion

In this review, a brief overview of the production of biofuels through microbial consortia is provided. Utilizing biofuels to lessen reliance on fossil fuels as an energy source is necessary to achieve a sustainable society. With increasing bioenergy demands in the transportation sector, sustainable biofuel production is also under consideration. When producing biofuels, microorganisms offer a great alternative with high productivity and high yields. The primary objective of subsequent research has been to optimize high output and energy value at a lower manufacturing cost by using molecular techniques to model strain development. Metabolic engineering and integrated engineering for biofuel production are a great source for efficient yield gains. Evolutionary engineering, in conjunction with metabolic engineering, is also receiving great attention. Moreover, as metagenomics and synthetic biology techniques continue to advance, there is a strong probability that further distinct metabolic routes for the synthesis of biofuels may emerge in the near future. These pathways may include screening and synthesis of novel enzymes. In addition to facilitating an increase in the titres, yields, and productive capacities of biofuels, further developments in metabolic engineering and synthetic biology will facilitate the creation of designed cell factories that will enable self-adaptation for biotechnological purposes. The advancements in CRISPR-CAS9 and genome editing technologies in addition to in-depth gene-scale studies are opening new doors to develop model microorganisms for biofuel productivity and commercial-scale biofuel usage.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ABE, acetone-n-butanol-ethanol; BACs, bacterial artificial chromosomes; GDL, glucan degradation locus; GHs, glycoside hydrolases; YACs, yeast artificial chromosomes.

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