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Synthetic biology and metabolic engineering paving the way for sustainable next-gen biofuels: a comprehensive review

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Biofuels are pivotal in transitioning to sustainable energy systems, offering renewable alternatives to fossil fuels with reduced emissions. This review examines the evolution of biofuel production, contrasting first-generation biofuels derived from food crops with second-generation biofuels from non-food lignocellulosic feedstock. This review evaluates social and environmental impacts, with a focus on land use, energy efficiency, and scalability. Advances in synthetic biology and metabolic engineering have revolutionized biofuel production by optimizing microorganisms like bacteria, yeast, and algae for enhanced substrate processing and industrial resilience. Key enzymes, such as cellulases, hemicellulases, and ligninases, facilitate the conversion of lignocellulosic biomass into fermentable sugars. CRISPR-Cas systems enable precise genome editing, while *de novo* pathway engineering produces advanced biofuels such as butanol, isoprenoids, and jet fuel analogs, boasting superior energy density and compatibility with existing infrastructure. Notable achievements include 91% biodiesel conversion efficiency from lipids and a 3-fold butanol yield increase in engineered Clostridium spp., alongside ~85% xylose-to-ethanol conversion in *S. cerevisiae*. However, commercial scalability is hindered by biomass recalcitrance, limited yields, and economic challenges. Emerging strategies, including consolidated bioprocessing, adaptive laboratory evolution, and AI-driven strain optimization, address these barriers. This review also explores biofuel integration within circular economy frameworks, emphasizing waste recycling and carbon-neutral operations. Multidisciplinary research is essential to enhance economic viability and environmental sustainability, ensuring biofuels play a central role in global renewable energy systems.

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1. Introduction

In the global efforts to move towards sustainable energy systems, using biofuels has been identified as a way to provide a renewable energy source and at the same time decrease GHG emissions.[1] According to the IEA (2023), biofuels accounted for approximately 3% of global transport fuel in 2022, with projections demanding a threefold increase to meet the Sustainable Development Scenario (SDS) by 2030.[2] Landmark studies such as the Bio-future Platform (2022) emphasize integrated biorefineries and waste valorisation as key enablers of this expansion. Biofuels, which include biodiesel, bioethanol, and biogas, are forms of bioenergy obtained from biological sources such as plants, algae, and waste.[3] In contrast to the conventional energy sources, which are exhaustible resources, these are renewable, which makes biofuels a fundamental element for a circular economy in energy.[4] Their contribution in decreasing ecological impacts is most important, since emissions from burning biofuels tend to have less net CO_2 due to the carbon sequestration that occurs during biomass maturation. Furthermore, the decentralized production of biofuels can improve energy security and resilience, especially in rural regions, as highlighted by recent IRENA policy analyses and national bioeconomy strategies.[5,6] However, conventional biofuel production has its drawbacks, which are both in its scale and sustainability. First-generation biofuels, produced from food crops like corn, sugarcane, and soybeans, are known to directly compete with food and thus fuel food insecurity, especially in regions experiencing a shortage of food. As illustrated in Fig. 1, the annual publication trends in biofuels and biofuel generation methods showed a significant increase until 2023, followed by a decline. The right graph presents the global distribution of biofuel-related research, with China, the United States, and India as leading contributors (data retrieved from the Scopus search).

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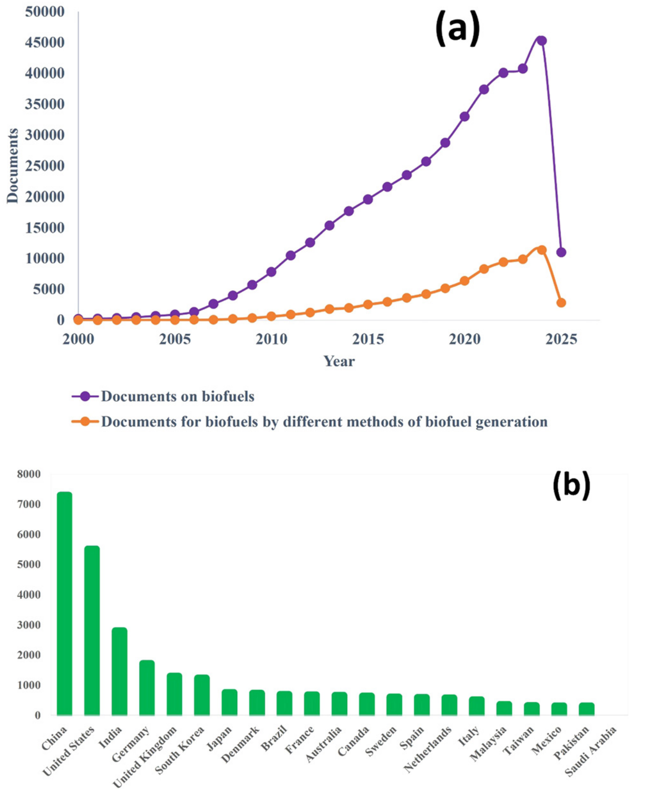

Fig. 1 (a) Annual publication trends in biofuels, along with different methods of generation based on scopus data and advances in biofuel production through synthetic biology and metabolic engineering, (b) the global distribution of biofuel-related research, with China, the United States, and India as leading contributors (source: data retrieved from the scopus).

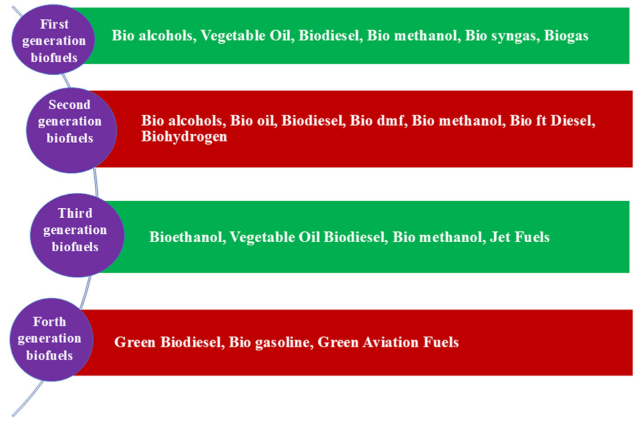

Fig. 2 An in-depth classification of biofuels: advancements from first to fourth generation, exploring feedstock, production technologies, and sustainability aspects.

Additionally, the demands for large, and sometimes excessive, amounts of land and water for the growth of these energy crops necessarily lead to deforestation, decline of soil quality, and thus, loss of biodiversity.[7,8] The production of biofuels entails high energy inputs, and the low energy returns of first-generation technologies add to their doubtful sustainability. Second-generation biofuels, which employ non-food feedstock like crop waste and straw, wood, and grass, are also not without difficulties: feedstock preparation is often elaborate; production costs are high; and conversion is not very efficient (as shown in Fig. 2).[9,10] These constraints show why new strategies must be developed to address the issues of biofuel efficiency and sustainability as well as its scalability. It has been realized that overcoming these limitations requires the advancement of genetic engineering as a technique for the next phase of advancement in the production of biofuels.[11,12] Using molecular biology tools, it is possible to genetically optimise microorganisms, algae, and energy crops that will increase the efficiency in biofuel production. Biotechnology means that microbial metabolism can be engineered to efficiently convert sugars to bioethanol or lipids to biodiesel.[13,14] For instance, new bacterial and yeast hosts with enhanced sugar conversion rate and tolerance to inhibitors generated during hydrolysate production. In the same way, genetic engineering to increase the lipid content of algae has made biodiesel production from algae a prospect that can occupy comparatively less land than terrestrial crops. In energy crops, the ability to edit genes at will using CRISPR-Cas9 is making it possible to improve biomass production, increase photosynthesis rates, and reduce the amount of lignin, which make it easier to turn energy crops into biofuels.[15,16]

Biofuel production depends on the feedstock type, which results in four distinct generations that have specific strengths and weaknesses. This part reviews the technological systems behind biofuels, along with their environmental effects and their practicality against traditional fossil fuel usage.

1.1 First-generation biofuels

First generation biofuels were produced from food crops, including sugarcane, corn, and vegetable oils, using established methods such as fermentation and distillation, and transesterification. The established production methods for biofuels receive criticism because edible biomass consumption creates conflicts with food production and land allocation. The fuel-centered production method results in decreased resource efficiency because non-fuel waste products often end up discarded as waste. First-generation biofuels continue to be popular because they are produced using mature technology and benefit from existing production facilities.

1.2 Second-generation biofuels

Biofuels of the second generation use microbes and non-edible crops as materials instead of traditional food plants to present a more environmentally friendly approach. Modern technologies, including membrane filtration and integrated biorefineries, increase fuel production and reduce both energy needs and waste formation. Biofuel production processes use thermophilic and mesophilic microorganisms through batch or continuous operations for biofuel synthesis as well as organic acid and amino acid production. Second-generation biofuels maintain their development phase because proven research shows they outperform traditional biofuels in terms of environmental impact alongside improved economic benefits.

1.3 Third-generation biofuels

Microalgae serve as the source for third-generation biofuels because they generate more biomass and oil content than conventional crops. The production of algal biofuels requires two main methods, which combine algal oil transesterification with hydrotreatment processes. Biofuels resolve the food-*versus*-fuel dilemma by using unsuitable land and wastewater sources, which decreases environmental pressure. The assimilation of pollutants through microalgae-based systems creates twofold environmental advantages for waste management. Scale-up issues and production expenses create obstacles for the widespread implementation of this technology.

1.4 Fourth-generation biofuels

Fourth-generation biofuels represent the latest biofuel technology through the use of genetically modified (GM) algae and photobiological solar fuels and electro-fuels. The genetic modification of GM algae leads to improved photosynthetic efficiency and enhanced lipid accumulation and cell rupture through autolysis, which simplifies oil extraction processes. A suite of advanced genome-editing instruments, which include CRISPR/Cas9, TALEN, and ZFN, make it possible to perform exact adjustments to metabolic pathway networks. Solar-derived feedstock provides large quantities and affordable costs, which make fourth-generation biofuels a viable, sustainable energy option for a long time. Genetic modification needs additional study along with appropriate policies because of its related ethical and regulatory issues.

These generational approaches demonstrate that higher technological advancement comes at the expense of less sustainable characteristics. Current biofuel markets are led by first-generation products, but their environmental and socioeconomic problems push the industry toward developing more advanced generations. The development of sustainable biofuels in their second and third generations still needs to improve their operational readiness for production-scale deployment. Biofuels of the fourth generation demonstrate tremendous potential even though they are still under development because they integrate renewable energy and synthetic biology. Development in biofuels should concentrate on developing affordable production solutions, integrating improved algal growth facilities together with waste-to-energy conversions and hybrid biorefinery system designs. Biofuel energy economy needs R&D funding alongside effective policy decisions to move past fossil fuels. In summary, while first-generation biofuels rely on food crops and conventional processes, second- and third-generation biofuels emphasize using non-food lignocellulosic and algal feedstock, respectively. Fourth-generation and next-gen biofuels integrate synthetic biology to create drop-in fuels, hydrocarbons that are fully compatible with existing engines and infrastructure, produced from engineered microbes capable of utilizing carbon dioxide or industrial waste streams (in Table 1(A)).

Table 1 A. Comparison of biofuel generations: feedstock, technologies, yields, and sustainability metrics. B. Progress, issues, and prospects of biofuel production technologies[17]

Generation	Feedstock type	Technology	Yield (per ton feedstock)	Sustainability
A				
First	Food crops (corn, sugarcane)	Fermentation and transesterification	Ethanol: 300–400 L	Competes with food and high land use
Second	Crop residues and lignocellulose	Enzymatic hydrolysis and fermentation	Ethanol: 250–300 L	Better land use and moderate GHG savings
Third	Algae	Photobioreactors and hydrothermal liquefaction	Biodiesel: 400–500 L	High GHG savings and scalability issues
Fourth	GMOs and synthetic systems	CRISPR, electrofuels and synthetic biology	Varies (hydrocarbons, isoprenoids)	High potential and regulatory concerns

Aspect	Key roles	Impact
B		
Genetically engineered microorganisms (GEMs)	Tailoring metabolic pathways for enhanced biofuel production. Utilizing diverse feedstock like lignocellulosic biomass. Reducing byproduct formation.	Improved biofuel yields. reduced ecological footprint. Scalable and sustainable energy solutions.
Enzymatic innovations	Development of thermostable and pH-tolerant enzymes. Optimization of lignin-degrading enzymes. Co-catalytic systems.	Efficient hydrolysis of cellulose. Utilization of recalcitrant feedstock. Cost reduction in bioconversion processes.
Synthetic biology approaches	Precision manipulation of pathways using tools like CRISPR-Cas9. Design of biosynthetic circuits for CO_2 conversion.	Production of advanced biofuels. Alignment with decarbonization goals. High energy density fuels.
Challenges	Economic feasibility, reliance on agricultural feedstock, technical bottlenecks, and regulatory hurdles.	Need for innovation in process optimization, feedstock diversity, and societal acceptance.
Future directions	Leveraging AI for enzyme and pathway discovery. Expanding non-food feedstock. Enhancing policy and international cooperation.	Accelerated R&D, improved sustainability, and broader biofuel adoption.

In addition, synthetic biology has given rise to designing new metabolic pathways whereby microorganisms can produce the next generation of biofuels, such as butanol, isopropanol, and hydrocarbons that are similar to petroleum products.[18] These are next-generation biofuels with higher energy content and a better fit with existing fuel systems and networks. Another aspect of genetic engineering is used to minimize the impact of the environment on the production of biofuel.[19] Specific engineered strains of microorganisms can metabolize unconventional substrates, including industrial and municipal waste, which adds up to the waste value-added while minimizing feed competition with food sources. Furthermore, scientists are trying to optimize microorganisms to capture carbon during biofuel production processes, which would turn the biofuel systems carbon negative.[20,21] A schematic illustrates strain improvement strategies for biofuel and valuable chemical production through biochemical and metabolic engineering approaches. Biochemical engineering optimizes growth conditions by regulating CO_2, temperature, salinity, and nutrient availability, while metabolic engineering employs genetic modifications to enhance biosynthetic pathways. The integration of these approaches enhances microbial efficiency, facilitating sustainable biofuel production and industrial biochemical synthesis (Fig. 3).

However, some challenges define the application of genetic engineering in biofuel development also discussed in Table 1(B). These include: regulation, biosafety, and acceptance of GMOs as a source of bioenergy.[22] The key challenge that remains is scaling these engineered systems to industrial levels while staying affordable. However, the prospects of integrating genetic engineering with other related technologies like artificial intelligence and bioprocess optimization provide a clue to the future of biofuel production.[23–25]

Thus, the use of biofuels as part of the sustainable energy mix is one of the most important key aspects to stabilize the climate and decrease the usage of fossil fuels. However, the problems that are inherent to the conventional biofuel production process must be addressed to afford the full potential of biofuels.[26] These seemingly insurmountable challenges can be addressed by genetic engineering, which provides a toolbox to produce better, cleaner, and superior biofuels.[27] Biofuel production will be able to bring a significant change to energy transition and environmental conservation since it combines genetic modification, waste utilization, and unique bioprocessing strategies. This review analyses the scientific basis for biofuels, the challenges in traditional processes, and the potential of genetic engineering for creating the biofuel of the future.

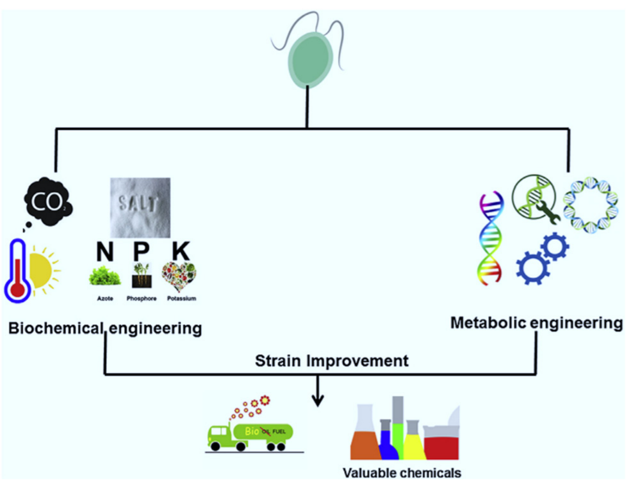

Fig. 3 Biochemical and metabolic engineering in microalgae for the production of biofuels and valuable chemicals. Reproduced from ref. 21 with permission from [Elsevier], copyright [2019].

2. Genetically engineered microorganisms for biofuel production

Microorganisms as a tool for biofuel production: a renewed focus on cleaner energy solutions has accelerated the search for new substitutes to conventional sources of energy, such as fossil fuels, and biofuels are viewed as a perfect solution to the problem. GEMs play a significant role in enhancing the ability to produce higher yields,[28,29] efficiency, and resource sustainability in biofuel production. This section discusses the engineering of bacteria, yeast, and algae for biofuel production, their features and capabilities, the issues arising, and the scientific developments that make them viable large-scale solutions, also shown in Fig. 4.[30,31]

Escherichia coli and Clostridium spp. have attracted more interest due to their capability to produce bioethanol and biobutanol, respectively.[32] *E. coli* has been used in synthetic biology for a long time due to its easy genetic manipulation and short doubling time. The manipulation of the *E. coli* genetic code has made it possible to produce non-indigenous enzymes and pathways for the transformation of lignocellulosic materials into bioethanol and biobutanol.[33] A key activity to enhance cellular economy is the fine regulation of metabolic pathways that have enhanced processes and minimized byproduct formation and substrate misutilization shown in Fig. 4. Likewise, butanol-producing Clostridium spp. has also been a subject of interest for metabolic engineering to enhance production of the desired product.[34,35] Optimization strategies are also concerned with the redox potential, distribution of carbon, and the tolerance of organisms to toxic byproducts such as butanol, which is toxic to the microbes. The engineering of these bacteria[36] by multiple genetic manipulations has been made easier by the modern genetic techniques, including CRISPR-Cas9 and multiplexed genome editing.[37] Fig. 5 illustrates the integration of genetic engineering approaches for optimizing microbial biomass productivity, utilizing cyanobacteria and bacteria for biofuel synthesis. The process involves DNA modification and biowaste utilization, followed by chemical processing to enhance biofuel yield, supporting sustainable energy production.

Among the microorganisms used in industrial biotechnology, yeasts, especially *S. cerevisiae*, are perhaps the most popular because of their efficient fermentation characteristic.[38] However, native yeast strains have some drawbacks in the degradation of

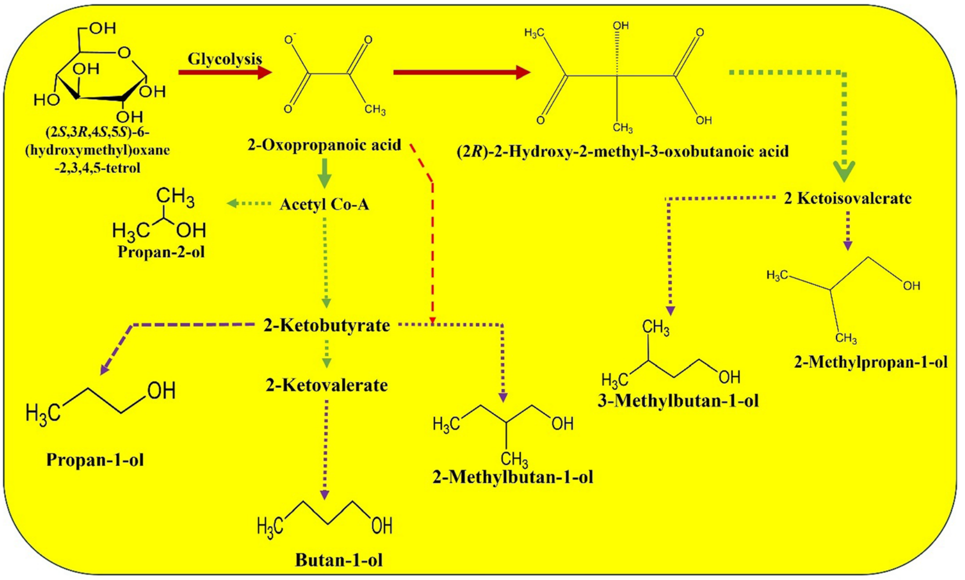

Fig. 4 Metabolic pathways for the biosynthesis of higher alcohols: analyzing glycolytic intermediates, acetyl-CoA derivatives, and their conversion into industrially relevant biofuels. Under the terms of creative commons attribution-non-commercial-share alike 3.0 unported license.[31]

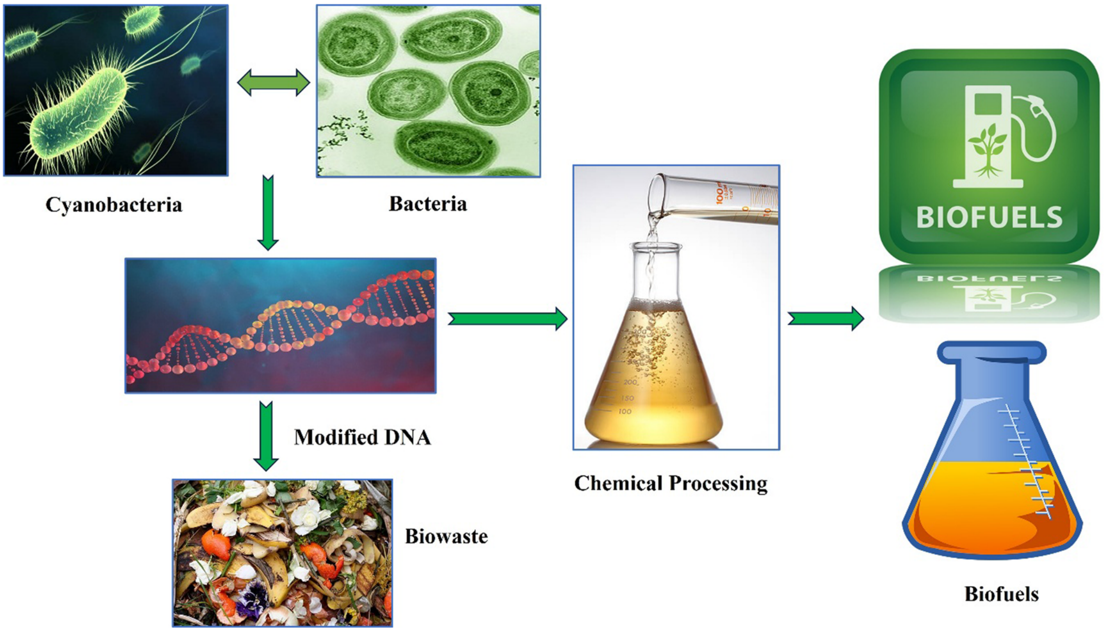

Fig. 5 Advanced genetic engineering strategies for enhancing microbial biomass productivity: a sustainable approach to optimizing biofuel generation and industrial biochemical synthesis.

lignocellulosic biomass, which is one of the most used feedstock for bioethanol production. The ability of engineering *S. cerevisiae* to metabolize pentoses such as xylose and arabinose, and hexoses such as glucose, has greatly boosted its use in bioethanol production. The metabolic engineering approaches have included the expression of heterologous sugar utilization pathways, increasing the efficiency of transporters, and optimizing the regeneration of cofactors to increase ethanol production.[39,40] In addition, enhancements for genetic stress tolerance for furfural inhibitors, acetic acid, and high ethanol concentrations have broadened the use of yeast in the industrial sector (in Fig. 6). The engineered yeast strains contain stress-responsive regulatory networks and overproduction of protective proteins, which allows large-scale fermentation to be cost-effective for biofuel production.[41]

The current methods of genetic engineering used for these microorganisms are presented regarding synthetic biology

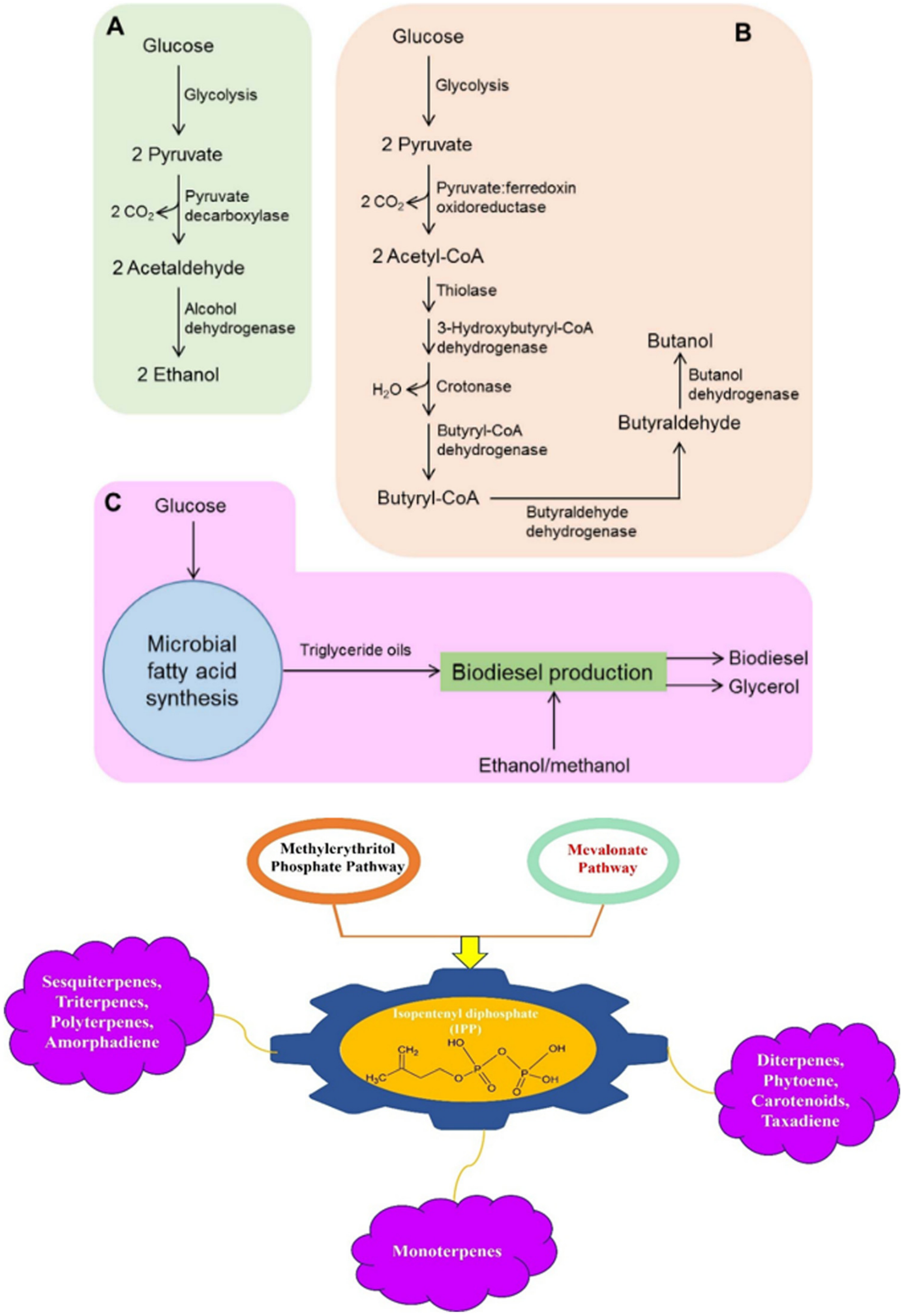

Fig. 6 Major pathways for the production of: ethanol (A); butanol (B); biodiesel (C); and isoprenoid fuel precursors. Reproduced from ref. 40 with permission from [Elsevier], copyright [2018].

tools, omics, and metabolic modelling based on machine learning. The industrial applications of engineered strains, the issues of scale-up, substrate specificity, and biotechnological impacts are also looked into.[42] Genetically engineered microorganisms offer the potential to make the biofuel production process more effective, environmentally friendly, and profitable with the help of the enhanced knowledge and capabilities in the control of microbial systems.[43]

3. Enzymatic innovations in biofuel development

The increasing concern for the availability of sustainable energy resources has led to an increased exploration of enzymatic processes for biomass conversion and the development of improved biofuels.[44] These enzymes are of great importance in the conversion of lignocellulosic biomass into higher value products for better energy and fuel security. Among these enzymes, cellulases, hemicellulases, and ligninases are some of the most important enzymes that can effectively degrade the difficult-to-break-down polysaccharides and lignin present in plant biomass, such that there will be effective release of fermentable sugars.[45]

Among these enzymes, cellulases are particularly important for the hydrolysis of cellulose, which is one of the principal constituents of LCB materials. But their efficiency can be hampered by factors such as thermal instability, sensitivity to acidic environments, and substrate complexity.[46,47] To improve the efficiency of cellulases, methods of protein engineering and directed evolution are used to optimize the activity, stability, and substrate selectivity of the enzymes. New developments have been aimed at the development of thermostable and acid-stable cellulases, which have the ability to operate in industrial environments. These engineered enzymes are characterized by their ability to function effectively under high temperature and low pH conditions that are typical in industrial biomass pretreatment.[48] Such advancements have greatly improved the effectiveness of enzymatic hydrolysis, making the cost of biofuel production affordable. Besides cellulases, hemicelluloses and ligninases are also important components of the plant cell wall degradation. Hemicelluloses act on hemicellulose, a non-uniform polysaccharide; they cleave the latter into simple sugars. Ligninases, however, break down lignin, an aromatic polymer whose presence limits the availability of cellulose and hemicellulose to the enzymes. The biotechnological modification of ligninases has recently become an attractive strategy to enhance lignin degradation, thus facilitating the release of fermentable sugars.[45] The synergistic use of enzyme complexes, including cellulases, hemicellulases, and ligninases in optimum ratios, has also boosted the general efficiency of biomass conversion. This integrated strategy tackles the refractoriness of lignocellulosic biomass and optimises the sugar recoveries to enhance the viability of biofuels at an industrial scale.

Apart from biomass degradation, enzymes have been crucial in the synthesis of advanced biofuels. For example, lipases that are glycerol ester hydrolases are used in the conversion of biodiesel by transesterification reactions.[49] These enzymes help to catalyse the transesterification of triglycerides from vegetable oil and animal fats into fatty acid methyl esters, the composition of biodiesel. The advantages of using lipases include the following: mild reaction conditions, high selectivity, and low formation of unwanted products. Recent improvements in the methods of enzyme anchoring and protein modification have improved the stability and recyclability of lipases, which has made the process more economical for the large-scale biodiesel production depicted in Fig. 7.[50] Decarboxylases and dehydrogenases are among the enzymes used in the biosynthesis of alcohol-type biofuels, including ethanol and butanol. Decarboxylases are enzymes that eliminate carboxyl groups from organic acids, and dehydrogenation enzymes need to act on the important redox processes involved in alcohol synthesis.[51] These enzymes play a crucial role in the engineered microbial pathways for producing biofuels; the sugars are converted directly to alcohol fuels. Such enzymatic pathways have been targeted in metabolic engineering and synthetic biology to improve the yield and productivity of alcohol fuels. These developments also help in the diversification of biofuel feedstock as well as fit well in the shift towards sustainable energy systems worldwide.[52]

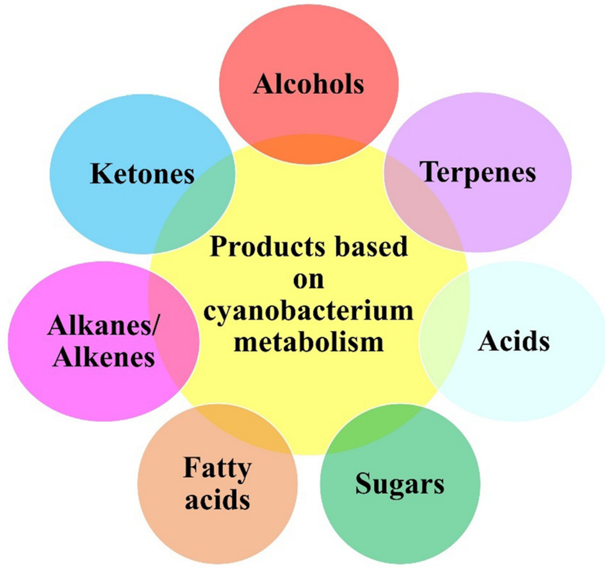

Fig. 7 List of natural products metabolized by cyanobacterial cells.

3.1 Enzyme engineering and directed evolution

Protein engineering today produces durable and efficient enzymes to support efficient biofuel creation.[53] Scientific teams improve enzymes by using both directed evolution and rational design methods, which boost enzyme capabilities. The procedure of directed evolution combines repeated changes in enzyme genes with performance selection under plant operations to achieve better results.[54] Rational design works with computer models of structures to apply specific edits that improve enzyme performance. Scientists succeed in engineering enzymes for efficient biomass hydrolysis and biodiesel synthesis by developing stronger cellulases, hemicelluloses, and lipases. Better enzyme engineering methods for cellulases and hemicelluloses enhance their ability to convert lignocellulosic biomass into simple sugars, which then produce more ethanol and butanol. Enhanced lipase variants help make more biodiesel from plant and algal oil through transesterification. Biofuel production benefits more from today's enzyme research and development progress.

3.2 Enzyme immobilization for enhanced efficiency

Researchers use enzyme immobilization to boost industrial biofuel production because it protects enzymes from damage while making them reusable.[55] By placing enzymes into capsules or bonding them to solid surfaces, our method prevents them from losing their natural function and denaturing. Enzyme protection in a special environment helps the enzyme stay active during long, ongoing bioconversion processes. New materials and polymer supports enhance enzyme performance because they help substrates move freely and make better enzyme-substrate connections.[56] The use of silica nanoparticles, MOFs, and carbon-based supports shows remarkable potential since they offer large surface areas, tunable properties, and high enzyme loading capacity. Enzymes attached to these carriers maintain better stability and work more effectively in the production process. Despite the many benefits of enzyme immobilization discussed previously, this technology still faces major obstacles that affect its general use.[57] The enzyme activity often decreases during immobilization, especially when enzymes are connected to macromolecular carriers, which reduces their ability to function properly. The effectiveness of immobilized enzymes remains limited by four major problems, including slow movement of reactants, enzyme release from the support, expensive production costs, and issues in large-scale production. Scientists must keep studying enzyme immobilization methods to enhance performance while making these systems work better in multiple industries. Fig. 8 shows all the benefits and challenges associated with enzyme immobilization.

The process of enzyme attachment to carriers lowers production expenses and protects enzymes from loss during long-term manufacturing operations, which helps make large-scale biofuel production financially viable and eco-friendly.[58,59] The biofuel industry gains better results and reduces its resource usage through advanced immobilization processes. Fig. 9 shows an illustration of the strategies of enzyme immobilisation using agrowaste nanocarriers.[57]

Protein engineering helps industrial biotechnology by improving enzyme functions to handle tough industrial conditions. This article studies how rational design and directed evolution work together with semi-rational methods to enhance enzyme performance.[60] Machine learning now helps these methods produce better results. Engineered PETases show how protein engineering helps solve important environmental cleanup objectives, like plastic recycling and benefits.[61,62] Our standard ways of making enzymes have produced solid progress, yet they have controlling aspects like: rational design needs structural data, while directed evolution struggles with large variant space and experimental issues, plus semi-rational strategies demand complete evolutionary knowledge. Machine learning can solve important problems, but it needs extensive data and strong computing power to work efficiently. The large-scale production of enzymes faces practical problems because it requires expensive facilities and makes the process too expensive to use. Research teams combining computer enzymes and

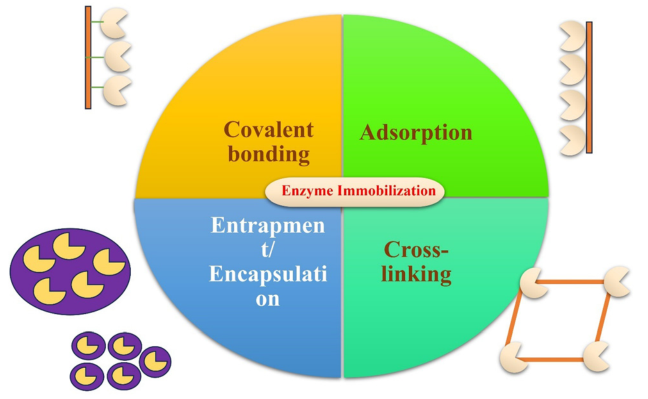

Fig. 9 Advanced strategies for sustainable enzyme immobilization on agro-waste-derived nanomaterials: enhancing catalytic efficiency, stability, and reusability for biofuel production and green energy applications.

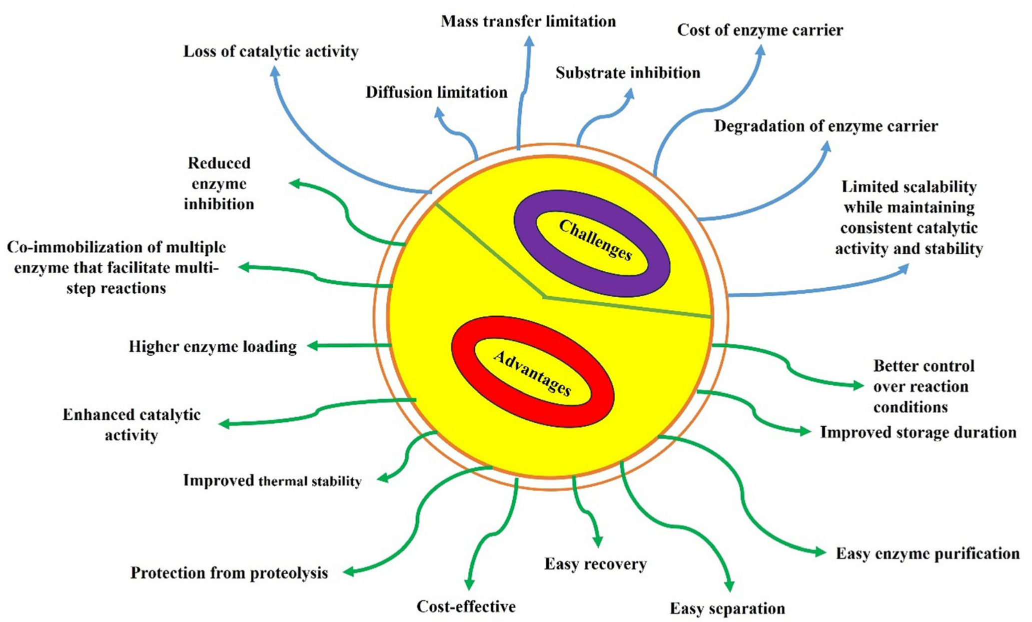

Fig. 8 Comprehensive evaluation of the challenges and advantages of enzyme immobilization utilizing agrowaste-derived nanocarriers for enhanced catalytic efficiency, stability, and sustainable industrial applications.

experiments with AI systems, such as deep learning, can speed up enzyme ancestry development and design industrial applications.[63] Future work must make protein engineering more cost-effective and scalable, plus improve its life-cycle assessment to make protein engineering the core of industrial biotechnology and produce new sustainable biocatalysis methods.[64]

3.3 Metabolic engineering of microbial systems

Synthetic biology and metabolic engineering technologies now allow us to use microorganisms better for making biofuels. Engineers use genetic methods to improve enzyme production and biofuel output in yeast and bacterial microorganisms. Scientists use CRISPR-Cas9 technology to edit metabolic networks and make both fermentations better and metabolic pathways direct more resources toward biofuel components.[65] Ethanol, butanol, and advanced biofuel production improved through research that modified enzyme systems affecting carbohydrate metabolism, lipid creation, and energy equilibrium in microorganisms. Studying metabolic flux helps find weak points in metabolic pathways while determining gene targets for genetic modification.[66] These methods help create efficient microorganisms for industry that produces more fuel while staying strong under production conditions. Metabolic engineering of microorganisms offers an effective way to use CO_2 because it helps fight greenhouse gas emissions in a renewable manner. This report studies how engineering both natural and synthetic CO_2-fixation pathways improves their ability to capture carbon dioxide.[67,68] Researchers boost metabolic processes by managing metabolic pathways and making cofactors more available, together with controlling genes that manage stability and effectiveness. The process of CO_2 fixation moves forward through evolutionary changes, while enzyme enhancement plus additional CO_2 gathering systems contribute to its development. Research now allows for creating useful materials like fuels, plastics, and chemicals from CO_2 using both natural and modified natural microbial organisms to support environmental sustainability.[69,70]

3.4 Microbial consortia for synergistic bioconversion

Companies create engineered microbe groups to improve their work to transform biomass into fuels. When several microorganisms work together, they utilize many metabolic pathways that achieve faster conversion of complex biomass to fermentable sugars.[71] Researchers achieve better performance in enzyme treatments through co-culturing and build higher biofuel production levels. A series of microbial strains working together enable the network-like breakdown of plant waste by passing needed steps between members. A group of microorganisms work together to produce different enzymes, which help increase the process capacity without requiring extra enzyme input. These systems withstand changes in production materials and process settings which makes them useful in industrial processes. Our better understanding of how microbes work together enhances our chances of making biofuel production more effective and sustainable at the same time.[72]

With the growing global energy demand, the lack of fossil fuels, and the increasing carbon emissions, there is a need to find alternative sustainable energy sources. Advanced technologies that turn biomass into biofuels instead of relying on fossil fuels provide a promising way to use biorefineries. To study bacterial biomass and biodiesel production using an integrated biorefinery approach, this study has sampled 96, 93 and 98% for CO_2, SO_2, and NO, respectively, and cultivated 274 g of bacterial biomass in a 20 L bioreactor. Biodiesel was produced at 91% w/w conversion efficiency from the extracted lipids (58% w/w). To improve lipid production, metabolic pathway analysis was carried out, and a life cycle assessment of the process was performed. Alternative and safer chemicals were also incorporated to mitigate adverse effects and to decrease global warming potential (GWP100). In addition, the capital investment of a bacterial biorefinery was compared with conventional fuel refineries based on a techno-economic analysis, which showed a net present value of $193 per liter of biodiesel. Bacterial biorefineries are demonstrated as a key component in the creation of a circular economy through the promotion of sustainable practices, reduction of waste, efficient use of resources, and reuse and recycling of materials in order to create a more sustainable energy landscape.[73] A recent study by Yadav *et al.* demonstrated a 91% biodiesel conversion efficiency using bacterial biomass in a 20 L bioreactor with a lifecycle GHG reduction of 65% compared to fossil diesel. Furthermore, a techno-economic analysis indicated a net present value (NPV) of $193 per liter, demonstrating economic feasibility (optimization of bacterial biorefineries for sustainable biodiesel production and flue gas reduction: a holistic approach to climate change mitigation and a circular economy). Comprehensive techno-economic analyses (TEA) and life cycle assessments (LCA) are essential tools for evaluating the commercial feasibility and environmental impacts of biofuel technologies. These methods enable stakeholders to balance cost-efficiency with sustainability metrics, guiding investment and policy decisions.

Researchers use multiple methods to enhance biofuel output by modifying enzymes, trapping them in specific areas, altering metabolic processes, and combining different bacteria types. Research teams and industry partners can solve biofuel production problems by using these advanced methods to develop renewable energy better.[74,75]

Therefore, it can be concluded that the use of enhanced enzymatic technologies in biomass conversion and biofuel generation is a revolutionary solution to the global energy challenge. Engineering cellulases for better performance, creating thermostable and acid-stable enzymes, and, more importantly, synergistic enzyme combinations have come a long way in improving the enzymatic hydrolysis of lignocellulosic biomass.[57,76] At the same time, lipases, decarboxylases, and dehydrogenases involved in biodiesel, alcohol fuel synthesis just show that enzymes are perfect to promote biofuel synthesis. Further advancements in enzyme engineering and process optimization may well usher in new changes to the biofuel industry and set the stage for a more environmentally friendly world.[50,77]

4. Synthetic biology approaches

Synthetic biology refers to the systematic application of engineering concepts to design new biological systems, or redesign existing systems to perform a set function or task.[78,79] The foundation of synthetic biology is the application of sophisticated tools and techniques that control the biological components at the genetic and metabolic levels in order to achieve particular goals, including increasing efficiency, output, or even the ability to create brand-new functions. However, three pivotal aspects of synthetic biology have to be explored: CRISPR-Cas systems, artificial metabolic pathways, and metabolic engineering in host organisms and systems that are revolutionizing the biotechnology and industrial sectors.[80]

While genome editing tools such as CRISPR-Cas systems have become the latest tools in synthetic biology. CRISPR-Cas systems that are derived from bacterial innate defenses are highly specific, efficient, and inexpensive platforms for genome editing. These systems consist of two primary components: a guide RNA (gRNA) that guides the Cas nuclease to a particular DNA sequence and the Cas enzyme that then makes clean cuts on the DNA.[81] Additional cellular repair mechanisms make it possible to carry out selective changes such as gene addition, deletion, or substitution. CRISPR-Cas not only works for gene editing but also for transcription and epigenetic control, and base editing. Enabling further enhancement of elucidation is the development of variations to include CRISPRa (activation) and CRISPRi (interference) that afford more comprehensive control of gene manifestation. Furthermore, new advances in the CRISPR tools, namely prime editing and base editing, are promising to provide much more control and finesse. These advancements enable the researchers to develop genetically engineered organisms for use in particular applications, including enhancing the quality of the crops, and designing microbes for biofuel production.[82]

The synthetic metabolic paths represent another substantial domain of synthetic biology that allows the synthesis of valuable compounds and increases biological output (Table 2). These pathways are intended to overcome natural constraints and include fresh enzymatic reactions to increase the yield of the target products. This is attained by synthetic biologists, who insert enzymes from various organisms into a single host organism to give it a desired metabolic network.[83] For example, in the synthesis of biofuels, pharmaceuticals, and fine chemicals, artificial pathways have greatly enhanced the precursor feed and intermediate conversion. In addition, there are several important applications of computational tools in the design of metabolic pathways, with the ability to predict the rates of metabolic conversion, to determine the limitations, and to model possible scenarios before practicing the experiment. One successful application in this area is the genetic engineering of microorganisms for the large-scale synthesis of artemisinin, an essential antimalarial compound. This was achieved by reconstructing and enhancing the metabolic pathway for artemisinin synthesis in yeast, thus providing a proof of concept for using synthetic pathways to solve some of the major problems faced by humanity today, including drug availability and sustainability.[84]

The remaining metabolic engineering approaches in host organisms also support genome editing and artificial pathways. Bacteria, yeasts, plant cells, and many other organisms are used as host organisms to produce many valuable products. Metabolic engineering is the process of designing and constructing changes to an organism's metabolic pathways to improve the synthesis of target products or to generate entirely new capabilities. This is done through a simultaneous design of

Table 2 Advancements in metabolic engineering for biofuel production: synthetic biology view[42,85]

Approach	Description	Key organisms	Advantages	Challenges
Metabolic engineering	Modifying metabolic pathways in microorganisms to enhance biofuel production.	*E. coli*, Saccharomyces cerevisiae	Increased yield and productivity.	Balancing metabolic flux and potential toxicity to host cells.
Synthetic pathways	Designing and introducing entirely new biosynthetic pathways for novel biofuels.	Cyanobacteria, Yeast	Enables the production of non-natural biofuels.	Complexity of pathway integration and need for extensive pathway optimization.
Gene editing (CRISPR/Cas9)	Precise modification of genetic materials to enhance traits related to biofuel production.	Various bacteria and algae	High precision, reduced off-target effects.	Regulatory hurdles and ethical concerns.
Synthetic genomes	Constructing fully synthetic genomes to create optimized microorganisms for biofuel production.	Mycoplasma mycoides	Full control over genetic content and elimination of non-essential genes.	Technical complexity, high cost, and ethical considerations.
Directed evolution	Iterative rounds of mutagenesis and selection to evolve enzymes with improved biofuel production efficiency.	Various microbes	No need for prior knowledge of enzyme structure and broad applicability.	Time-consuming and may require high-throughput screening facilities.
Chassis organisms development	Engineering robust microbial platforms (chassis) that can host synthetic biofuel pathways.	*E. coli*, Yeast	Customizable and scalable and supports diverse metabolic pathways.	Developing versatile and resilient chassis organisms can be resource-intensive.
Synthetic ecology	Engineering microbial consortia to cooperatively produce biofuels.	Mixed microbial cultures	Exploits the natural division of labor and resilience to environmental changes.	Managing interactions and stability within consortia.
Phototrophic systems	Utilizing light-driven synthetic pathways in photosynthetic organisms for biofuel production.	Cyanobacteria, Algae	Direct conversion of sunlight into biofuels and renewable and sustainable.	Low conversion efficiency and dependency on light conditions.

experimental approaches, based on systems biology, and cycles of design and synthesis. The three main strategies are the overexpression of enzymes, which act as rate-limiting enzymes, knocking out competing pathways, and the control of metabolic fluxes in the network.[86] Recent advances in synthetic biology tools, including CRISPR-Cas systems, artificial promoters, and RNA-based regulators, have greatly improved metabolic engineering tools and strategies. Furthermore, given that omics technologies include genomics, transcriptomics, proteomics, and metabolomics, various host organisms can be quantitatively characterized, and the data thus obtained can be used for metabolic reprogramming. This combination of approaches has produced advances in many areas, including green chemistry for chemical production, bioremediation, and agricultural-biotechnology.

The synergy between genome editing, artificial pathways, and metabolic engineering is putting innovation in the synthetic biology system in motion.[87] When these approaches are integrated, researchers can build optimised, bespoke organisms to tackle problems that the world is facing today. For instance, carbon capture and conversion using microbial cell factories for the production of valuable chemicals and materials are applications that benefit from the synergistic action of these strategies. In the same way, synthetic biology has great potential to revolutionize different spheres of healthcare, biopharmaceutical production, the application of an individualized approach, and the creation of living drugs. However, all these advancements come with ethical and biosafety issues yielded by new science and technologies, and therefore they call for ethical and biosafety practices of research.[88]

Therefore, synthetic biology is a new approach in engineering life at the molecular level. Instrumental systems such as CRISPR-Cas systems, artificial metabolic pathways, and modern metabolic engineering approaches offer a powerful arsenal to address many of the most significant issues in science and industry. It provides additional information on these dynamical strategies and sheds light on their fundamental concepts, development in the recent past, and uses in different disciplines. Synthetic biology is the way to find a new approach to the sustainable development of technologies and innovations.[89,90]

5. Global biofuel sustainable development scenario (SDS)

SDS from the International Energy Agency presents an approach to fight climate change through transitioning energy systems while decreasing air contaminants (IEEFA, 2022). SDS objectives need biofuel production to expand three-fold through 2030, resulting in 280 million tonnes of oil equivalent (Mtoe) or 10% of total transportation fuel demand, which exceeds the current 3% (IEA, 2019). The recent 6% growth of biofuel production to 96 Mtoe (161 billion liters) in 2019 did not reflect the necessary 10% annual expansion required to reach the 2030 targets. The transport sector maintained its long-term yearly growth rate of 1.7% starting from 1990, while adding 3% more CO_2 emissions in 2022 because of pandemic recovery activities. According to the Net Zero Emissions by 2050 Scenario, emissions need to be reduced by more than 3% annually between 2030 and 2050, which requires strict regulations together with fiscal incentives and infrastructure investments. Massive development occurred in clean renewable energy sectors, where electric vehicle sales surged by 55% and nuclear capacity increased by 40%, and electrolyser capacity surged dramatically. The adoption of new technologies is most prominent in sectors with established technologies, yet long-distance transport and heavy industry need faster innovation to reach their abatement goals. Currently, different areas exhibit varying degrees of development, thus requiring enhanced worldwide policy backing and international mutual assistance. The world needs additional well-planned initiatives to fulfill the 2030 SDS goals while closing the existing production deficit (IEA, 2023), (Tracking Clean Energy Progress 2023, IEA, Paris, https://www.iea.org/reports/tracking-clean-energy-progress-2023, Licence: CC BY 4.0).[2,91]

5.1 Scaling up biofuels to achieve SDS targets

Biofuel production for the sustainable development scenario (SDS) requires countries to boost their biofuel output through increased fuel use, along with transportation fuel blending requirements and national policy alignment with SDS standards.[92] The wide-scale biofuel manufacturing process depends on multiple linked elements between biomass feedstock availability and composition, and innovative conversion technology development and deployed production pathways, together with supportive policy frameworks for advanced biofuel installations.[93]

All organic materials that originate from living organisms function as a flexible and plentiful source for bioenergy and biofuel production.[94] They are broadly categorized based on their origin into three main types: (i) primary biomass resources, which are harvested directly from agricultural activities or non-agricultural lands such as rice and wheat straw, corn stover, and sugarcane tops;[95] (ii) secondary biomass resources, generated as by-products during the processing of primary biomass such as sawdust, woodchips, paper pulp residues, and animal waste; and (iii) tertiary biomass resources, which include post-consumer and industrial waste like food-processing waste, packaging materials, and demolition debris.[6] The biomass streams from agriculture and food processing and forestry, and municipal waste sectors present significant potential for biofuel production, according to global annual estimates that range between 50 and 150 exajoules (EJ).[96] The theoretical maximum biomass energy potential extends from 200 to 500 EJ annually. The worldwide quantity of crop residues grew from 6411 million tonnes (Mt) in 1991 to 6973 Mt in 2001. FAO (2018) reports that agriculture controls approximately one-third of the worldwide land territory and will remain essential for future biomass delivery. The open burning of crop residues by Brazil, India, the United States, and China results in carbon emissions that total 15.8 Mt of CO_2 while simultaneously wasting valuable resources because these countries collectively burn approximately 182 Mt of crop residues.

The production of biodiesel utilizes two categories of important biomass feedstock, including edible oils from palm, soybean, sunflower, and rapeseed, and non-edible oils from neem, castor, jatropha, pongamia, and rubber seed oil.[97,98] Waste cooking oil stands out as a biodiesel feedstock because it contains significant amounts of fatty acids and methyl esters, which make it suitable for transesterification-based biodiesel synthesis. Microalgae function as an exceptionally promising sustainable raw material base for biofuel production. Microalgae cultivation does not require terrestrial land or freshwater supplies because they do not compete with other crops.[99] The annual production of fresh biomass by selected saltwater microalgae reaches 86 Mt, along with their ability to capture and store 211 Mt of CO_2 through photosynthesis. The dual advantages of microalgae serve renewable energy generation and GHG reduction purposes, thus presenting an effective solution for handling energy security alongside environmental sustainability challenges.[100]

5.2 Lignocellulosic biomass composition and biofuel production pathways

The biofuel industry depends on lignocellulosic biomass, which originates from plant-based materials as a sustainable and versatile feedstock. The three primary biopolymeric components of its complex structure include cellulose, hemicellulose, and lignin. The plant cell wall contains cellulose microfibrils embedded in a hemicellulose matrix, which creates an open network that provides both stiffness and elasticity to biomass.[99,101] The biofuel production of ethanol and hydrogen from cellulose and hemicellulose materials is possible through hydrolysis and fermentation of their hexose and pentose sugar components. The complex nature of lignin makes it non-fermentable, but it contains valuable energy potential since it works as a solid fuel for heat and electricity production.[102] The collection of lignocellulosic biomass material occurs through dedicated energy crops and forest residues as well as agricultural residues and grasses. Biofuels can be produced from these feedstock through biochemical and thermochemical processes because they demonstrate substantial production potential. Seed-bearing crops, together with edible and non-edible tree species, contain oil contents that reach between 30% and 60% when measured on a dry weight basis.[103,104] The biodiesel production potential of microalgae depends on their different strains, which contain between 20% and 50% lipids according to Baskar and Solomon.[105]

Scientists now view biodiesel synthesis as suitable for woody plants and forest trees, and shrubs because these non-edible oils provide valuable solutions for food security regions. The production of biofuels from biomass depends on two main technological conversion processes, which are thermochemical and biochemical.[106,107] The thermochemical conversion pathway includes five different processes, which include gasification and pyrolysis and liquefaction and combustion, and torrefaction. The conversion technologies transform lignocellulosic biomass through different process conditions to produce multiple energy products such as syngas, bio-oil, heat, and biochar. The thermochemical conversion methods show special compatibility with dry biomass while providing quick conversion capabilities that allow existing energy systems to integrate (Fig. 10). The biochemical conversion pathway uses either microbial processes or enzymatic activities to transform organic biomass into clean biofuels that include ethanol, biodiesel, and methane, along with biohydrogen.

The conversion of biomass into biochemical products uses agricultural residues together with industrial and food waste and municipal organic materials. The process efficiency relies on both the biomass selection and pretreatment approaches, along with the chosen microbial consortium. Table 3 presents different biochemical methods for biofuel manufacturing, which demonstrate the wide range of possibilities for this transformation process to supply sustainable global energy solutions.[13,108] These processes collectively form a complete method for developing valuable biofuels from renewable

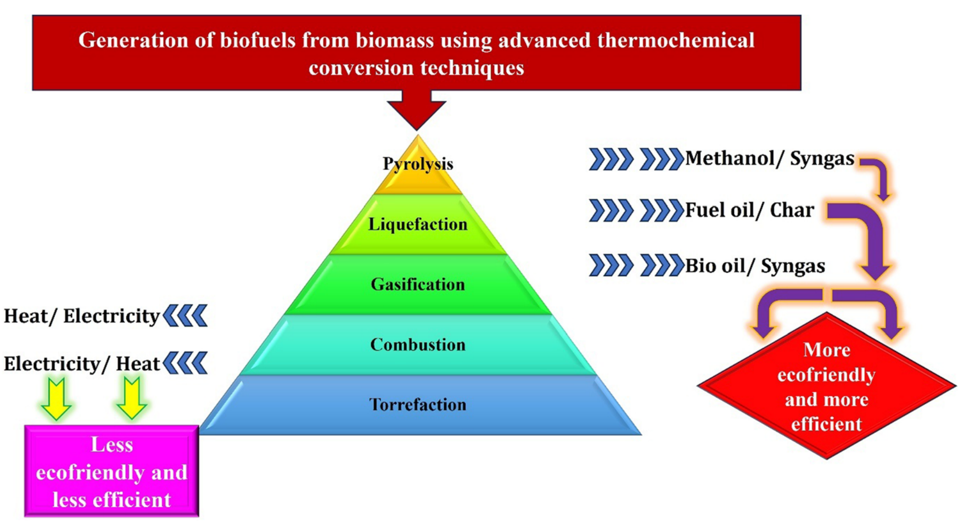

Fig. 10 Production of biofuels from biomass *via* thermochemical conversion processes: advancing sustainable energy solutions.

biomass to accelerate the worldwide shift toward zero-carbon energy systems.

6. Biofuel use: environmental and climate change perspectives

The transportation industry looks to biofuels as a practical, sustainable option because they deliver substantial greenhouse gas (GHG) emission reduction potential. The shift from fossil-based energy systems to biofuel usage significantly decreases atmospheric pollutants and carbon emissions to help address climate change adaptation requirements.[120]

6.1 Biofuels and air quality improvement

Fossil fuel combustion through combustion of gasoline and diesel results in dangerous environmental pollutants consisting of carbon monoxide (CO), sulfur dioxide (SO_2), unburned hydrocarbons (HCs) and nitrogen oxides (NO_x), and particulate matter (PM). The emissions from these sources damage air quality while creating photochemical smog and lead to major public health problems because of exposure to fine particulate matter ($PM_{2.5}$).[121] The combination of ethanol with gasoline enhances combustion efficiency because ethanol contains more oxygen, which leads to decreased emissions of major pollutants. Research on ethanol fuel blends E10, E15, E22, and E100 with respective anhydrous ethanol contents of 10%, 15%, 22%, and 100% shows these combinations cut down air pollutants, thereby making ethanol essential for cleaner transportation fuels.[122–124] Studies have proved that diesel engines running on biodiesel blends meet positive expectations for emission reductions. Biodiesel contains a natural oxygen content of 11% that enhances complete combustion to reduce CO, HC, SO_x, smoke opacity, and particulate emissions.[125] Biodiesel holds two environmental advantages since it breaks down naturally and contains no sulfur content like petroleum-based diesel. The environmental advantages of biodiesel appear across different blend options from B10 to B100 and B15 to B20, which represent the biodiesel content in diesel from 10% to 100%, respectively.[126,127]

6.2 Biofuels and climate change mitigation

The production of biofuels has substantial climate change benefits through achieving carbon-neutral status. During biofuel production, biomass substances capture atmospheric CO_2 through photosynthesis, and this stored carbon offsets emissions when the biofuel is burned, thus lowering the total environmental impact. The carbon dioxide sequestration capabilities of ethanol made from corn reach 1.8 tons per hectare per year, and switchgrass surpasses this number with an annual sequestration of 8.6 tons per hectare. The conversion of land for biofuel crop cultivation may impact the total amount of GHG emissions. The conversion of grasslands and forests into biofuel production sites results in major carbon releases reaching between 300 tons per ha and up to 600–1000 tons per ha, which emphasizes the need for sustainable land management practices.[128]

Biofuel adoption, together with GHG reduction, depends heavily on existing policy frameworks. Life cycle assessments (LCAs) establish that biofuels restore carbon in the air while fossil fuels create new emissions. Biofuel blending methods provide an effective path to displace conventional fuel usage. The low carbon fuel standard (LCFS) in California serves as an interesting policy example. The combined use of biodiesel and renewable diesel in 2018 resulted in a 4.3 million metric ton (Mt) reduction of CO_2 emissions, which exceeded ethanol-based reductions. Renewable biodiesel has proven its effectiveness for reducing transportation-related emissions through

Table 3 Biofuel production matrix: feedstock diversity, biochemical strategies, and optimal process conditions

Biochemical route	Feedstock and biofuels produced	Optimal operating conditions	Ref.
Anaerobic digestion	Rice straw with cow dung; methane yield of 189 ± 37 L kg^{-1}; digestion efficiency 70–90%	Mesophilic: 45 °C; hyper-thermophilic: 65–70 °C; total solids: ∼89.7–89.8%	108
	Algae and wheat straw (1 : 1 ratio); the methane yield increased by 77%	Pretreatment with 10% CaO at 75 °C for 24 h; digestion at 35 °C	109
	Monoraphidium and stigeoclonium species biomass, 72% CH4 yield enhancement	Hydrothermal biomass pretreatments, 130 °C; 15 min, pH 7.0, reaction time 10 h	110
Alcohol fermentation	Olive stones (moist endocarps); ethanol yield of 6.4 L/100 kg	Hydrothermal pretreatment at 225 °C; fermentation at 30 °C and pH 4.5 with pachysolen tannophilus	111
	Rice straw; ethanol yield of 25.3 g L^{-1}	2% NaOH microwave pretreatment; pichia stipitis fermentation for 72 h	112
	Wild-type cyclotella microalgae ethanol yields 0.18 kg^{-1} of algal biomass.	Pretreatment at 190 °C for 1 h, fermented by. bruxellensis, incubated at 37 °C, pH 5.5	113
Biological H_2 production	*Chlorella vulgaris*; H_2 recovery of 11.6 mL L^{-1}	Photo-fermentation; light intensity: 48 μmol photon m^{-2} s^{-1}; pH: 6.8; duration: 24 h	114
	Chlamydomonas and *Pseudomonas* species; H_2 recovery ≈10 mL L^{-1}	TAP-S medium; Light intensity: 50 μmol m^{-2} s^{-1}; duration: 12 days	115
	Mixed food waste, sewage sludge, 3% glycerol; H_2 yield of 179.3 mL g^{-1} VS	Pretreatment at 100 °C for 30 min; dark fermentation at 30 °C, pH 5.5	116
Transesterification	Waste vegetable oil; biodiesel yield of 94%	Reaction at 80 °C for 1 h; close reflux condenser; bimetallic tungsten-zirconia catalyst	117
	Microalgae biomass; biodiesel recovery >90%	Methanol/algae ratio: 10 : 1; supercritical methanol at 245–370 °C, 200 bar; time: 10–80 min	118
	Waste cooking oil; biodiesel recovery of 91%	Methanol/oil ratio: 37 : 1; temperature: 253.5 °C; pressure: 198.5 bar; time: 14.8 min	119

CO_2 emission reductions, which have surpassed 18 Mt since the program started in 2011.[129,130] The California Energy Commission (CEC) states that renewable biodiesel produced in the United States provides both economic sustainability and extensive emission reductions without necessitating changes to current vehicle engines. According to the National Biodiesel Board, the implementation of clean biofuels annually reduces CO_2 emissions by 20 million metric tons, which establishes a socially advantageous climate change mitigation approach (Calif, 2021).[131,132]

7. Challenges in biofuel development

The production of biofuels has become a potentially viable solution to the problem of utilizing fossil fuels, which are regarded as a major source of greenhouse gases. However, it is important to note that despite these enhancements, several essential issues pose as barriers to the biofuel commercialization. Perhaps one of the major challenges is the low rate of substrate turnover. Some of the biofuel production processes use hard-to-break feedstock like lignocellulosic biomass feedstock.[133] The nature of such materials is highly resistant to biodegradation, which requires expensive pretreatment steps and unique enzymes to further the process; this raises the cost of production and lowers yields. Furthermore, the conversion of these substrates to biofuels by microbes is characterized by low yields because of metabolic limitations and inefficiency in the utilization of the substrates, which requires the application of efficient metabolic engineering techniques.[134,135]

Another major problem is the ability of microorganisms to produce biofuels to tolerate toxic compounds that are formed during biomass processing and fermentation. HPL, which occurs during the breakdown of lignocellulosic biomass, generates inhibitors like furans, phenolic components, and organic acids toxic and lethal to microbes. As will be discussed in more detail below, these toxic compounds hinder cellular functions and inhibit the overall efficiency of biofuel production.[136] To enhance the feasibility of biofuel production, it is critical to create strong microbial strains that are resistant to high inhibitor concentrations or to design engineering solutions that prevent such inhibitors' formation. Moreover, the very accumulation of biofuels themselves can be toxic to the producing microorganisms, thus making the production process even more challenging and requiring the development of new bioprocessing strategies to overcome these problems.[137]

7.1 Challenges in genome editing and AI integration

CRISPR-Cas genome editing. Despite their power and specificity, CRISPR-Cas systems are subject to some of the shortcomings such as off-target mutations, mosaicism, and species and cell-type-dependent efficiency. These issues present biosafety issues, specifically with the editing of photosynthetic microorganisms and crop plants used to make biofuels. Also, the quick commercialization of CRISPR-edited biofuel strains is still constrained by regulatory vigilance and the public's reservations surrounding genetically modified organisms (GMOs).

Artificial intelligence (AI). ML and AI software programs are beginning to be used in the design and modeling of strains; the application of these tools is currently hindered by access to high-quality, curated experimental data. Traditional predictability in most AI models is trained on model organisms, including *E. coli* and *S. cerevisiae*, which limit their predictive potential in novel and diverse biofuel-producing microorganisms. Efficiency in generating common interface platforms that can integrate the AI outputs into the synthetic biology workflows is also lacking.

Synthetic biology regulation. There is also the issue of legal and ethical implications in the development of synthetic biology as the biofuel industry grows, particularly about the release of engineered microbes to the open systems. Commercialization is complicated by international inconsistencies in the regulation of GMOs (*e.g.*, strict laws on GMOs in the EU contrasted with lax norms in the US and India). The creation of internationally consistent guidelines and risk analysis models is, therefore, necessary to reduce these challenges and ensure safe innovation.

Despite high precision, CRISPR systems face limitations such as off-target effects, potential gene drive risks, and ethical concerns. AI models for strain design rely on vast training data, often unavailable for non-model microbes. Furthermore, synthetic biology tools face regulatory hurdles, especially in regions with stringent GMO legislation.

Another cluster of challenges can be described as the financial and operational viability of the approach, including scalability. While the technologies of biofuel production have been successfully tested in the laboratory and pilot plants, their scaling up to a large industrial scale is accompanied by some technical and economic issues.[138] Expensive feedstock and its preparation, cost of enzymes for hydrolysis, and cost involved in downstream processing are major challenges for cost-effective production. Also, glorying the particular feedstock, corn or sugarcane, has implications on resource utilization, choice of feed, and competition for land. Some of these problems may be overcome by creating efficient and inexpensive processes that can use non-food feedstock such as agricultural residues or algae. But this comes with a cost, hence extensive research and development are required in order to minimize the capital and operational expenditures in the production systems shown in Table 4. Moreover, the ability to introduce biofuel production into already existing networks and logistics systems presents another challenge, which requires the identification of better ways to accomplish this with less expense.[139,140]

The problem is compounded by regulatory and ethical factors. Government policies and regulations have a central function in the formation and use of biofuels, yet those policies may differ significantly from one country to another, which has the effect of making the field uneven for business entities.[142] Such provisions as high environmental standards and certifications slow down the production and commercialization

Table 4 Challenges in scaling biofuel production[141]

Challenges	Description
Feedstock availability	Limited availability of sustainable and cost-effective feedstock.
Land use competition	Competition with food production and land for agricultural use.
High production costs	Expensive production processes compared to fossil fuels.
Technological barriers	Inefficient conversion technologies and a lack of scalable processes.
Energy balance	Achieving a positive energy balance where energy output exceeds input.
Environmental impact	Potential negative environmental impacts, such as deforestation and water use.
Policy and regulation	Inconsistent policies and regulations are hindering market development.
Market acceptance	Limited consumer acceptance and infrastructure for biofuels.
Genetic modification concerns	Ethical and ecological concerns related to genetically modified organisms (GMOs) in biofuel crops.
Storage and distribution	Challenges in storing and distributing biofuels due to their chemical properties.

processes and lead to a rise in production costs; uncertain policy frameworks have adverse effects on investment and research. There are also questions that concern ethical issues connected with the RTFO, relating to the use of biofuels and biofuel production that leads to deforestation, thus destruction of habitats, and excessive use of water. Stakeholders have a difficult task of ensuring that energy production and distribution are achieved without compromising the ecosystems and resources in society. The process of achieving this is through policy formulation and the implementation of sustainability principles.[143]

Solving such problems requires a *trans*-disciplinary solution that incorporates engineering, economics, policy, and the latest developments in biotechnology. Owing to these strategies of metabolic engineering and novel approaches to substrate utilization, biofuel production may be more cost-effective and environmentally friendly. Microbial tolerance to toxic by-products and biofuels can be improved through genetic engineering and adaptive laboratory evolution, therefore increasing productivity and resilience. Economic feasibility can therefore be attained by integrating novel feedstock, enhancing process designs, and strengthening the relationships between academia, industry, and government. Finally, the integration of legal requirements and the advocacy of integrity can create the foundation for the creation of responsible and sustainable biofuels. As the global energy consumption increases, there is a need to overcome these challenges to realize the full potential of biofuels as a critical component for the transition to a low-carbon economy.[144]

8. Future perspectives

This review demonstrates how the application of artificial intelligence to genetic engineering constitutes a new era in the biotechnology field by enhancing the precision and speed at which genetic materials can be manipulated. As with other applications of bioinformatics, AI tools and algorithms are employed for target identification, off-target effect prediction, as well as for refining the CRISPR-Cas9 systems for their intended use.[145] This computational assistance saves time in experimentation, controls error, and speeds up the production of GMOs that possess the required characteristics, be it increased crop resistance, better therapeutic proteins, or specially adapted microbial strains for industrial applications. Furthermore, AI can perform big genomic data processing to find new gene roles, which helps researchers to investigate new areas of synthetic biology and metabolic engineering. When machine learning is integrated with omics-based approaches, scientists can develop models that dictate genetic alterations for innovation in agriculture, medicine, and bioremediation.[146]

Of equal interest is the development of new systems based on microbial and enzymatic pathways in which the integration of the two platforms can provide a synergistic benefit for the efficient production of biochemicals. Microorganisms, which can be engineered bacteria or yeast, are suitable for obtaining valuable substances in the course of fermentation, while enzymatic conversions are characterized by high selectivity and activity. When combined, these two approaches can eliminate the drawbacks of both systems: the metabolic stress affecting host microbes or the expensive process of enzyme production. Biochemical systems make it possible to convert renewable resources like lignocellulosic biomass or agricultural wastes into biofuels, bioplastics, and pharmaceuticals. For example, microbial consortia can be tailor-made to metabolise intricate substrata into middleman metabolites that are further metabolised to the final products through biosystem-immobilised enzymes. These systems are further improved by developments in protein engineering, which enables the fine tuning of enzymes for effective functioning under certain conditions, and by synthetic biology platforms that enable the fine control of metabolic pathways in microorganisms.[147] Integrating TEA and LCA frameworks early in the development pipeline ensures that biofuel technologies not only scale economically but also meet stringent environmental and regulatory benchmarks.

Bio-economy and sustainability of biofuels and the notion of a circular economy are central to combating global environmental problems and shifting to a lower-carbon economy. The circular bioeconomy aims at using renewable biological resources and waste materials in order to create closed-loop systems that harm the environment little or not at all. Regarding biofuels, the concept can touch on the utilisation of biomass, algae, and agricultural residues as feedstock, so that these crops would not have to compete with the food industry.[148] Technological developments in production processes, for example, the CBP and AD, increase the feasibility and environmental friendliness of the biofuel industry. In addition, the incorporation of life cycle assessment (LCA) in the production of biofuels guarantees the consideration of the sustainability effects on the production system from feedstock production to fuel use. More and more attention is paid to the production of biofuels with the added value co-products like biochar or biopolymers, which would improve the economics of bio-refineries. Moreover, the use of cellulosic ethanol as a biofuel, biodiesel from algae, and a hydrogen biofuel is a good example of how innovation can be attained in the quest for energy security and climate change.[149]

Altogether, these leading-edge AI, symbiotic biotechnological systems, and circular-bioeconomy paradigms provide excellent indication of how modern biotechnology holds enormous promise with regard to building a sustainable future. Using the approaches that integrate different disciplines, researchers and practitioners can work on the most urgent issues of world development, such as food safety, access to medicines, energy efficiency, and environmental protection.

The future of biofuels lies not only in the optimization of these technologies but also in the development of second and third-generation biofuels derived from non-food feedstock such as algae, lignocellulosic biomass, and waste oils. These feedstock offer a more sustainable alternative to traditional biofuels, as they do not compete with food production and can be sourced from waste materials. The integration of AI and hybrid systems will play a crucial role in advancing these technologies by optimizing metabolic pathways for improved yields and efficiency. Additionally, the implementation of a circular bioeconomy approach ensures that biofuel production is not just sustainable but also restorative, contributing to the overall resilience of our ecological and economic systems. In conclusion, the integration of AI in genetic engineering, the development of hybrid microbial-enzymatic systems, and the advancement of a circular bioeconomy are poised to revolutionize the biotechnology and biofuel industries. Integrating TEA and LCA frameworks early in the development pipeline ensures that biofuel technologies not only scale economically but also meet stringent environmental and regulatory benchmarks. These approaches offer a holistic and sustainable path forward, one that minimizes waste, optimizes resource use, and provides renewable energy solutions. By leveraging these innovations, we can address pressing global challenges such as climate change, resource scarcity, and environmental degradation while fostering the growth of a sustainable and regenerative bio-based economy. These developments represent not only technological progress but also a paradigm shift in how we approach the relationship between science, nature, and industry, with far-reaching implications for the future of biotechnology and sustainability.

8.1 Policy and commercialization outlook

Biofuel implementation and commercialization largely depend on the favorable policy regimes and market drivers. Some of the outstanding ones are:

California Low Carbon Fuel Standard (LCFS). This provides carbon intensity reduction targets and incentives for advanced biofuels, which reduce 4.3 million tons of carbon dioxide every year.

Renewable Energy Directive II (EU RED II). This requires 14 percent of transportation fuels to be made up of renewable sources by 2030, with the focus of second- and third-generation biofuels supporting this mandate.

The National Bio-Energy Mission of India. This targets the production of 15 billion liters of biofuels by 2026, with the emphasis on the use of non-edible oil seeds and agricultural waste valorization.

Even in the face of these policy drivers, commercialization of next-gen biofuels is met with some challenges:

• The low efficiency in production costs and productivity prevents cost parity against fossil fuels.

• Enforced blending standards (*e.g.*, E10, B20) also differ around the world, producing factionalized markets.

• Storage, transportation, and dispensing systems of biofuels continue to be underdeveloped in numerous areas.

Nevertheless, the global biofuel market is estimated to grow at a compound annual growth rate (CAGR) of around 7 percent between the year 2023 and 2030. Green financing models and carbon credit mechanisms, as well as public–private partnerships, will probably be the most important drivers of the accelerated market penetration.

9. Conclusion

In recent years, the convergence of cutting-edge technologies like Artificial Intelligence (AI), synthetic biology, and bioengineering has opened up new frontiers in genetic engineering and biotechnology. These innovations lead to transformative advances in various sectors, including environmental sustainability, energy production, and health. Nonetheless, there are still significant gaps in research. One of the most exciting avenues of exploration is the integration of AI in genetic engineering, where machine learning algorithms and computational models are being employed to predict gene interactions, optimize metabolic pathways, and enhance the precision of genetic modifications. Another promising area in biotechnology is the development of hybrid systems that combine microbial and enzymatic pathways. A third key area in modern biotechnology is the emphasis on a circular bioeconomy and biofuel sustainability. A circular bioeconomy represents a shift away from traditional linear economic models that depend on extraction, production, and disposal, toward systems that emphasize reuse, recycling, and the sustainable management of biological resources. The convergence of synthetic biology and metabolic engineering is central to realizing the vision of scalable, economically viable, and sustainable biofuel production. By precisely redesigning microbial systems and biosynthetic pathways, these tools together enable high-yield biofuel generation from diverse and renewable substrates.

These are the establishment of a microbial chassis with strong tolerance to toxic byproducts, the improvement of lignocellulosic biomass conversion efficiencies, and the reduction of energy requirements during pretreatment. The industrial scale-up of engineered microbial and enzymatic systems, especially of third- and fourth-generation biofuels, is the subject of future research. It is also essential to develop perfect methods of genome editing to minimize off-target effects and enhance safety and acceptability by society. Moreover, the design of strains and the modeling of processes using AI need to be scaled by developing large and diverse training sets and by establishing open-access databases available to biofuel scientists. Finally, harmonizing international policy frameworks, advancing techno-economic assessments, and fostering interdisciplinary collaboration will be key to transitioning from proof-of-concept to commercialization. It is possible to overcome these obstacles strategically and enable next-generation biofuels to be a foundation for the global low-carbon economy and the future of the circular bioeconomy.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions

Jiten Yadav and Harneet Marwah: methodology, project administration, writing – original draft, data curation, validation, software, and funding acquisition. Chandra Kumar: data curation, formal analysis, software, and writing – original draft. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

This is a review article. All data discussed are from previously published sources, which are cited in the manuscript.

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