

Paradigm of Integrative OMICS of Microbial Technology Towards Biorefinery Prospects

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Abstract

Climate change, finite natural resources, and increasing population necessitate producing sustainable energy with positive economic growth. Recent advances in OMICS coupled with genome editing and synthetic biology have paved the way for the development of sustainable technologies. These techniques help identify critical genes/pathways and re-construct and redesign biological pathways to develop eco-friendly and economically viable industrial metabolites. With the help of microbial technology, biorefinery-related research is actively pursued in many countries to develop microbial strains, producing varied value-added biochemicals and biofuels. The application of multi-omics data in deciphering key genes, their manipulation, and outcomes implies the domain's potential to find new horizons in biorefineries using microbial factories to produce various biofuels and biorefinery products. This review illustrates OMICS's role in developing industrial chemicals and microbes biorefineries. Besides, prospects for genome editing and synthetic biology have been elucidated.

Keywords: Biorefinery; Metabolic Engineering; Microbial Technology; Multi-omics; Synthetic Biology

1. Introduction

A burgeoning population, shrinkage of natural resources, global warming, and ever-increasing demands for non-renewable sources of energy like fossil fuels kindle to look for lignocellulosic biorefineries, which are considered adequate ‘green’ alternatives for the production of chemicals and fuels from various lignocellulosic biomass (Kumar et al. 2022; Dragone et al. 2020; Li et al. 2019). These lignocellulosic biomasses comprise lignin, a complex phenolic polymer, celluloses, and hemicelluloses. Being a significant part of plant cell walls, these are abundantly available on Earth, with a worldwide production of around 181.5 billion tonnes (Paul and Dutta, 2018). Lignocellulosic biomasses are often by-products from various agro-industries. Its composition provides appropriate substrates for biofuel production through fermentation by various microorganisms through the conversion of carbohydrates into sugars and finally to biofuels (Fig. 1). However, to access the inner cellulose layer, the outer complex lignin layers need to be depolymerized or separated. This process is called delignification, which aims to improve the sugar digestibility of the microbes (Sánchez et al. 2011). It was also found that most biorefineries target the cellulosic part due to its ease of degradation and conversion while leaving behind the recalcitrant part lignin (Li et al. 2019). Lignin can be an excellent source of various value-added chemicals; however, it is often neglected by industries because of its complex, organic nature, making its degradation challenging. For effective valorization and conversion into other chemicals, the lignin needs to be depolymerized into smaller fragments of monomeric or short-chained oligomeric aromatic compounds (Li et al. 2019).

Depolymerization of lignin is conventionally carried out by various physicochemical methods that involve extreme conditions, negatively affecting the enzyme production capacity and biofuel production efficiency by microorganisms. The harsher conditions associated with the

physicochemical methods can be easily overcome by exploiting the ability of certain bacteria and fungi to produce lignin-degrading enzymes like laccase, manganese peroxidase, etc. (Linares et al. 2018). However, one of the reasons the biorefinery industries are reluctant to use such biological methods is due to the slow rates of lignin degradation owing to limited enzyme production and exorbitant costs.

Finding amicable, eco-friendly solutions are required to overcome the setbacks associated with conventional processes. OMICS techniques such as genomics, transcriptomics, proteomics, epigenomics, metabolomics, metagenomics, meta-transcriptomics, meta-proteomics and meta-metabolomics are high-throughput technologies, which generate vast chunks of data about essential genes, regulatory pathways and elements, proteins, RNAs, microbes at a meager cost. Extrapolating this data and desired traits could help develop cost-effective lignocellulosic biorefinery technologies. Besides, embracing promising techniques like genome editing and synthetic biology would provide an excellent platform for lignocellulose biorefinery.

Synthetic biology can combine with transgenes in a combinatorial and modular manner and offer improved tolerance of solvents, eventually resulting in the effective conversion of biomass into profitable products (French, 2009). Precisely engineering designer enzymes and metabolic pathways in synthetic biology will substantially increase the lignocellulosic biomass properties (Kalluri et al. 2014). Synthetic biology routes utilize hemicellulosic hydrolysates obtained from biomass, which are converted to pentose sugars by bacteria and yeast. This process is environmentally friendly and can be performed under mild conditions such as atmospheric pressure and ambient temperature, thus avoiding the need to purify xylose, making this process more economically viable. A microorganism must be easily cultivated, show a high capacity for xylitol production, and have excellent tolerance to stress and toxic compounds to produce xylitol

(Guo et al. 2006). In similar areas, various studies in molecular engineering are being conducted to discover and formulate ideal conditions for the economical production of xylitol. *Saccharomyces cerevisiae* was first identified as an ideal candidate to be genetically modified to optimize xylitol production.

Optimization of metabolic pathways and simultaneous utilization of sugars are some genetic modifications being studied (Su et al. 2013). Amongst the naturally occurring D-xylose fermenting yeasts, *Candida* sp. is attributed as one of the best producers of xylitol. Unfortunately, due to its pathogenic nature, it is not commercially viable. The improvement of xylitol production by overexpressing the homologous xylose reductase (XR) gene in *Kluyveromyces marxianus* HPE453 (KSIHPE453) has been reported. This approach was adopted upon the consideration that the deletion of the xylitol dehydrogenase (XDH) gene would result in the accumulation of xylitol, resulting in yeast's ability to produce cell biomass lost. This method proves to be a promising improvement with the increase in the xylitol yield from sugarcane bagasse. Nevertheless, using genetically modified strains poses some challenges to overcome, such as increased metabolic stability and tolerance to inhibitors (Pal et al. 2016). ABE (acetone-butanol-ethanol) fermentation process produces biobutanol from various substrates, including lignocellulosic materials.

The transcriptional repression of *solR* and the overexpression of aldehyde dehydrogenase produce the best results in higher production rates (Harris et al. 2001). There was an increase of 8.2 g/L acetone, 2.2 g/L ethanol, and 17.6 g/L butanol, which was 66%, 194%, and 51% higher than what was obtained using the wild-type strain. Increasing selective butanol production was unsuccessful because of the overexpression of enzymes, which are directly related to its production rate. Hence, an antisense RNA strategy was employed for the knock-down of CoA transferase (*ctfB*), an acetone-producing enzyme, in addition to the overexpression of the butanol-producing enzyme

alcohol/aldehyde dehydrogenase (aad). The adopted approach resulted in a two-fold increase in the butanol/acetone increase with a minimal decrease in butanol titer of approximately 9.8 g/L compared to the wild-type, which reduced roughly 10.2 g/L (Weber et al. 2010).

1.1 CRISPR/Cas9-mediated genome editing in microbial cell factories

Genome engineering is an effective process of altering the native genome of an individual microbe to introduce the desirable character for augmenting the particular metabolite production (Singhania et al. 2017; Ulaganathan et al. 2017). In contrast to the conventional genome modification technique, CRISPR/Cas9 is a distinctive genome editing approach that involves the upregulation or downregulation of microbial genes and the introduction or deletion of a gene at a specific site of a microbial cell (Ulaganathan et al. 2017). CRISPR comprises the palindromic DNA sequence repeats flanked by a spacer DNA associated with Cas9. It is a defense system of prokaryotes against bacteriophages employed for site-directed mutagenesis as an influential instrument (Zaidi et al. 2017).

Cas 9 (Protein 9) isolates *streptococcus pyogenes*, an unwinding or cleaving enzyme from CRISPR-associated genes translated into helicase or nuclease proteins to cleave the targeted active sites of a double helix DNA strand (Schaeffer and Nakata, 2015; Rodriguez, 2016). In detail, the CRISPR system integrates plasmid DNA or phage DNA into its bacterial genome. It later uses the transcribed RNA to identify the phage in subsequent encounters, directing the Cas 9 for targeted DNA cleavage (Louwen et al. 2014). Moreover, thus, a non-coding auxiliary tracrRNA base pairs with crRNA and leads to the formation of the Cas9-crRNA complex (Niewoehner et al. 2014). CRISPRa system has been used for the specific gene activation, where dead Cas9 (dCas9) was fused with RNA Polymerase III (transcription activators subunit) of *Escherichia coli*. Besides, CRISPRi is used to unwanted knockout genes by forming a dCas9-sgRNA complex and binding

to the target gene sequence that blocks the transcription by inhibiting the RNA polymerase (Adegboye et al. 2021; Qi et al. 2013; Senturk et al. 2017; Luo et al. 2015).

This innovative genome editing tool has been widely implemented in various applied research for redesigning diverse microbial cells for improved characteristics such as spore formation resistance (*Bacillus subtilis*), increasing xylose utilization, and (R-R)-2,3-butanediol or mevalonate production (*S. cerevisiae*), enhancing the production of α -cyclodextrin glycosyltransferase, improving the biofuel production, biofuel tolerance, and inhibitor tolerance, modifying hemicellulase/cellulase and developing thermotolerant microbial strains (Vervoort et al. 2017). On the other hand, applying this genome engineering tool in non-conventional yeast (*K. lactis* and *S. pombe*) would be a rapid technique to target many genes and their metabolic pathway alternation for enhanced production of commercial value-added products (Horwitz et al. 2015).

For instance, the industrially important *K. lactis* strain has been re-engineered by integrating the six genes to enhance muonic acid production by manipulating the pathway toward muconic acid precursor molecule production. Nevertheless, the production of guide RNA in non-conventional yeast strains' CRISPR/Cas9 system remains a significant challenge owing to its undefined RNA polymerase III promoters (Madhavan et al. 2017).

A successful CRISPR/Cas9 system of non-conventional yeast establishes genetic circuits, transcriptional regulation, heterologous expression host, and metabolic networks for the biofuel system. The site-specific breakdown of DNA in non-conventional yeast could alleviate the challenges accompanied by homologous recombination. Whereas, in the case of several bacterial genera such as *Corynebacterium*, *Bacillus*, *Lactobacillus*, *Clostridium*, *Escherichia coli*, *Pseudomonas*, *Mycobacterium*, *Streptomyces*, and *Staphylococcus* CRISPR-Cas 9 mediated genome editing has been employed for gene manipulation for improving the production of biofuels

and commercial value-added products (Cho et al. 2018). Recently, *E. coli* strain MG1655 was engineered based on the CRISPR/Cas 9 system to introduce the dual operon-synthetic pathway to produce n-butanol (5.4 g/L) with glucose as the carbon source. Similarly, a synthetic cassette of butanol from xylose (redox balanced pathway) was incorporated into the ethanologenic *E. coli* SSK42 strain genome via the CRISPR/Cas9 system, preceded by the endogenous gene encoding ethanol (Abdelaal et al. 2019).

The efficiency of *Clostridium saccharoper butylaceticum* N1-4 in butanol production was achieved by expressing guide RNA using a specific promoter gene (PJ23119) (Wang et al. 2017). Thus, the genome engineering strategy in the microbial cell, such as CRISPR/Cas9, could be the ideal solution to overcome the limitations in bioconversion by gene manipulation for enhanced biofuel production and augmentation of other desired physical characteristics. Though CRISPR/Cas9 possesses several advantages in the genome engineering of microbial cell factories, it is in the nascent stage of developing products from the technology. Thus, more research work in this direction is anticipated. So far, several reports have aimed to describe the lignocellulosic biorefinery; here in this review, OMICS-based interventions made for the generation of biofuels, chemicals, and biorefinery compounds have been elucidated in subsequent sections.

2. Chemicals and fuels synthesized through synthetic biology and metabolic engineering

Fossil fuels like natural gas, coal, and products obtained from crude oil are indisputably the primary source of energy that powers the world. According to the Ministry of Petroleum and Natural Gas, in India, more than 220 million metric tons of crude oil was imported in the financial year 2019-20 (Press Information Bureau, 2019). Even though there is an increasing energy demand, it is undeniable that fossil fuels are non-renewable, known to cause pollution, and thus need to be substituted with renewable energy sources. The Government of India's 2018 National

biofuel policy visualizes a goal of 20% ethanol blending in petrol and 5% biodiesel blending in diesel by 2030 (Press Information Bureau, 2021). While the commercial production of first-generation and second-generation biofuels happens, on the one hand, the research and development of third and fourth-generation biofuels happen on the other with the help of various omics sciences (Mehmood et al. 2021). Mass spectrometry and computational analysis have allowed accurate quantification of microbial intracellular metabolites and their metabolic fluxes, thereby revealing the complex cellular metabolism in rational bioengineering (Fig. 2)

The current strategies employed for producing various biofuels and chemicals were illustrated in Table S1. For example, several researchers have performed knockout of genes associated with the acetate and lactate formation in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485, *Clostridium thermocellum*, and *Thermoanaerobacter mathrani* BG1L1 to enhance the ethanol yield (Georgieva et al. 2008; Shaw et al. 2009; Tripathi et al. 2010). Recently, Velmurugan and Incharoensakd (2020) have investigated the co-cultivation system for two different genetically engineered *Synechocystis* strains for ethanol production. The co-cultivation system comprising strains with *pdc-adh* overexpression (Δ APX) and *glgC-phaA* knockout (Δ GBK) has resulted in 4708 mg/L of ethanol. Similarly, the overexpression of lipase-specific foldase, acyl-CoA synthetase, and lipases together with the deletion of 7 Acyl-CoA dehydrogenase (*fad*) genes has enhanced the FAME yield by 21.3 g/L in *Rhodococcus opacus* sp. (Kim et al. 2019). Whereas, gene fusion technology was widely opted in *E.coli* and *Corynebacterium glutamicum* for the production of value-added chemicals like α -Farnesene, and 3-Hydroxypropic acid respectively (Chen et al. 2017).

Even though synthetic biology lacks a clear-cut definition, the primary aim of this field of science is to engineer living systems by optimizing the genetic code, redesigning metabolic pathways, and

manipulating the organism's proteome using various genetic tools to achieve maximum yield of the desired product (NHGRI, 2019). Thus, synthetic biology can also be used to help broaden the available options of biofuels, viz., the production of advanced biofuels like long-chain alcohols, alkene/alkanes, fatty acids, and isoprenoid-based biofuels (Fig. S1).

Table S1

2.1 Long Chain Alcohols

The long-chain alcohols (i.e., alcohols with more than four carbon atoms) like 1-butanol, 2-methyl-1-butanol, isobutanol, 1-pentanol, isopentanol, 3- and 4-methyl-1-pentanol and 1-hexanol are known as higher alcohols that have similar biofuel properties (Clomburg and Gonzalez, 2010; Wang et al. 2017). Several clostridium strains are employed for producing butanol yet are not robust candidates for industrial applications owing to the lower titer and yield (Abdelaal et al. 2019). Hence, recent studies are focused on the genetic engineering of *E.coli* strains for enhanced alcohol production using crude glycerol and several agricultural wastes (Liang et al. 2020).

The pathway genes of butanol namely *hbd*, *ter*, *atoB*, *adhE2*, *phaA*, and *crt* was inserted into *E.coli* (SSK42 and BL21) and the engineered strain yielded 4.3 g/L and 6.9 g/L of butanol using xylose and glycerol as a carbon source, respectively (Saini et al. 2017; Abdelaal et al. 2019). Nitta et al. (2019) stated that resolving the imbalance of free CoA and maintaining the cofactor balance is crucial for the CoA-dependent pathway for butanol production. Dong et al. (2017) improved the NADH regeneration by modifying the *fdh* expression in *E.coli* BW25113 host integrated with genes associated with the butanol pathway which in turn enhanced the butanol production (20 g/L) with 83% of theoretical yield. Other than *E. coli*, this pathway has also been expressed in *P. putida*, *B. subtilis*, and *S. cerevisiae* (Rabinovitch-Deere et al. 2013). On the other hand, iterative keto-acid elongation has resulted in the production of longer-chain alcohols such as 2- and 3-

methyl-1-butanol and 1-pentanol (C5 alcohols), and 3- and 4-methyl-1-pentanol and 1-hexanol (C6 alcohols) in various *E. coli* strains (Wang et al. 2017). Recently, Tian et al. (2019) improved the precursor metabolites (dimethylallyl pyrophosphate and isopentenyl pyrophosphate) availability by CRISPRi-mediated knockdown of several genes *gldA* (glycerol utilization), *prpE* (propionyl-CoA synthesis) and *asnA* (asparagine production) to increase isopentenol synthesis by 18-24%. In addition, adaptive laboratory evolution and upregulation of the *RpoS* gene have increased the tolerance of microbial strains to various alcohols which could enable the economic viability of long-chain alcohols (Wang et al. 2020).

2.2 Fatty Acids

Fatty acids are a class of molecules used by organisms to store excess carbon and generate high amounts of energy. Its metabolism is well-studied in both prokaryotes and eukaryotes. Biodiesel, an efficient and clean diesel alternative, is usually produced from plant oils and animal fats primarily made up of acylglycerols by transesterification to form fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE). Petroleum-based diesel shares similar properties with biodiesel because it contains long-chain and aromatic hydrocarbon molecules. Thus, biodiesel can replace petroleum-based diesel directly without manufacturing new engines or infrastructure. FAMES or FAEEs are not reported to be naturally generated by any microorganism. A preliminary study for producing FAEEs in *E. coli* involved the transfer of *adhB* and *pdc* genes from *Zymomonas mobilis* and the gene encoding WS/DGAT from *Acinetobacter baylyi* strain ADP1 as well as the addition of exogenous oleic acid while growing the culture in glucose medium. Later, the production of FAEEs without adding exogenous acid was achieved by overexpression of *fadD* and *tesA* from *Escherichia coli* and two copies of *atfA* from *Acinetobacter baylyi* and deletion of the *fadE* gene (Janben and Steinbuchel, 2014; Zhang and Agarwal, 2012). Whereas,

in yeast, overexpression of genes (6-phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase (G6PD)) associated with the NADPH supply has increased the fatty acid production by 1.7 times (Hao et al. 2016). On the other hand, a 70% increase in fatty acid production (460 to 780 mg/L) was observed by performing the cloning of ATP citrate lyase, eliminating the glycerol-3-phosphate dehydrogenase and downregulating the malate synthase (Ghosh et al. 2016). However, the higher concentration of fatty acids that are toxic to the yeast which affects the cell growth remains a major limiting factor in fatty acid production (Ullah et al. 2021).

2.3 Alkanes and Alkenes

Alkanes and alkenes are a significant part of petrol, diesel, and jet fuels. A wide variety of organisms also produces alkanes, and their function is not well-understood, but it is postulated that they play a role in membrane dynamics and cell protection. Metabolic production occurs in two ways: either elongating an acyl CoA or its subsequent decarboxylation resulting in an odd chain hydrocarbon or aldol condensation of two acyl CoA followed by decarboxylation and decarbonylation to form an odd chain hydrocarbon. The significant hosts for metabolic engineering to produce alkanes and alkenes are *E. coli*, *S. cerevisiae*, the cyanobacterium *Synechocystis sp.*, and *Streptomyces globisporus*. Some of the strategies used for alkane and alkene production are co-expression of *Drosophila* CYP4G1 with cytochrome P450 reductase (CPR), co-expression of *Arabidopsis* CER1–CER3, deletion of hexadecenal dehydrogenase gene *HFD1* in *S.cerevisiae*, elimination of competing pathways, enhancement of aldehyde deformylating oxygenase (ADO) expression (ADOs from *Synechococcus elongatus* and *Nostoc punctiforme*) (Luo et al. 2019; Kang and Nielsen, 2017). Among the different strategies, combining the co-expression of multi-enzymes and recombinant DNA technology in *E.coli* with model optimization by flux balance analysis has

remarkably improved the overall alkane production by 36-fold (Fatma et al. 2018). Metabolic engineering of *Y. lipolytica* followed by fermentation optimization has yielded a high lipid content of about 99 g/L for which the NADH was supplied by the pentose phosphate pathway (Wasylenko et al. 2015; Yang et al. 2019). Similarly, another study by Li et al. (2020) reported 1.47 g/L of hydrocarbon yield in the engineered *Y. lipolytica* strain which was higher than the yield attained in *E. coli* (1.31 g/L). Therefore, genetic modification and fermentation optimization play a significant role in improving the efficiencies and yield of long-chain hydrocarbon (alkanes or alkenes) in *E. coli* and yeast.

2.4 Isoprenoids

Isoprenoids or terpenoids are a class of organic compounds found in plants and animals and have isoprene, a five-carbon compound, as their building block. They are classified based on the number of isoprene units, and a few of their categories are mentioned: hemiterpenes (C₅), monoterpenes (C₁₀), sesquiterpenes (C₁₅), diterpenes (C₂₀), triterpenes (C₃₀) and tetraterpenes (carotenoids, C₄₀) (Isoprenoid, 2018). Biosynthesis of isoprenoids occurs through the mevalonate pathway (MVA) or the 1-deoxy-D-xylulose-5-phosphate pathway (DXP) and is derived from isopentyl diphosphate and dimethylallyl diphosphate. Among the microbes, *E. coli* and *S. cerevisiae* are the common heterologous hosts for the genetic manipulation and metabolic engineering of isoprenoids. The genes for synthesis are taken from a wide range of plants and fungi. Isoprenoids have a common characteristic, i.e., cyclic structure and methyl branching. The methyl branching decreases the freezing point, while the cyclic structure increases energy density, making them suitable as diesel and jet fuel substitutes (Niu et al. 2017). The synergistic effect of MVA and DXP pathway has improved the isoprene production (60 g/L) in *E. coli* under fed-batch fermentation (Yang et al. 2016). The enzyme genes such as ISP-DEFGH, *DXR*, and *DXS* are the common

overexpression targets in *E. coli* to enhance isoprenoid biosynthesis (Lv et al. 2013). For instance, overexpression of *IDI*, *DXR*, and *DXS* genes in *E. coli* has resulted in 17.5 mg/L of isoprene in a shake flask cultivation. Whereas, Lv et al. (2016) explored the dual metabolic engineering of mitochondrial and cytoplasmic acetyl-CoA and MVA pathway modification in *S. cerevisiae* resulting in 2.5 g/L and 37 mg/L of isoprene in fed-batch and shake-flask fermentation respectively. Recently, Navale et al. (2021) reported that the overall production of isoprenoids could be increased by opting for the fusion enzyme strategy. Future, modular control systems, chromosomal integration, and CLIVA-pathway assembly techniques could aid in the enhancement of the isoprenoid biosynthesis.

3. An integrative OMICS for biomanufacturing of CAzymes for biomass processing

Biorefineries and biomass processing are centered on the prime utilization of the biomass obtained from plant feedstock present in ample proportions, which proves to be an excellent source of complex carbohydrates. Plant biomass is primarily composed of carbohydrate polymers i.e., hemicellulose (20-35%) and cellulose (35-50%) which prerequisites a large arsenal of enzymes for its breakdown (Sun and Cheng, 2002). CAzymes (Carbohydrate-active enzymes) are a kind of enzyme family that targets these carbohydrates, which undergo biotransformation, i.e., synthesis, metabolism, modification, and delivery (Zerva et al. 2021; André et al. 2014). These CAzymes can degrade lignocelluloses into simple sugar compounds further usable for biofuel production and other value-added products (Bredon et al. 2018).

The CAZymes are classified into 5 major classes namely glycoside hydrolases (GH's), carbohydrate esterase(CE's), polysaccharide lyases (PL's), glycosyltransferases (GT's), and auxiliary activities (AA's) based on the sequence similarities of amino acids (Henrissat, 1991; Park, 2018). Among them, GH has a vital role in breaking the β -1,4 glycosidic bond during the

hydrolysis of polysaccharides into simple sugar monomers. Also, the utilization of sugar monomers for the production of various biofuels and value-added chemicals such as organic acids and biosurfactants have been reported (polypeptides, glycolipids, phospholipids, polymers, and fatty acids) (Paulino et al. 2017). In this regard, microbial-based glycolipids are predominantly emerging as an alternative for synthetic surfactants that find application in the bioremediation of hydrocarbon and heavy metal-contaminated soil. Glycolipids are possessed with similar properties of surfactants and act as effective anti-biofilm forming and anti-microbial agents (Ng et al. 2023). CBM's presence in many CAzymes accelerates the propinquity of the enzyme to its substrate, especially for the highly insoluble ones (carbohydrate-binding molecules), which help in their conversion to simpler molecules (Contesini et al. 2021). Although the present CAzymes and their families are well-acknowledged for their line of action, there are still constraints that exist in the transformation of cellulose which is highly recalcitrant and which also proves to be the most significant polysaccharide and an industrially worthy sugar substrate obtained from the lignocellulosic biomass. To effectively degrade these lignocellulosic compounds, a cocktail of CAzymes posing in a complementary and cooperative manner is utilized, which converts the trapped carbohydrate molecules such as cellulose and hemicellulose to various usable products (Bredon et al. 2018).

Different technologies, like multi-omics measurements, direct measurement of enzyme activity, cell isolation, cell sorting, and NMR, can be used to develop insights into CAzymes and their application. To screen novel CAzymes, rapidly advanced ORFs and operons can be used for proximate screening of required enzymes. Algorithms like IDBA-UD and MEGAHIT are based on the de Bruijn graph that is involved in assembling the sequence reads from a complex metagenomic or single-cell sequencing and aids in the reconstruction of contigs with high

accuracy. Whereas, metaSPAdes, a metagenomic assembler, exploits the computational ideas to address the limitations of complex metagenomic assembly and has also proven to be beneficial in polymorphic diploid genomes or single cell assemblies (Peng et al. 2012; Li et al. 2015; Nurk et al. 2017).

T-RFLP has also been exploited for other studies (Deutsch et al. 2015). To engineer these novel microorganisms producing CAzymes, strategies require the discovery of enzymes and their engineering to understand the complexity of the recalcitrance barrier of biomass. This includes metabolic engineering and synthetic biology, including OMICS, to produce tailor-made microbes with the required attributes, to understand the underlying cellular function (Heux et al. 2015).

An effective metagenomic technology called PCR-DCGE can study anaerobic CAzymes, which produce bacteria and archaea in the digester (Idalia and Bernardo, 2017). To identify and amplify novel CAzymes to degrade lignocellulosic compounds, genome-related metaproteome, and metatranscriptome are administered on anaerobic fungi where their CAzymes-related bioinformation indicates domain-specific specialization, and it is found that both endo, as well as exo acting enzymes, are used to target cellulose structure (Hagen et al. 2021).

The microbial enzyme evolved along with its other strains and has co-evolved into hydrolase xylanases. It comprises arabinofuranosidase, glucosidases, and galacturonisidases which undergo hydrolyzation of ester bonds to link acetyl and phenolic acid moieties.

Bioactive compounds such as alginate oligosaccharides and non-carbohydrate related fractions such as lignin are transformed into ferulic acid and vanillin with the help of lignin modifying enzymes. One method of gaining insights into the enzymatic arsenal is via the transcriptomics analysis of the organism (Fig. 3). Another approach is the secretomics analysis that can provide insight into the CAzymes arsenal established by microorganisms (Heux et al. 2015).

Transcriptomics has been an advantage over metagenomics as its main focus is on metabolically effective microorganisms and their related genes (Soccol et al. 2022). It also helps recognize active genes involved in lignocellulosic degradation and marker exploration to control the industrial manufacture of biogas (Raghavachari, 2013; Stark et al. 2014). The importance of xylan-active enzymes has become highly recognized because xylooligosaccharides are also potent inhibitors of celluloses which can accelerate the bio-refining process (Qing et al. 2010; Qing and Wyman, 2011).

A few applications of genetic engineering approaches focused on promoting bioconversion and enhancing value-added compound production are discussed in detail. *Rhodococcus jostii* RHA1, an ancestral strain was widely studied owing to its peculiar ability to utilize lignin-derived aromatic compounds as well as the lignocellulose-derived sugar monomers (DeLorenzo et al. 2017). Genetic and metabolic engineering of *R. jostii* RHA1 has optimized the lignin bioconversion into various products like pyridine-dicarboxylic acids (PDCA), cis,cis-muconate (CCMA), and triacylglycerols (TAGs) (Xie et al. 2019; Cai et al. 2020; Spence et al. 2021). For example, homologous recombination of *pcaHG* genes (*R. jostii* RHA1) with *praA* gene (*Paenibacillus* sp. JJ-1b) and *ligAB* genes (*Sphingobium* SYK-6) has blocked the β -ketoadipate pathway by the deletion of *pcaHG*. And simultaneously expressed the enzymes associated with the PDCA production.

In addition, oxidation rate of lignin and subsequent accumulation of PDCA was drastically increased by the heterologous expression of *dyp2* (*Amycolatopsis* sp. 75iv2) using a different lignin model compound as a sole carbon source (Spence et al. 2021). Similarly, heterologous recombination of xylulokinase and D-xylene isomerase encoding *xylB* and *xylA* from *Streptomyces padanus* MITKK-103 in *R. jostii* RHA1 has enhanced lipid accumulation using

xylose (Kurosawa et al. 2013). A recombinant *R. jostii* RHA1 cell utilizing L-arabinose was also obtained by expressing the genes (*araD*, *araB*, and *araA*) from *E.coli* K12 MG1655 and *Streptomyces cattleya* NRRL 8057, resulting in 39.7% of lipid accumulation in engineered strain (Xiong et al. 2016). Another one is the fabrication of a low MW aromatic product using an *R. jostii* RHA1 gene deletion mutant which generates vanillin, an excellent chemical product utilized in the food and flavor industry. Lately, *in-silico* modeling of *Synechocystis sp.* PGC6803 for ethanol production evolved from data combining genomics, transcriptomics, proteomics, and metabolomics (Qing et al. 2010; Qing and Wyman, 2011; Nishiguchi et al. 2019).

4. Transcriptomics for algal and yeast Biorefinery

Current development in the integrated transcriptomics of algal and yeast strains significantly aids in recognizing several genes and provides a basic understanding of carbohydrate and lipid biosynthesis. Some of synthetic biology's gene manipulation processes opted for producing microalgae and yeast-based bioengineering products such as biodiesel, bioethanol, biomethane, biohydrogen, and single-cell oil are discussed in the subsequent section.

4.1 Integration of transcriptomics in biorefinery

Recently, studies have primarily focused on genetic modifications of microalgae encompassing complex cellular metabolism to upgrade carbon fixation, high value-added chemical formation, and lipid accretion (Raheja et al. 2022; Bhattacharjya et al. 2021). Genome sequencing and transformation systems have been more beneficial and usable in recent years. Some of synthetic biology's basic gene manipulation processes (gene target, selection system, plasmid construction, DNA editing tools, and host selection) remain the same (Chakdar et al. 2021). Transformation and selection methods are major objectives to achieve the target gene modifications. Recently, TALEN, CRISPR Cas9, and ZFN (new genome-editing tools) have been used in gene

modification. CRISPR-dCas9, miRNA, and siRNA (gene-interfering tools) are used to suppress or initiate the gene expression for desired microalgae phenotype without modification of genes (Kang et al., 2022; Brar et al. 2021; Mishra et al. 2019). *Chlamydomonas reinhardtii* (model microalgae), the first model to exhibit the CRISPR-Cas9 gene modification, showed clear evidence that Cas9 and sgRNA can accomplish the purpose of algae (Jakhwal et al. 2022). Apart from understanding the genome information, transcriptomes give out an approach to identifying mutant strains.

In *Dunaliella tertiolecta* (non-model microalgae), lipid content is increased by random insertion of plasmid pGreenI1000, which helps in gene upregulation in the photosynthesis pathway. Besides random insertion, a mutant *Chlamydomonas reinhardtii* produces a 5.2-fold enhancement in H₂ production than by exposure of microalgae to ARTP (atmospheric and room temperature plasma). Additionally, a comparative transcriptome revealed the gene upregulation of photosynthesis, including photosystem I, II, cytochrome b6/f complex, and ATPase to improve hydrogen production. Many successful genetic transformations have been done for algae. To date, more than 30 different strains of algae have been transformed effectively. The stability of expression can be enhanced through proper usage of the codon, sequencing of introns, and the use of endogenous promoters. Different methods are used to transfer DNA into microalgal cells, including electroporation, agitation of glass beads, biolistic microparticle bombardment, and gene transfer. Various genetic engineering strategies adopted for microalgae bioengineering were deciphered in Fig. 4.

4.2. Microalgae and yeast-based biofuel engineering

Microalgae serves as a promising source for biofuel production owing to the high lipid or carbohydrate yield and faster growth rate. However, the cost-intensive cultivation system for

microalgae necessitates other feasible processes concerning the production of lipids or carbohydrates. From the aspects of engineering actions, attaining high lipid or carbohydrate productivity of algae is consistently the crucial issue while estimating commercial potential. In many reports, the productivity of lipids or carbohydrates of the microalgae is calculated by multiplying biomass with lipid content. This may not give exact results as productivity content is generally not linearly correlated.

Yeast is an authentic little factory worthy of completely changing plant sugars into fuel. The magic works through an anaerobic method known as fermentation. Sugars (specifically glucose) contained in cellulose or starch are transformed into ethanol under the action of yeast enzymes. Yeast already helps make bread and beer out of the biofuel ethanol, but scientists believe it can produce an excellent economical fuel called isobutanol. Normally, yeast alone creates a minimal quantity of isobutanol. Currently, researchers at Princeton are attempting to extend isobutanol production by roughly five times that of normal yeast strains by making the yeast more tolerant to isobutanol's noxious effects. Isobutanol has a greater energy density than ethanol by almost 25% and is a lot better suited for use in vehicles than current ethanol-based fuels. Recently, Bisht et al. (2023) studied the efficiency of microalgae and yeast consortium-based biofilm for biofuel production which resulted in a high amount of lipid (36%) with 47.63 g/m² and biomass yield and productivity. Therefore, the application of a co-cultivation strategy using a microalgal-yeast-based consortium could aid in COD removal from wastewater through its synergistic action and enhance the biomass productivity for biofuel production (Satpati et al. 2023). Table S2 represents the various omics strategies adopted in microalgae and yeast for the enhanced production of various biofuels and value-added chemicals. In microalgae-based biorefinery, the titer of various biofuels (ethanol, butanol, isobutanol, FAME) and value-added chemicals (carotenoid) is enhanced by the

overexpression of Pdc, phaJ, ter nphT7, bldH, phaB, yqhD, adhA, bHLH and Phytoene synthase gene in *Synechocystis* sp. PCC 7002 (Wang et al. 2019), *Synechococcus elongatus* PCC 7942 (Lan and Liao, 2012), *Synechocystis* sp. PCC 6803 (Miao et al. 2018), *Nannochloropsis salina* (Kang et al. 2015), and *Chlamydomonas reinhardtii* respectively (Couso et al. 2011). Whereas, the knockout of the Phospholipase A2 gene in *Chlamydomonas reinhardtii* has increased the lipid yield by 64.25% (Shin et al. 2019). On the other hand, the gene fusion (bisabolene synthase and ERG20 gene from *Abiesgrandis*) and overexpression (MCE2 and gapC gene from *Mucorcircinelloides* and *Clostridium acetobutylicum*) were studied in *S. cerevisiae* and *Yarrowia lipolytica* for enhanced Bisabolene (150 mg/L) and FAME (98.9 g/L) production respectively using glucose as a carbon source (Qiao et al. 2017).

Table S2

4.3. Products obtained from algal and yeast Biorefineries

4.3.1. Products obtained from algal biorefinery

Microalgae are compounds that are rich in energy in large amounts (starch and triacylglycerol). These compounds can be converted into Bioethanol and Biodiesel. Production of energy from microalgae has the advantage of fast mass production of carbon dioxide. Biomass obtained from microalgae can be used to produce ethanol, biogas, Biodiesel, and hydrogen. Biofuels obtained from microalgae are presently perceived as the third generation addressing an altogether new measurement in the option bioenergy field.

4.3.1.1. Biodiesel

Microalgae are considered an elective energy source without the downsides of the first- and second-generation biofuels. Biodiesel is a likely replacement for ordinary diesel fuel. Biodiesel is non-toxic and biodegradable with little CO₂ release. Transesterification is the process by which

Biodiesel can be obtained from several raw materials. This process uses sodium hydroxide and potassium hydroxide in the presence of a catalyst. One of the fundamental factors that impact microalgae's lipid and fatty acid content as far as development is CO₂ fixation. The essential microalgae species utilized for Biodiesel are *Chlorella*, *Chlamydomonas*, *Nannochloropsis*, and *Botryococcus*. The quality of Biodiesel is incredibly reliant on the composition of fatty acids. There are many advantages of microalgae-based Biodiesel, which include high oil content and high growth rate.

4.3.1.2. Bioethanol

Another popular fuel is Bioethanol, which is fundamentally derived from sugar- and starch-based food crops. Several fermentation processes produce ethanol. For the production of bioethanol, lignocellulosic materials can be best because they are high in lignin and can also have vast mass amounts of carbohydrates primarily composed of starch and cellulose. Accordingly, even though the quantity of studies concerning the utilization of microalgae for bioethanol creation is generally restricted contrasted and studies concerning their utilization for biodiesel creation, numerous scientists accept that soon, microalgae-based bioethanol creation will end up being a possible option for traditional sugar-rich yields or lignocellulosic biomass feedstocks for creating bioethanol.

4.3.1.3. Biomethane

Anaerobic digestion of microalgae is one of the intriguing strategies in biomethane production that involves a series of conversion processes like hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Ma et al. 2018; Kannah et al. 2019). Microalgae species such as *Laminaria*, *Saccharina*, *Genera Ulva*, and *Fucus* are widely explored for biomethane production where the methane amount ranges from 143 L to 400 L per kg of volatile solids (VS) (Perazzoli et al. 2016).

The cell wall composition of microalgae has a crucial role in methane production, where the methane yield was considerably higher for algal biomass with high lipid content (1014 L/kgVS) than the carbohydrate (415 L/kgVS) or protein-rich (851 L/kgVS) biomass (Angelidaki and Sanders, 2004; Becker, 2007). Recently, Hu et al. (2021) have studied the efficiency of microalgal consortium as a co-substrate in biomethane production which results in improving the biomethane yield by 27.4% with 81% of energy recovery. In addition, cultivation conditions, carbon-to-nitrogen ratio, operating parameters, and salt concentration also influence the anaerobic digestion of algae (Kumar et al. 2016).

4.3.1.4. Biohydrogen

Biohydrogen production from microalgae like green algae and cyanobacteria is a promising alternative strategy that produces environmentally benign renewable energy to achieve sustainability and carbon neutrality. Photosynthesis (direct and indirect biophotolysis) and fermentative (photo and dark fermentation) production are the two different biohydrogen generation routes by microalgae (Wang et al. 2021). Studies have shown that among different routes, microbial dark fermentation is of great potential as it is capable of successfully generating biohydrogen without relying on solar energy (Foong et al. 2021). Several microalgae feedstocks such as *Saccharina sp.*, *Chlorella sp.*, and *Scenedesmus sp.* are widely studied (Wang and Yin, 2018). In addition, the fermentative biohydrogen is obtained primarily from the carbohydrate fraction of microalgae. Hence a mild pretreatment of algal biomass enhances the hydrogen productivity by 50-70% upon destructing the other cell wall components like algaenan and pectin (Nagarajan et al. 2020; Griffiths et al. 2021; Barati et al. 2021). Recently, Abdel-Kader et al. (2022) reported that co-pretreatment of *Chlorella* biomass with macerozyme and cellulase has resulted in 2350 mg/L of biohydrogen. Whereas, the efficiency of biohydrogen production was found to be

4200 mL/L using yeast (*Rhodobacter sp.* and *Rhodopseudomonas palustris*) as a source of hydrolases. On the other hand, genetic engineering approaches such as gene silencing, gene overexpression, gene disruption, and RNA interference are opted to either enhance the electron flow or modify the vital enzymes involved in biohydrogen production (Chaturvedi et al. 2020; Fayyaz et al. 2020). For example, genetic manipulation of *C. reinhardtii* (expression of pyruvate oxidase genes (POX) and catalase (CAT) from *E.coli* and *Synechococcus elongatus*) has increased biohydrogen productivity by 100 times after 48 h of fermentation. Similarly, the expression of the methanotrophs gene in microalgae to intensify oxygen tolerance also improves biohydrogen production (Xu et al. 2011). Recently, techno-economic feasibility analysis has revealed that the cost of algal biohydrogen production is found to be 7.61 kg⁻¹ and 7.24 kg⁻¹ US dollars for fermentative and photolysis. Future, research should focus on designing an efficient bioreactor that has a pivotal role in the overall production cost (Shokravi et al. 2021).

4.3.2. Products obtained from yeast biorefinery

After extensive engineering and thorough research, various oleochemicals have been produced from yeast cell factories specializing in central carbon metabolism and lipid metabolism. For oleochemical production, oleaginous yeast hosts, like *Yarrowia lipolytica*, *Rhodospiridium toruloides*, and *Cryptococcus curvatus*, are typically chosen as industrial workhouses for their efficient lipid synthesis and accumulation (Zhang et al. 2021). Vast amounts of ethanol (the alcohol in wine and beer) and carbon dioxide (a gas that makes bread rise) are made using yeast. Ethanol as a fuel is produced by the yeast *Saccharomyces cerevisiae* (baker's yeast) mainly from sugarcane sucrose in Brazil and corn starch in the United States, which manufacture around 85% of the worldwide yearly production of 109.8 million m³. Around 80% of the ethanol produced in North America, including strains that express amylases, are genetically engineered (GE) strains which

help to provide higher ethanol yields; whereas more significant than 90% of non-GE strains in South America are used in ethanol production along with cell recycling primarily as starters in non-aseptic fermentation systems. Even after substantial research on lignocellulosic ethanol (or second-generation ethanol), this option still accounts for less than 1% of global ethanol production (Jacobus et al. 2021).

4.4. Challenges and feasibility of microbial lipid-based lignocellulosic biorefinery

Lipid production from lignocellulosic biomass has received substantial attention only in recent years, even though single-cell oil (SCO) has been studied for decades as biofuel research moves toward producing drop-in fuels. The challenge currently facing research is twofold: increasing yeast's resistance to alcohol and extending yeast's assimilation capability to other forms of sugar aside from glucose, like pentoses. These pentoses are available in plant fibers, making up 30% instead of 70% of glucose. The range of renewable energy sources available will increase if we rise to this challenge and promote sustainable development. We can propose many alternatives to fossil fuels, specifically in the transport sector, to reduce greenhouse gases. So far, microalgae engineering is now fronting many restrictions. In *Chlorella sp.*, a shortage of genomic materials and poor efficiency of transformation technique has blocked the enlargement of genetic engineering in microalgae. Take scale-up and industrial applications, for example, require enormous research targets. While microalgae can openly change CO₂ into chemicals, the amount concentration was a lot less than the particular value. For example, in *E.coli* and cyanobacteria, the maximum production yield of ethanol could be easily attained.

5. Bottlenecks and future prospects for multi-omics

The main hurdle that creates barriers to the utilization of lignocellulosic feedstock in biorefinery is the phenomenon of lignocellulose depolymerizing sugar. CAzymes are specifically designed to

act upon such recalcitrant biomass. According to previous studies and the CAzymes database, a mixture of cellulases such as EG's and CBM can synergistically hydrolyze these lignocelluloses. Other efficient enzymes such as ferulic acid and acetyl xylanesterases can be employed to cleave hemicellulose. The molecular approaches that are conducted on cellulolytic microbes have quickened the rapid development of NGS (Next Generation Sequencing) of metagenomes and transcriptomes of cellulolytic microbes using tools based on mass spectrometry and high-performance proteomics tools.

With the help of techniques such as NGS and OMICS, several novel species involved in microalgal lipid metabolism are discovered, such as *Ostreococcus taris*, *Volvox carteri*, *Chlamydomonas reinhardtii*, *NC64A*, *Dunaliella salina*. Complete sequencing takes place, and the database obtained helps annotate the genes that participate in microalgal lipid metabolism. (Benmoussa, 2016). Microalgae are an excellent feedstock for third-generation biofuels and many valuable co-products (Chisti, 2007). Although much is not known about the algal genome and its regulatory mechanisms, functional genomics (OMICS) can be used to get more insights into it (Benmoussa, 2016). For enhanced biofuel production, functional lipids in certain microalgae create a significant aspect of the ever-growing economy and the development of third-generation biofuel. Comparative proteomics analysis is an efficient way to understand cell metabolism and identify key proteins in biosynthesis pathways and trafficking systems in other organisms such as fungi. Expansins found in aerobic bacteria, including *Bacillus* sp., have potent cellulose enhancers for hydrolysis cellulose substrates, particularly at low dosages (Bunterngsook, 2014). The genome sequencing of other cellulolytic fungi belonging to the phyla *Basidiomycetes* and *Actinomycetes* has revealed that a total of 218 genes are associated with lignocellulose degradation.

RNAi-mediated, si-RNAs and artificial miRNA silencing can be used to understand other essential metabolic pathways. These pathways can be further engineered to synthesize essential biomolecules to obtain valuable products and for cost-effective processing of by-products (Cerutti et al. 2011). Certain omics technologies are independent of culture media that follow specific metagenomics ideas to reveal the convolution of cellulolytic enzymes. Through high-throughput sequencing, recognizing genes with different functions can help identify new enzymes for such processes. The progression of recent high throughput screening techniques for CAzymes assessment, which undergo in vivo optimization for the design of pathways, is the current hindrance in developing novel CAzymes. Using phylogenetic analyses can be helpful in the detection of preserved genes.

A study recognized and mapped QTLs for seed oil content and yield in *Jatropha curcas* by designing SNP markers using SSR markers for genomic data. The search for the discovery of effective enzymes will be supplemented by enhancement in enzyme engineering and heterologous hosts for the production of high-quality cellulases. The productivity of synonyms can be further accelerated by chemical additives like surfactants in cellulase blends (Li et al. 2016; Garvey et al. 2013). In the future, genome sequence and analysis that can predict promoters' strength and inducibility can be studied. Enzyme extraction from *Actinomycetes* and Thermophiles is another promising sector in the development of CAzymes. Other high-value components such as carotenoids and limonene can be used as a secondary fuel in biorefineries. Thus, gene annotation, along with CRISPRi and CRISPRa technologies, can provide new opportunities for synthetic biology and OMICS, which in turn can fasten the process of identifying carbohydrate-degrading enzymes and a promising tool for biorefineries (Lin et al. 2019). In a nutshell, NGS and OMICS techniques based functional characterization of microbial species and elucidation of metabolic

pathways through RNAi technologies provide deep insights for engineering the strains towards valuable biomolecule production. Genomics together with advanced synthetic biology tools could aid in establishing a microbial refinery.

6. Conclusion

Synthetic biology coupled with the integration of omics techniques (genomics and transcriptomics) using different sources is advanced to meet the energy demands. Microalgae and yeasts are also being researched for producing other bio-based fuels and biorefinery products. Thus, synthetic biology integrated OMICS help broaden the available options of biofuels, the production of long-chain alcohols, alkene/alkanes, fatty acids, and isoprenoid-based biofuels. CAzymes can degrade lignocellulosics into simpler sugar compounds used for biofuel and value-added products. Therefore, the review encompasses synthetic biology with OMICS approaches that could result in the sustainable development of biofuels and biorefinery products.

Conflict of Interest

The authors declare that they have no conflict of interest in publishing the article.

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Declaration of generative AI in scientific writing

During the preparation of this work, the author(s) did not use any AI tool/service.

List of Abbreviations

The list of abbreviations kept under supplementary Material

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Figure Captions:

Fig. 1. Generation of biofuel and high value-added chemicals from different biomass.

Fig. 2. Schematic diagram depicting workflow of metabolomics pipeline pertinent to microbial metabolite extraction, purification and characterization

Fig. 3. Overview of the key steps involved in the microbial transcriptomics with various bioinformatics tools.

Fig. 4. Schematic representation of various genetic engineering strategies for bioengineering of microalgae

Figures

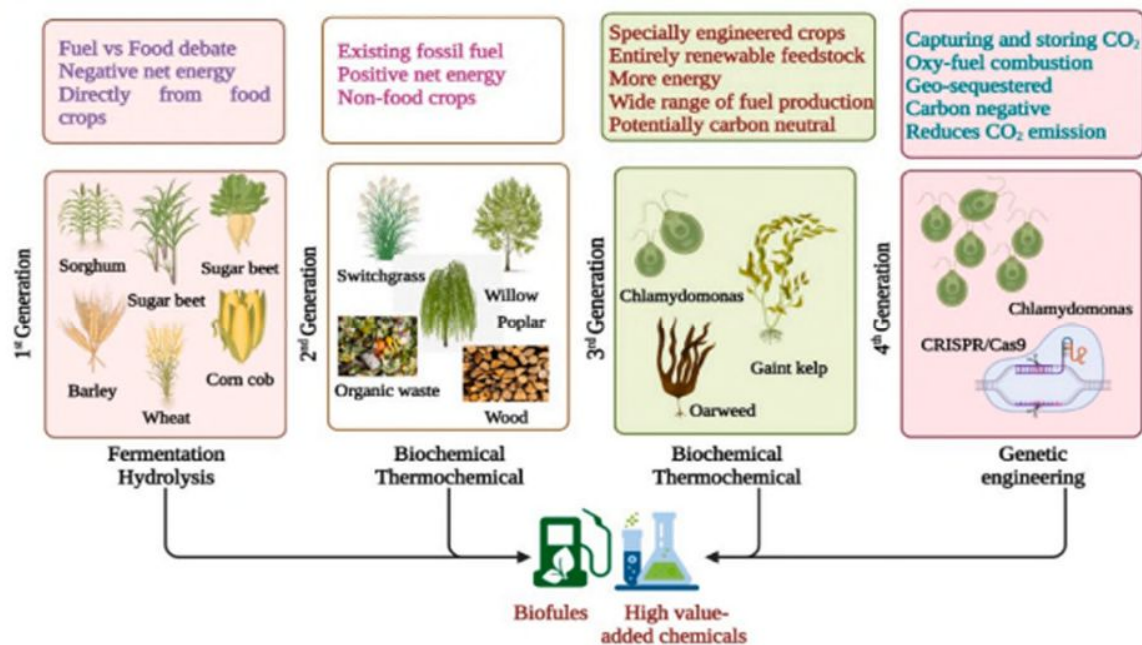


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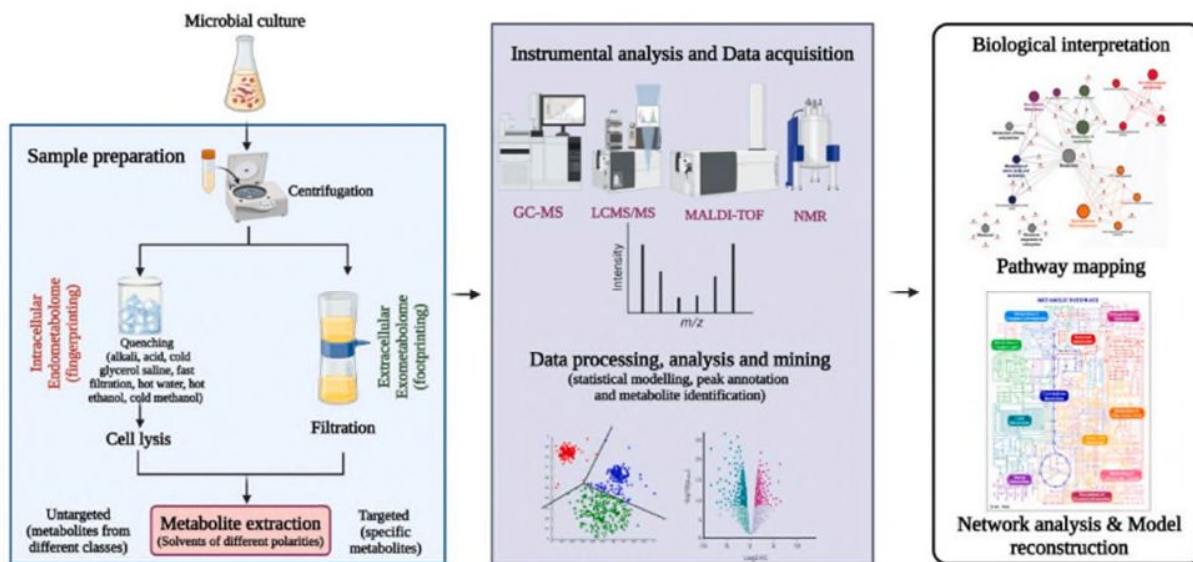


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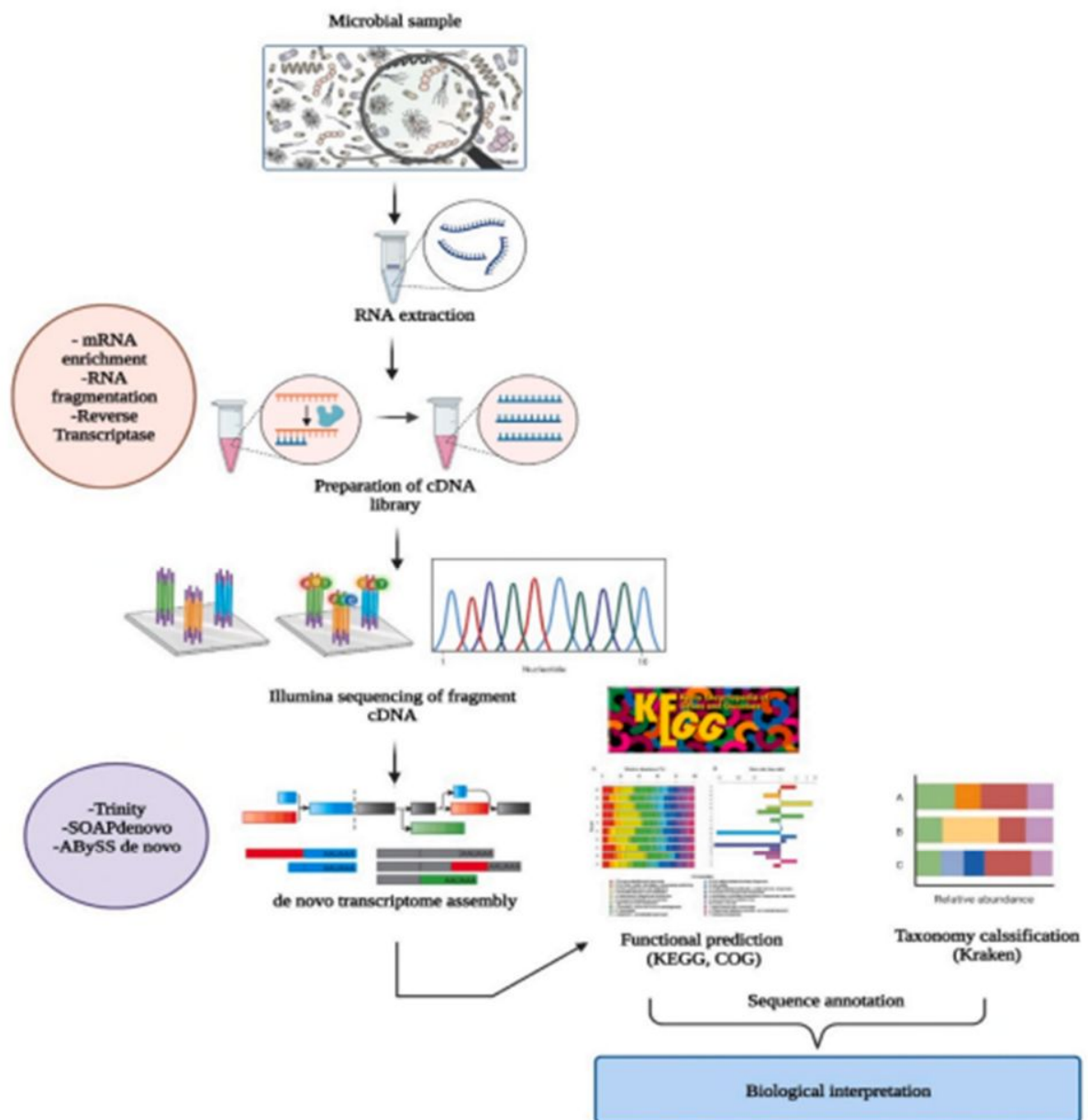


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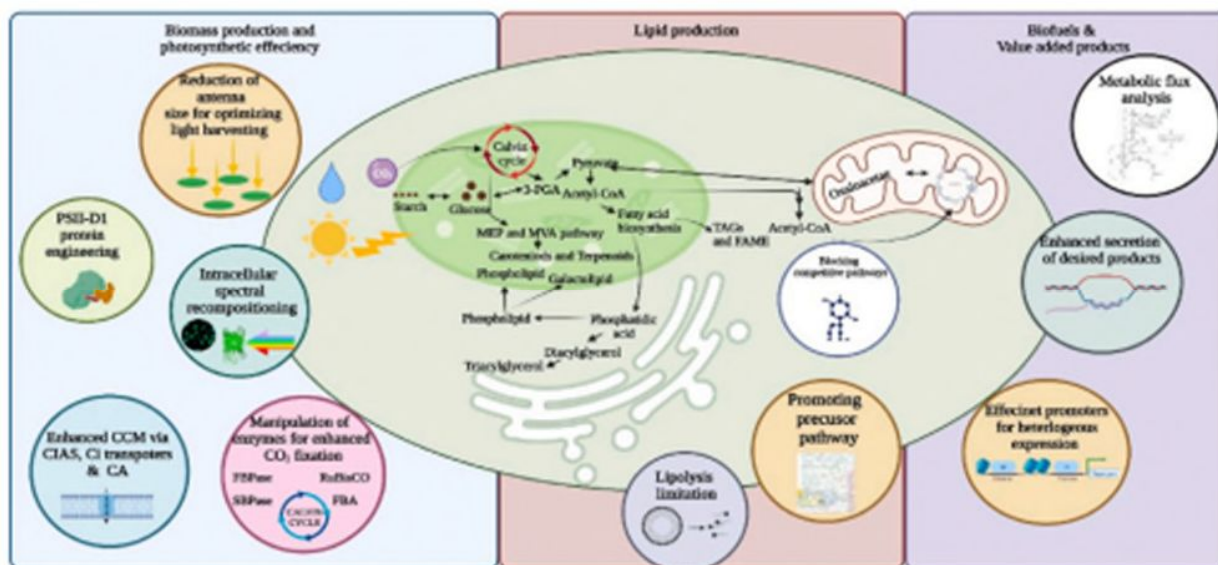


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Paradigm of integrative OMICS of microbial technology towards biorefinery prospects

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