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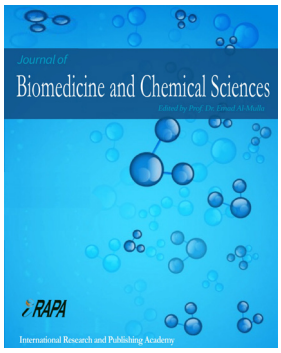

Metabolic Engineering of Microbial Cell Factories for Sustainable Biomanufacturing

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ABSTRACT

Metabolic engineering is essential for the development of microbial cell factories to produce biomolecules from low-value renewable substrates. This role helps advance the development of ecologically responsible and commercially robust chemical industries, including biofuels and high-value compounds like medicines. The ability of microbial cell factories to generate a wide variety of substances sustainably, therefore satisfying modern commodity needs, has piqued the scientific community's attention. The goal of metabolic engineering is to convert different microorganisms into efficient cell factories to produce desired products, and it has been used for decades to develop novel metabolic pathways and alter pre-existing ones with the help of system biology, synthetic biology, and evolutionary engineering.

INTRODUCTION

The advent of the idea of metabolic engineering in the early 1990s sparked the development of a new scientific discipline and greatly accelerated the rate at which cells capable of producing chemicals could multiply (Koffas et al., 1999; G. Lee et al., 2023). Metabolic engineering is a method that uses recombinant DNA technology to alter a living organism such that it produces an engineered metabolite (Rahmat & Kang, 2020). Methods like genome editing and evolution, tolerance engineering, and metabolic flux rewiring have been created to help us get there (Munro & Kell, 2021). Both prokaryotes like Corynebacterium glutamicum and Escherichia coli and eukaryotes like yeast have been used as cell factories to create the necessary compounds using these methods (Onda et al., 2003).

The use of synthetic biology advances and the growth of information about metabolite damage and its mitigation or repair were causing improvements in the processes and efficiency of the area of metabolic engineering (Withers & Keasling, 2007). The first experiments in metabolic engineering showed that if the relevant damage control systems are insufficient or missing, a buildup of reactive intermediates might hinder the flow of constructed pathways and threaten the host cells (Watstein et al., 2015). Synthetic biologists aim to improve genetic pathways (Benner & Sismour, 2005), changing the metabolic outputs of

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cells consequently (Andrianantoandro et al., 2006). Progress in genetic circuitry and the dramatic decrease in the cost of synthesized DNA have both influenced the efficacy of metabolic engineering (Shih et al., 2016).

Metabolic engineering is the study of manipulating metabolic pathways in live organisms to achieve certain chemical outcomes. It involves optimizing the synthesis of a product by changing genes, enzymes, and biochemical pathways (J. W. Lee et al., 2012). Metabolic engineering often also involves determining which metabolic route generates a target molecule and then tweaking that pathway to boost output or efficiency (K. R. Choi et al., 2019).

Recombinant DNA technology may be used to alter the metabolic pathways of different species, improving the genetic and regulatory activities of cells and so, increasing the production of a desired chemical (Acevedo-Rocha et al., 2019). Through a series of enzyme reactions and biochemical processes, chemical networks promote the conversion of basic resources into critical molecules required for cellular life (Z. Yang et al., 2008). The goal of Metabolic engineering is to create mathematical models of these networks, evaluate the effectiveness of producing valuable products, and identify the components of the network that limit their development. Genetic engineering techniques could be potentially employed to manipulate the network (Rabara et al., 2014), offering a means to mitigate the current constraints. This opens the possibility of modeling the modified network to ascertain the yield of the new product. Traditional metabolic engineering has aimed to either replace or supplement the laborious process of strain formation using computer modeling, informed by a select few essential studies (S. Y. Lee & Kim, 2015).

The capacity of metabolic engineering to expedite the synthesis of various compounds, fuels, and natural products from renewable biomass has led to significant progress (Nevoigt, 2008). This has resulted in less reliance on fossil fuels and a neutral carbon footprint (Salazar & Meil, 2009). Recent advancements in synthetic biology technologies have paved the way for the creation of microbial cell factories, capable of producing high-value molecules with high titer, yield, and productivity. These advancements encompass the comprehension and rewiring of metabolic networks, along with the development of genetic components and circuits (Lowry et al., 2012).

Metabolic Engineering Methods and Tools

The main objectives of metabolic engineering include enhancing chemical and fuel production, diversifying substrates (Ko et al., 2020), and bolstering the host organism (Yoon et al., 2013). This is achieved by conducting a detailed analysis of individual case studies. To fully appreciate the potential of metabolic engineering, also known as systems biology, it's crucial to comprehend the value of these resources. Research provides a foundational understanding of Metabolic Engineering's core principles, along with a few examples of its application in the systematic and quantitative analysis of cellular physiology. As a result of these advancements, improved technological processes have emerged (Ko et al., 2020).

In contemporary times, metabolic engineers utilize a broad spectrum of software tools. These tools are designed to support various experimental and analytical methodologies. They allow extraction and interpretation of information from large datasets, simplification of complex models into more digestible forms, and the proposition of effective network design strategies using computational methods integral to metabolic engineering. Techniques such as post-structural network analysis, pathway exploration, metabolic flux analysis, network visualization, nucleic acid and protein engineering, culture optimization, and attempts at metabolic reconstruction are all examples of technologies used for manipulating and understanding cellular metabolic networks (S. Y. Choi et al., 2020).

Methods for the metabolic engineering of microbe-based factories

The insertion or modification of genes involved in cellular metabolic pathways may improve metabolic pathways (Schuster et al., 2000). To further enable the creation of new substances, researchers have used synthetic biology techniques to design and build novel metabolic pathways inside cells. Protein engineering methods may be used to increase the production of desired chemicals by modifying enzymes that play a role in metabolic processes (Bilal et al., 2018).

In addition, metabolomics applications enable the monitoring of metabolite levels associated with a specific metabolic route, providing insightful new views on how best to control and optimize that process (Beger et al., 2016).

Researchers use synthetic biology methods to build new metabolic pathways in cells. Scientists have altered the genetic makeup of E. coli to produce thebaine, an opiate precursor (Cravens et al., 2019). Synthetic biology refers to the study of engineering innovative biological systems to perform certain

tasks (Rollié et al., 2012). Synthetic biology was used to develop a strain of yeast to produce cannabinoids, which have medicinal promise but were previously unavailable (Walker & Pretorius, 2018). Enzymes in metabolic pathways may have their specificity and efficiency improved by using directed evolution techniques (Hibbert & Dalby, 2005).

Use of system biology and other omics-based techniques

Metabolomics and transcriptomics are two examples of omics-based approaches that may be used to study cellular gene expression and regulation. Metabolomics can be used to monitor the concentrations of metabolites associated with a metabolic pathway, providing valuable insights into the regulation and improvement of the pathway (as referenced), while transcriptomics can be used to identify genes implicated in metabolic pathways (Jendoubi, 2021). Through the integration of data from various omics-based approaches, the area of systems biology seeks to get a comprehensive understanding of the operation of complex biological systems.

This discussion centers on sustainable bio-manufacturing via the application of metabolic engineering to microbial cell factories. Metabolic engineering and microbial cell factories have proven instrumental in the successful production of various environmentally sustainable bio-manufactured commodities, including but not limited to biofuels, biochemicals, bioplastics, biomaterials, medicines, and nutraceuticals (Cho et al., 2022). The production of artemisinin, an essential medication for treating malaria, involved the transfer of the metabolic pathway from the plant Artemisia annua to the yeast Saccharomyces cerevisiae (Zhao et al., 2022). Biofuels like isobutanol and the amino acid lysine were produced in E. coli by metabolic engineering (Pu et al., 2023). Enzymes like chymosin are often used in the cheesemaking process, and they were first cultivated from the fungus Aspergillus niger (Hellmuth, 2006). Polyhydroxyalkanoates (PHAs) have been produced using a wide variety of bacteria, including Pseudomonas putida and Cupriavidus necato, these bioplastics are seen as a potential replacement for petroleum-based plastics (Sehgal & Gupta, 2020). Enhancing metabolic pathway efficiency and developing innovative synthetic biology approaches to increase the range of synthesizable molecules are the primary foci of current research in this area (Zhu et al., 2021).

Exemplary Products and Applications of Metabolic Engineering

Artemisinin, an efficient antimalarial compound, is derived from the Artemisia annua plant, however, its natural synthesis is limited and expensive, prompting researchers to explore alternative approaches. By employing metabolic engineering, various organisms such as yeast and bacteria can be harnessed to produce artemisinin (Hommel, 2008). This is achieved by introducing the necessary DNA elements responsible for artemisinin synthesis into these organisms.

In 2013, the first successful demonstration of artemisinin production using metabolic engineering was accomplished in yeast. In this study, the yeast strain Saccharomyces cerevisiae was subjected to genetic modification in order to enable the synthesis of artemisinic acid, which serves as a precursor to artemisinin, utilizing glucose as the primary substrate. Genes sourced from Artemisia annua and other organisms were integrated into the yeast genome, establishing a biosynthetic pathway for artemisinic acid production. Subsequently, this compound could be chemically converted into artemisinin (Paddon et al., 2013).

In recent years, advancements in this methodology have led to improved artemisinin yield and reduced production costs. Notably, scientists have optimized the biosynthetic pathway by enhancing the expression of specific genes and modifying the growth conditions of the yeast (Zhao et al., 2022).

Chymosin: Through the utilization of metabolic engineering techniques, the production of chymosin, a crucial enzyme utilized in cheese production, can be achieved (Luerce et al., 2014). The conventional source of chymosin was derived from the abomasum (fourth stomach) of young ruminant animals. However, this method presents limitations in terms of accessibility, animal welfare concerns, and batch-to-batch variation. To overcome these challenges, metabolic engineering has been employed to produce chymosin in a more sustainable and controllable manner (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) et al., 2022). The conventional approach involves the incorporation of the bovine chymosin gene into the genetic makeup of a compatible host organism, as is often observed in the context of chymosin. Commonly, microorganisms such as Escherichia coli bacteria or Aspergillus niger fungi are utilized for this objective (Silano et al., 2022).

Through genetic engineering techniques, the metabolic pathways of the host organism are altered to enable the synthesis of active chymosin (Pickens et al., 2011). By integrating the gene encoding bovine chymosin into the genome of the host organism, the

chymosin protein can be expressed and synthesized. The genetically modified host organism is then cultivated under controlled conditions to optimize chymosin production (Wei et al., 2016).

The metabolic engineering approach for chymosin production offers several advantages. It facilitates large-scale production, ensuring a constant and readily available supply of chymosin (Noseda et al., 2013). Additionally, it reduces reliance on animal-derived sources, addressing concerns related to animal welfare and batch-to-batch variation (Prache et al., 2022). Furthermore, by optimizing the metabolic pathways, it becomes feasible to enhance the efficiency and yield of chymosin production (Luerce et al., 2014).

Metabolic engineering provides a promising strategy for the sustainable and scalable production of chymosin, presenting a valuable alternative to conventional techniques and fulfilling the requirements of the cheese industry (Daboussi & Lindley, 2023).

Lysine: Lysine is an essential amino acid, as it is unable to be biosynthesized within the human body and must be obtained through dietary sources (Tomé & Bos, 2007). The significance of lysine resides in its utilization for the synthesis of proteins, facilitation of enzymatic activity, and involvement in diverse metabolic processes. Furthermore, lysine finds extensive application in animal feed due to its ability to enhance nutritional value (Liao et al., 2015). Within the realm of metabolic engineering, researchers possess the capability to manipulate the metabolic pathways of specific microorganisms with the aim of augmenting lysine production (Pickens et al., 2011). Typically, the microorganism Corynebacterium glutamicum serves as the preferred candidate for this purpose, as it naturally produces lysine. However, scientists have discovered approaches to heighten lysine production by modifying its metabolic pathways.

This endeavor may encompass diverse strategies, such as:

- The organism 49 exhibits increased production of lysine-synthesizing proteins as a consequence of the overexpression of genes associated with lysine synthesis. (Jakobsen et al., 2009).
- By suppressing the expression of genes associated with the enzymatic conversion of lysine into alternative compounds, it is possible to enhance the intracellular accumulation of lysine by 50% (Hallen et al., 2013).
- Modifying the organism's metabolic processes in other manners to amplify lysine production (Korosh et al., 2017).

By employing these methodologies, lysine production can be significantly increased, rendering it a more economically viable additive for animal feed and various other applications.

Microbial Cell Factories

The biotechnology industry is notably interested in the concept of microbial cell factories (Cho et al., 2022). Metabolic engineering forms a critical part of the bioengineering approach known as "microbial cell factory," which perceives microbial cells as types of production facilities (Calero & Nikel, 2019). Metabolic engineering can be applied to restructure the fundamental metabolism of these cells and facilitate the production of a wide array of both endogenous and exogenous metabolites (Rasor et al., 2023).

This research focuses on the engineering of metabolic pathways in microorganism-based factories for the manufacture of bioactive chemicals. To create desired molecules, metabolic engineering involves the modification of genes and biochemical processes in live organisms (Pickens et al., 2011). Scientists utilize microbial cell factories, which are populations of microorganisms that have been genetically engineered to produce large quantities of a desired molecule, such as a drug, biofuel, or food additive (Vitorino & Bessa, 2017). By combining metabolic engineering with microbial cell factories, scientists have been able to produce anything from simple compounds to high-end medications (Cho et al., 2022). The modification of cellular metabolic pathways to maximize the output of a desired molecule while limiting the creation of undesirable byproducts is a major challenge in metabolic engineering (Pickens et al., 2011).

Metabolic engineering has gained significant attention in the production of nutraceuticals, which possess the potential to enhance health and prevent disease (Ullah et al., 2021; Yuan & Alper, 2019). This growing interest parallels the advancements in microbial engineering, which have addressed challenges related to chemical synthesis and low extraction yields. Researchers have utilized this approach to develop nutraceuticals that offer added value to the industry.

The Wide Variety of Microbial Hosts

Recent attention has been paid to the successes and failures of designing both native and heterologous microbial hosts to produce bacterial natural products (Cho et al., 2022). The focus has been on the genetic resources and methods used to better the strains. These advancements have made it possible to design

microbial hosts to overproduce a target natural product, speeding up the strain enhancement process that had previously taken a very long time. The taxonomy of microbial hosts encompasses various organisms, such as bacteria, archaea, fungi (including yeasts and molds), algae, protozoa, and viruses. (Bajić et al., 2022). The field of functional genomics and genetic engineering has witnessed significant progress in recent times, enabling the expedited enhancement of strains through the amplification of specific natural products within microbial hosts (Zhang et al., 2016).

Commercialisation Status of Metabolically Engineered Microbial Cell Factories

Scientists have shifted their attention to finding sustainable ways of chemical and fuel production in response to rising concerns about climate change and the depletion of fossil resources (Wang et al., 2021). For years, scientists have been looking at the possibility of using microbes to convert sustainable non-food biomass into chemicals and fuels. A century ago, simple sugars were converted into acetone, butanol, and ethanol as the first industrial attempt at this method. This strategy has been researched extensively in recent years. Technological advances in the previous twenty years have allowed for substantial progress in the area under examination (Zheng et al., 2022). Low-priced whole-genome sequencing, systems-level gene expression profiling, and other omics methods fall into this category. This revolution has also been aided by routine and improved recombinant DNA methods, as well as in silico metabolic modeling and simulation, enzyme/pathway engineering and evolution, and other similar approaches (Dai & Shen, 2022). However, there have only been a handful of successful commercialization of microbial processes that produce chemicals and fuels. This begs the question, "Is it possible to make the switch to a sustainable society based on widespread microbial biorefineries?" To achieve this goal, metabolic engineering projects must be conceived and carried out with a holistic techno-economic perspective that considers raw material availability, production scale, downstream processes, and potential applications across strain development (Van Dien, 2013).

Table 1
Status of commercialization of microbial cell factories

Product (Chemicals & Fuel)	Production Organism	Status	Feedstock
Acetone	*Clostridium acetobutylicum*	Commercialized	Corn
Isoprene	*S. cerevisiae*	Preparing	sugar, cellulose
Isobutene	*E.coli*	Demonstration	Glucose, sucrose
Citric acid	*Aspergillus niger*	Commercialised	
Succunic acid	*E. coli* *E. coli* *S. cerevisiae* *B. succiniproducens*	Commercialised Commercialised Commercialised Commercialised	Corn sugras Sucrose Starch, sugars Glycerol, sugars
Ethanol	*Clostridium autoethanogenum* *S. cerevisiae* *Zymomonas mobilis* *Kluyveromyces marxianus*	Demonstration Commercialised Commercialised Commercialised	
Isobutanol	Yeast	Commercialised	Sugars
Butanol	*Clostridium acetobutylicum*	Commercialised	Corn
Itaconic acid	*Aspergillus terreus*	Commercialised	Biochemistry
Famesene	*S. cerevisiae*	Commercialised	
Squalene	*S. cerevisiae*	Commercialised	Sugar cane
Sebacic acid	*Candida sp.*	Demonstration	Plant oils
Adipic acid	*Candida sp.*	Demonstration	Plant oils
Polyhydroxyalkanoates	*E. coli*	Commercialised	
Dodecanedioic acid	*Candida sp*	Under-commercialised	Plant oils
1,3-Propanediol	*E. coli*	Commercialised	Corn sugars
1,4-Butanediol	*E. coli*	Commercialised	Sugar

It is vital to consider product yield, productivity, and product titer to facilitate a seamless transition from laboratory-scale demonstration to large-scale commercial production (Müller et al., 2022). Due to the low-profit margins in bulk chemicals and fuels, maximizing these three parameters is

essential for remaining competitive with traditional and petrochemical processes. Increasing output via continuous cultivation is a practical option (Wang et al., 2021). It requires expensive equipment and places a heavy burden on the genetic stability of the production host, yet it is vulnerable to costly contamination and phage infection (Croughan et al., 2015). The properties of product production, such as growth-associated or non-growth-associated, should inform the development of the fed-batch cultivation strategy, and strains should be refined to meet the requirements of the process (Poontawee & Limtong, 2020). Avoiding the use of expensive inducers is advocated to maximize the efficient mass manufacturing of low-priced goods. As a result, metabolic genes are widely used in a constitutive expression setting (Pickens et al., 2011).

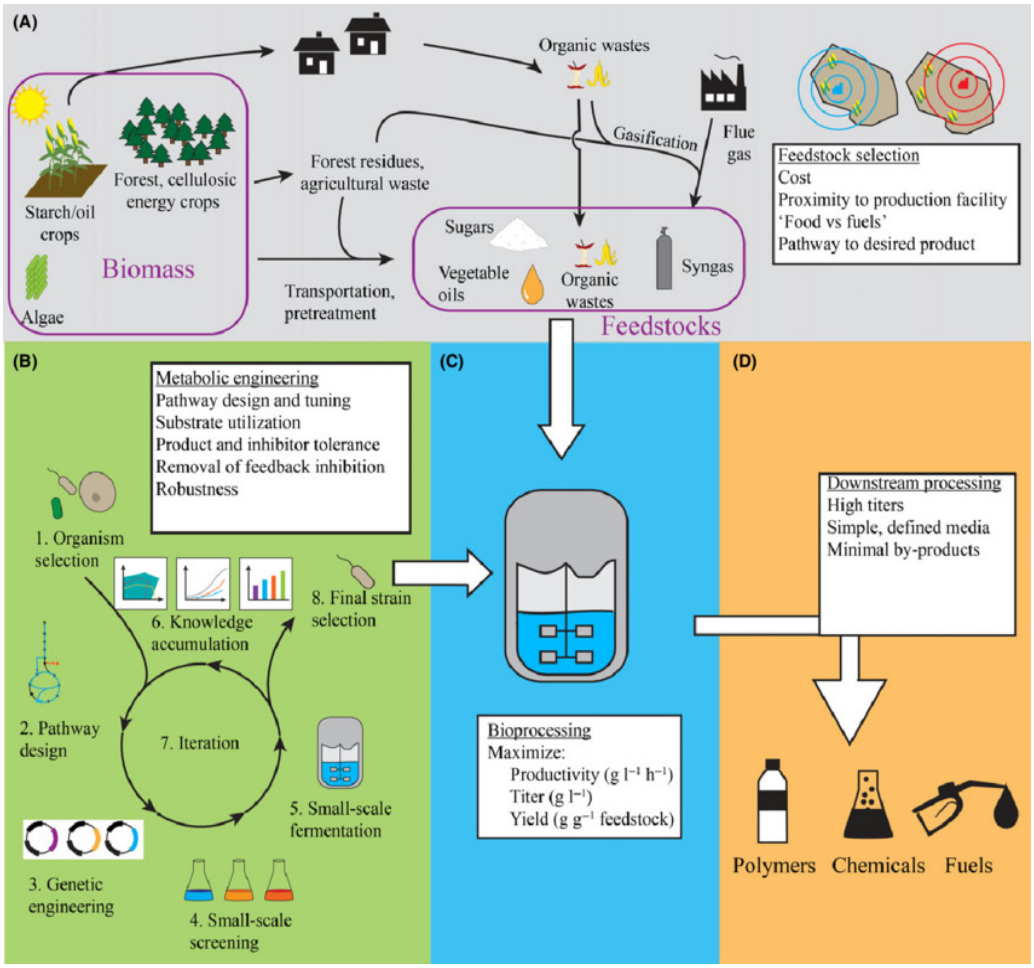

Fig 1. Overview of the microbial cell factory design process (S. Y. Lee & Kim, 2015)

The whole procedure for creating a microbial cell factory is shown in Figure 1. It is crucial to think about every step of the design process to guarantee a smooth commercial rollout (Kim & Ryu, 2017). A. It is important to think about how close the manufacturing facility is to the renewable feedstocks that may be used. The blue and red concentric rings represent the proper and erroneous locations, respectively. B. First, producing organisms are identified, and then metabolic engineering proceeds via a series of design-build-test-learn cycles until the process requirements are fulfilled. C and D-group elements. It is crucial to pay attention to the important factors involved in the manufacturing process and the downstream purification steps that lead to the final goods (Hsu, 2020).

During the strain generation phase (refer to Figure 1), it is crucial to think about the downstream techniques for isolating and refining material. The costs of downstream processing are very sensitive to the concentration of the product (Baumann & Hubbuch, 2017). Costs associated with handling and disposing of garbage, as well as the product itself, may be reduced when it is concentrated. Using a chemically defined medium not only simplifies the purification procedure that follows but also makes it easier to produce a specific output in a controlled environment (Ankit et al., 2021). One important part of strain improvement for lowering downstream processing costs is minimizing by-product generation (M. Yang et al., 2018). The downstream steps may be simplified by adjusting the fermentation conditions in tandem

with the generated strain. Since the protonated acid, rather than its salt form, is the desired output during commercial carboxylic acid synthesis, a low pH during the fermentation process is preferable (Gorden et al., 2017; Siew & Zhang, 2021).

For high-volume, low-margin products, the cost of feedstock may account for a significant portion of the bioprocessing budget. According to the Food and Agriculture Organization (FAO) in 2008, approximately 75% of the expenses associated with producing ethanol from maize in the United States were found to be directly linked to the cost of feedstock. Hence, the selection of appropriate feedstock stands as the paramount determinant in achieving optimal efficiency in the production of large quantities of commodities. To date, the majority of industrial processes have exhibited a preference for the utilization of uncomplicated sugars, such as those present in sugar cane and starch (refer to Table 1).

Systems metabolic engineering for the creation of microorganism-based cell factories.

As summarized above and as shown in Figure 1, metabolic engineers play a crucial role in the commercialization of microbial cell factories by creating strains that demonstrate optimal efficiency in the production of the desired product, meeting the needs of industrial bioprocessing on a grand scale (Cho et al., 2022).

Feedstock availability permitting, the requirements call for the use of a wide variety of carbon sources, including the most common 5- and 6-carbon monosaccharides found in lignocellulose (glucose, xylose, and arabinose), as well as disaccharides like sucrose and lactose and fatty acids (Geijer et al., 2022). In addition, the organism must produce high titers of the target product while being resistant to inhibitors found in the feedstocks (Du et al., 2011). The capability of quick growth in a simple medium is also desirable for the sake of process design. The organism also must be able to withstand the pH, temperature, and oxygen fluctuations that are common in industrial bioprocesses without showing signs of stress (Parapouli et al., 2020). When going from a 100 ml to a 10,000 m3 production scale, the organism must maintain genetic stability for at least 30 generations (Lande, 1976). The organism, among other things, should be resistant to infections induced by bacteriophage (Reygaert, 2018). In the end, the host organism used in manufacturing must possess the metabolic capacities necessary for the synthesis of the target product.

Challenges and Prospects in Microbial Cell Factories for Sustainable Bio-manufacturing

The rate of commercialization in industrial biotechnology has fallen short of expectations. It was formerly thought that replacing petrochemical goods with those made using microbes or enzymes would be less expensive and better for the environment than the conventional methods of making biofuels, polymeric materials, and chemical agents (Balan, 2014; Wackett, 2008). However, given the current situation, actualizing this undertaking looks to be difficult due to these

After the 2008 financial crisis, petroleum prices remained stable due to the discovery of abundant alternative energy sources like shale gas, natural gas hydrate, and sand oil. The fear of oil depletion has receded. However, agricultural raw materials used in bioprocessing are becoming more expensive, and cellulose may no longer be practical for microbial activities in 5-10 years. Bioprocessing costs are higher due to lower efficiency compared to chemical processing. Water-scarce areas are concerned about excessive freshwater use in bioprocessing. The chemical industry is shifting towards eco-friendly practices and renewable resources like biomass. Faster development of category-one chemical engineering products is necessary (Liang et al., 2012; Manfroni et al., 2022; Sehgal & Gupta, 2020).

Several reports show that the biopolyester family polyhydroxyalkanoates (PHA) have been put to use in the manufacturing sector (G.-Q. Chen, 2009; H. Chen, 2012). The difficulty in lowering manufacturing costs has stymied large-scale commercial production of PHA, especially for their use as bioplastics that are accepted as both biodegradable and bio-based (Montaño López et al., 2022). Although carbon dioxide might be used as a substrate, this is still a major roadblock.

To successfully introduce PHA to the commercial market, we must keep the “high volume and low price” strategy in mind as we work to improve PHA production strains and develop more cost-effective procedures. Products designed for use in biomedical applications, specialty polymers, chiral monomers, drug development, and other niche markets may benefit from “low volumes and high price”.

The following are the several challenges that contribute to this situation and solutions for sustainable biomanufacturing using microbial cell factories.

Table 2
challenges and solutions for sustainable biomanufacturing using microbial cell factories.

Challenges	Solutions
Microorganisms grow too slowly as production takes days.	minimizing the microbial cells.
Microbes cannot use mixed substrates.	Assembly pathways that can metabolize mixed substrates
Low conversion of substrates to products; Cell metabolism turn substrates into CO2, H2O & byproducts.	Removing unnecessary pathways consuming substrates.
High Consumption of fresh H2O; Fresh H2O as medium.	Utilization of seawater for cell growth.
Microbial cells grow to very low density.	Minimizing oxygen demand for aerobic cells and reducing Quorum sensing effect.
Discontinuous processing; contamination concerns.	Development of continuous process.
Difficulty to control the bioprocesses.	Artificial cells that contain only necessary metabolic pathways.

CONCLUSION

Progress in using microbial cell factories for the synthesis of biomolecules from low-cost and renewable substrates is summarized in this article. The difficulties of setting up commercial-scale microbial biorefineries for the manufacture of chemicals and fuels are also explored. The importance of metabolic engineering in developing sustainable chemical businesses is also highlighted, such as those producing biofuels and high-value pharmaceuticals. System biology, synthetic biology, and evolutionary engineering have all been discussed in relation to their potential use in tandem with metabolic engineering methodologies. These methods are used to convert microorganisms into efficient cell factories that can produce a wide variety of goods by constructing new metabolic pathways and modifying existing ones.

Competing Interest

The authors had no competing interests.

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