Punjab University Journal of Zoology

OPEN ACCESS

39(2): 213-230 (2024)
https://dx.doi.org/10.17582/journal.pujz/2024/39.2.213.230

Review Article

Cell-Free Systems: Platform for Sustainable Commercial Biomanufacturing of Biochemicals

Humaira[1], Afsana Huseynova Anvar[2], Shaukat Ali[3], Marcelo Franco[4], Muhammad Irfan[1*]

[1]*Department of Biotechnology, University of Sargodha, Sargodha Pakistan*
[2]*Department of Molecular Biology and Biotechnology, Baku State University, Baku, Azerbaijan*
[3]*Department of Zoology, Government College University Lahore Pakistan*
[4]*Department of Exact Sciences, State University of Santa Cruz (UESC), Ilhéus, 45662-900 Brazil*

Article History
Received: September 16, 2023
Revised: July 05, 2024
Accepted: July 18, 2024
Published: November 19, 2024

Authors' Contributions
Humaira wrote the original draft. AHA reviewed the literature. SA formatted figures and editing. MF did final editing. MI supervised the study, did final editing in the manuscript.

Keywords
Cell free systems, Biochemicals, Commercial production, Value added products

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Abstract | Increasing material consumption and associated environmental concerns demand a shift from current fossils-based commercial manufacturing practices to sustainable, ecofriendly production systems. Extensive metabolic engineering practices incorporating living systems for commercial manufacturing of cost-effective value-added products and biochemical from sustainable feed-stock show limited success as desired higher production rates run counter to life-sustaining metabolic activities. To address these challenges, cell-free approaches developed as promising platform to proceed towards sustainable bio-manufacturing through the development of self-sustaining, constantly operating systems employing renewable biomass to yield higher concentrations and productivity of extended-range of products. To-date established cell-free systems including extract-based and purified enzyme-based cell-free technologies have been widely exploited for producing commercially valuable biopolymers and biochemicals such as building-block chemical, biofuels, bioplastic and value-added products. However, implementation of these cell-free systems at industrial production scale requires key considerations of sustainable carbon source, co-factors and energy regeneration pathways and biocatalyst engineering and recycling strategies to proceed towards sustainable production. At present, extensive research is required to address challenges of high cost associated co-factor supply and optimization of protocols to exploit full-potential of these cell-free technologies.

Novelty Statement | This review article describe the potential utilization of cell free systems for the produc-tion of valuable chemicals through technological interventions which is the current demand of the society.

To cite this article: Humaira, Anvar, A.H., Ali, S., Franco, M. and Irfan, M., 2024. Cell-free systems: Platform for sustainable commercial biomanufacturing of biochemicals. *Punjab Univ. J. Zool.*, **39**(2): 213-230. https://dx.doi.org/10.17582/journal.pujz/2024/39.2.213.230

Introduction

The primary goal of metabolic engineering and industrial biotechnology is to fulfill ever increasing demand of sustainable and cost-effective chemicals and natural products (Dudley *et al*., 2015). At present, fossil fuels serve as almost sole source of materials and chemicals (Moulijn *et al*., 2014). Petrochemical industry has traditionally relied upon a few (xylene, benzene, methanol, propene, toluene, butadiene, ethylene) high-volume, low-cost commodity chemicals from which approximately all other products are derived. Some serious outcomes of increasing dependence on fossils include limited supply and price fluctuations due to finite availability (Höök and

Corresponding Author: Muhammad Irfan
irfan.ashraf@uos.edu.pk, irfan.biotechnologist@gmail.com

Tang, 2013), lack of novelty as materials are derived from limited number of building blocks (Bozell and Petersen 2010) and irreversible climatic changes (Karl *et al*., 2009). In current era, this petrochemical based material's synthesis cannot be substituted completely with other production systems; however, advances in engineering biological systems offer access to wide range of building-blocks for manufacturing novel chemicals and value-added products (Karl *et al*., 2009).

Currently, industrial biotechnology research mainly focused on bio-based systems that utilize specific, robust enzymes as catalyst of complex cascade of reactions in living systems to synthesize wide range of bio-molecules, polymers, biofuels, therapeutics, platform-chemicals and value-added products etc. (Lee *et al*., 2012; Curran and Alper, 2012; Nielsen *et al*., 2013). Microbes based bioconversions proved to be efficient because of their potential to utilize heterogeneous substrates, e.g., waste streams (BETO, 2017), biomass hydrolysates (Kim *et al*., 2012), hydrolyzed lignin (Linger *et al*., 2014) and mild operating parameters. Synthetic biology has arisen as growing scientific field that combine biological systems with engineering technologies (Khambhati *et al*., 2019). Synthetic biology holds great potential to engineer biological systems for extending range of applications from biofuels to biomedical research. High-throughput DNA sequencing and advanced biotechnology techniques (gene-editing) offer unlimited opportunities to alter cellular functioning for user-defined purposes (Lee *et al*., 2012).

Currently, microbes based manufacturing is still limited as cells inherently posed several problems (Green, 2011). For example, Engineering at cellular level is problematic particularly because of membrane barriers (hinder optimization of synthetic process, cause incompatibility, variability issues) and complicated metabolic pathways (Jiang *et al*., 2018; Yue *et al*., 2019). Moreover, either Suitable vectors are required for carrying genetic instructions to cells that should be maintained through chromosomal integration or selectable marker expression to allow instructions to be evaluated (Tinafar *et al*., 2019). Other limitations of cell-based systems are limited product yield due to toxicity effects, synthesis of competing by-products, difficulty in optimization of lab-scale cultures to commercial production-scale because of variable fermentation conditions and constraints in down-stream processing in case of intra-cellular accumulation of product (Takors, 2012).

Thus, to overcome these limitations synthetic biologists and biotechnologists have adopted an alternative approach cell-free synthesis. This strategy permits the activation of biological machinery through direct control of transcription, translation, and metabolism in cell-free environment. This *in-vitro* biomanufacturing offers several advantages as it separates target product's yield from cell-growth. Absence of cell boundary permits easy manipulation, standardization, sampling, and monitoring. Cell-free bioconversions eliminate requirements of long-term strain improvement programs thus, greatly enhance design-build-test-learn cycle speed (Takahashi *et al*., 2015). Other advantages include high production without any need to maintain cell biomass, eliminate unnecessary metabolic pathways which in turn limit the synthesis of undesired by-products, enhanced tolerance against toxins and high reaction rates due to enhanced biomass transfer-rates (Carlson *et al*., 2012; Dudley, 2015; Rollin *et al*., 2013). Cell-free systems offer opportunity to manipulate biological parts at four levels; DNA, RNA, Proteins, and metabolism to develop complex systems for synthesis of sustainable biochemicals, functional biomolecules and bioenergy. Currently, purified-enzyme system and crude-extract based system represent two well-established cell-free technologies (Zhang, 2015). This review highlights the features of these currently established cell-free approaches, potential applications for sustainable biomanufacturing of commercially valuable products, considerations for scaleup efforts, challenges that should be addressed to develop these cell-free technologies as competitive production platform and future prospects.

Cell-free systems

Currently, two well-developed and commonly exploited cell-free systems are: Extract-based systems and purified enzyme-based systems (Rollin *et al*., 2018; Taniguchi *et al*., 2017; Dudley *et al*., 2015). Both of these systems possess several common features i.e. both have potential to deliver ~100 theoretical yield as these are entirely orthogonal to biocatalyst production and accomplish primary goal of metabolic engineering through system design. For example, in vitro conversion of glycerol into 1,3-propanediol was reported to be 0.95 mol/mol that is much higher than reported in traditional fermentation (0.6mol/mol) mainly due to elimination of competing byproduct formation (Rieckenberg *et al*., 2014). As *in-vitro* bioconversions do not show homeostasis; thus, these systems possess potential to be manipulated rapidly like chemical pathway engineering and easy to scale-up (Takahashi *et al*., 2015). Furthermore, these systems offer opportunity to circumvent obstacles in situations where membrane barrier limit product removal or substrate uptake and ultimately leads to higher productivity (Jewett *et al*., 2008).

Extract-based system

Extract-based cell-free approach utilize cell-lysate along with other supplements *i.e.* template DNA, NTPs, energy generating substrates, transcription and translational factors, tRNAs, salts, amino-acids, co-factors and RNA-polymerase for *in-vitro* transcription and protein expression. Crude-extracts can be derived

both from prokaryotic and eukaryotic cells (Zemella *et al.*, 2015). Some extract-based cell-free systems developed from prokaryotes include *E. coli* (Shrestha *et al.*, 2012; Ninomiya *et al.*, 2014), *Sulfolobus solfataricus* (Nishimuar *et al.*, 2013), *Bacillus subtilis* (Kelwick *et al.*, 2016) and *Thermococcus kodakaraensis* (Yamaji *et al.*, 2009) extract-based systems. While reported eukaryotic extract-based systems include *Streptomyces lividans* (Li *et al.*, 2017), *Saccharomyces cerevisiae* (Gan and Jewett, 2014; Schoborg, 2014), tobacco BY-2 (Buntru *et al.*, 2015), wheat germ (Arumugam *et al.*, 2014), *Trichoplusia ni* (Ezure *et al.*, 2006), *Spodoptera frugiperda* (Ezure *et al.*, 2014), rabbit reticulocytes (Douthwaite, 2012), human K562 (Brodel *et al.*, 2015), Chinese Hamsters-Ovary (Brodal *et al.*, 2014), HeLa cell-lines (Yadavalli and Sam-Yellowe, 2015), HEK293 (Bradrick *et al.*, 2013), mouse embryonic fibroblast (Zeenko *et al.*, 2008) and *Leishmania tarentolae* (Mureev *et al.*, 2009). However, *E. coli* extract is the mostly widely exploited extract-based cell-free system (Lee *et al.*, 2012).

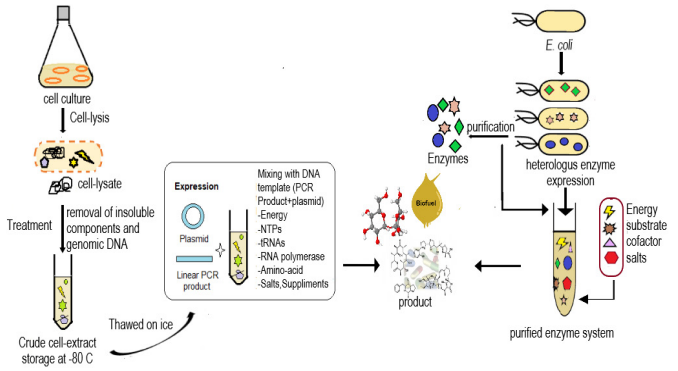

Figure 1: *In-vitro* established extract-based and purified enzyme-based cell-free systems. (Modified from: Lu, 2019 and Li *et al.*, 2018).

These extract-based systems provide cost-effective routes for protein synthesis and can be easily scalable for commercial fermentation (Cai *et al.*, 2015; Zawada *et al.*, 2011). Several protocols have been established for crude-extract preparation that rely on isolating the cells at exponential growth-phase when intra-cellular translation is at the highest level. After harvesting cell are washed, lysed (Kwon and Jewett, 2015), followed by run-off treatment to activate crude-extract in cell-free environment as shown in Figure 1. This process probably degrades genomic DNA and endogenous mRNA transcripts to considerably reduce *in-vitro* translational efficacy (Moore *et al.*, 2017). Further, small inhibitory molecules can be removed by dialysis, but it depends upon user preference (Kwon and Jewett, 2015). To-date several commercial extract-based batch systems have been established with improved energy-regeneration pathways that are designed by groups of jewett (Kwon and Jewett, 2015; Harris and Jewett, 2012), swartz (Yang *et al.*, 2012) and Noireaux (Gramela *et al.*, 2016; Caschera and Noireaux, 2104). Besides simplicity and ease to scale-up, key limitation of extract-based system cell-free system is their ability to sustain un-desired metabolic pathways; thus, additional engineering or purification may be required for their elimination (Moore *et al.*, 2017).

Purified enzyme-based cell-free system

This system incorporates enzymes that are first over-expressed and purified individually followed by their reassembling to reconstruct biosynthetic-pathways in open-environment (Dudley *et al.*, 2015). These purified enzyme-based cell-free systems proved to be extremely useful particularly if immobilization, encapsulation or engineered spatial-arrangement is required, also permit the easy optimization of each pathway enzyme and provide dynamic process control (Jin and Myung, 2019). Moreover, these enzymes-based approaches offer the opportunity to design novel synthetic pathways with aim of producing only the targeted product (Jinn and Myung, 2019). Researchers have also commenced commercial scale-up efforts and demonstrated the capacity of these systems up-to 20,000 L while maintaining high yield (~5g/L/h) (You *et al.*, 2017). To date, several purified enzyme-based systems have been reported for manufacturing of commercially valuable products including mono-terpenes (Korman *et al.*, 2017), iso-butanol (Sherkhanov *et al.*, 2020), polyhydroxy-butyrate (PHB) (Opgenorth *et al.*, 2016) styrene (Grubbe *et al.*, 2020), biohydrogen (Rollin *et al.*, 2015) and many others. However, absence of cellular biochemical regeneration system and high-cost associated with cofactors mainly limit scale-up of these enzyme-based cell-free technologies (Jinn and D-Myung, 2019; Dudley *et al.*, 2015).

Potential commercial applications

Currently, we are living in a material world (Callén-Moreu and López-Gómez, 2019). Approximately 15billion trees are cut-down each year (Crowther *et al.*, 2015) and worldwide fiber production is predicted to be more than 30million-tonns annually (Townsend and Sette, 2016). Our ecosystem is largely shaped by commercial manufacturing, processing, and disposal of extended range of synthetic materials. As we are proceeding towards irreparable climate change (Lenton *et al.*, 2019), it becomes essential to balance advancements in material-sciences against adverse environmental outcomes associated with material consumption. So, to-date main challenge is to shift manufacturing practices from global mass-manufacturing to localized sustainable manufacturing approaches (Kleer and Piller, 2019). For example, in-contrast with current fossils-based chemical-industries next generation building-block chemicals or value-added products may derive from ecofriendly, renewable feedstock through advancements in sustainable biotechnology, synthetic biology and growing bio-based economy (Freemont, 2019; French, 2019). As biological systems are extremely complicated and initial efforts to engineer these systems for user defined-purposed

show limited success (Kelwick *et al.*, 2020). Thus, cell-free manufacturing systems offers highly promising strategy for commercial manufacturing of sustainable, cost-effective industrially valuable platform-chemicals and value-added products. Potential applications of currently established cell-free systems for sustainable manufacturing of some commercially important products are listed here.

Isoprenoids

Isoprenoids are important class of compounds that have wide-range of applications in flavoring, disinfectants, pesticides, fragrances, chemical feedstock and pharmaceutical industry (Leavell *et al.*, 2016). Although, novel biosynthetic pathways for isoprenoid synthesis have been successfully expressed in microbes (George *et al.*, 2015) but efforts to design and characterize metabolic pathways in living systems is sufficiently time-consuming process (Du *et al.*, 2012; Biggs *et al.*, 2014). Thus, cell-free systems offer unique alternative strategy for commercial production of isoprenoids. To date, several cell-free systems has been reported for isoprenoid production. For example, Enzyme enriched *E. coli* extracts have been successfully utilized for cell-free synthesis of limonene (Dudley *et al.*, 2019). Study reported that cell-free biosynthetic pathway has been constructed for limonene synthesis by mixing several *E. coli* extracts each containing one overexpressed pathway enzyme. First, six-enriched lysates along with mevalonate (substrate), cofactors and energy-source were employed to synthesize limonene then system was extended further to utilize glucose as substrate. As extracts exhibit indigenous pathways to metabolize glucose to acetyl-CoA; hence, three additional enzymes were added to reaction mixture that catalyze the bioconversion of acetyl-CoA to mevalonate. Dudley *et al.* (2019) reported that by adjusting the concentration of co-factors, CoA and ATP this system has potential to produce 90.2mg/L limonene over 24 h. This enzyme-enriched extract-based system comprising of 20 metabolic-steps and incorporating 9 heterologous enzymes for conversion of glucose into limonene highlights the flexibility of extract-based system to sustain complex metabolic reactions in open environment (Dudley *et al.*, 2019).

Korman *et al.* (2017) have designed 27 enzyme containing biosynthetic-pathway for cell-free conversion of glucose into monoterpenes along with generation of ATP and NAD(P)H both of which serve as co-factors for terpene synthesis. Study reported that by altering the concentration of terpene-synthase enzyme this purified enzyme-based cell-free system have successfully synthesized pinene, sabinene and limonene with single glucose addition and maintained metabolic activity for ~ 5 days (Korman *et al.*, 2017). Moreover, Ward *et al.* (2019) reported cell-free synthesis of isoprenoids from isopentenol though construction of "isopentenol-utilization pathway" (IUP) in an open environment that appears to be promising substitute of native MVA (Mevalonate) and MEP (2C-methyl-D-erythritol-4-phosphate) pathways. This enzyme-based IUP utilize just four enzymes for bioconversion of isopentenol in presence of ATP into mono-, di-, and sesquiterpenoids; thus, offer highly flexible strategy for commercial production of isoprenoids. Metabolic-control analysis demonstrated that IUP is mainly regulated by Choline kinase (CK) and conversion efficacy is not influenced by any pathway intermediate. Study reported that this *in-vitro* established IUP can synthesize 220 mg/L of diterpene-taxadiene, over 9 h that is nearly 3-times greater than any other cell-free system reported for isoprenoid production (Ward *et al.*, 2019).

Recently, Niu *et al.* (2020) have developed cell-free system for biosynthesis of α-Pinene from glucose. Besides its widespread applications in pharmaceuticals, α-Pinene also represents promising substitute of jet-fuel due to its high-energy content, low hygroscopicity and high-fluidity at low-temperature (Niu *et al.*, 2017). Thus, biotechnological production of pinene has received much attention. Niu *et al.* (2020) reported that by using mixed-match approach concurrent addition of E. coli lysate containing overexpressed native 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, *Pinus taeda* pinene-synthase, SufBCD Fe-S cluster assembly-protein and isopentenyl-diphosphate isomerase significantly improved pinene production. NAD^+, NADH and ammonium-acetate were found to be the key parameters for regulation of pinene synthesis (Niu *et al.*, 2020). After optimizing the components of *in-vitro* reaction-mixture through Plackett-Burman experimental-design; pinene yield was further improved to 57 % by increasing enzyme-concentration in *E. coli*-extract to achieve overall productivity of 104.7 mg/L/ h (Niu *et al.*, 2020).

Platform chemicals

The European Commission's Circular Economy Strategy and Strategic Plan 2014 and Action-Plan 2015 have raised considerable debate regarding the role and affiliation of circular-bioeconomy and agricultural industries including forestry (DeBoer *et al.*, 2020), biofuel (Erickson, 2018) and plastic production (Blank *et al.*, 2020); animals (Horton, 2019) and textile industry (Aznar, 2019) along-with several other examples. This Circular-Bioeconomy demands absolute carbon-efficacy and development of alternative sources to replace fossils-feedstock on which chemical industry has traditionally relied as energy and carbon source. Biotechnological methods offer opportunities to utilize non-traditional feedstock (CO_2, agricultural waste, domestic waste, glycerol, methane etc.) along-with conventional substrates (starch, molasses) as carbon source (Bergquist *et al.*, 2020). Extensive research has been conducted to engineer living systems to produce cost-effective commodity-chemicals

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from biomass ranging from low-value products e.g., building-block chemicals, plastic, fuels to valuable natural products e.g., cannabinoids (Chae *et al*., 2017; Chubukov *et al*., 2016). Although, these metabolically engineered microbes are advantageous as they utilize renewable-biomass as feedstock, mitigate release of green-house gases and produce biodegradable-products but mainly low volumetric production because of cellular toxicity make these bio-derived products economically less-viable (Bowie *et al*., 2020).

Thus, to address this challenge cell-free systems received attention for sustainable production of platform chemicals and value-added products. For example, Kay and Jewett (2019) have designed *E. coli's* extract-based cell-free system to produce 2-3 Butane-diol (2,3-BDO) from glucose. 2,3-BDO is a promising aviation fuel and important platform-chemical that is widely used to produce industrially valuable chemicals e.g., acetoin, diacetyl, butadiene, methyl-ethyl-ketone. Single *E. coli* strain was metabolically engineered to overexpress three pathway enzymes required for conversion of pyruvate to 2,3-Butanediol that include 2,3-BD-dehydrogenase (BDH) from *Klebsiella pneumoniae*, acetolactate-decarboxylase (ALDC) and acetolactate-synthase (ALS) from Bacillus subtilis. Addition of cofactors (ATP, NAD^+) and glucose to *E. coli* extract containing endogenous glycolytic enzymes first metabolize glucose to pyruvate which then is subsequently converted to 2,3-BDO by heterologous enzymatic pathway expressed in *E. coli* extract (Kay and Jewett, 2019). Yi *et al*. (2018) have developed first hybrid cell-free system for metabolic conversion of starch into 2,3-BDO. Recombinant *E. coli* strain was developed to express 2,3-BDO synthetic pathway enzymes including acetolactate-synthase (*Mycobacterium tuberculosis*), acetolactate decarboxylase and butanediol-dehydrogenase (*Lactococcus lactis*). As, *E. coli* extract naturally has limited potential to metabolize starch into pyruvate; thus, addition of cyanobacteria-lysate into cell-free mixture significantly improves pyruvate synthesis from starch which is then subsequently converted into 2,3-BDO by heterologous enzymatic pathway. This hybrid system yields 1.6-folds higher titer of 2,3-BDO than concentration obtained by employing only *E. coli* extract (Yi *et al*., 2018). Gao *et al*. (2019) have reported purified-enzyme based cell-free system for metabolic conversion of biomass-derived D-xylose into D-1,2,4-buatnetriol (BT). Under optimized conditions this *in-vitro* constructed biosynthetic pathway produce 6.1 g/L BT from 18 g/L D-xylose over 40 h with an overall productivity of 48 % (Gao *et al*., 2019).

Generally, multiple sugar monomers in biomass hydrolysate adversely affect fermentation, results in lower productivity and by-product formation that subsequently influence downstream processing (Sutiono *et al*., 2021). Recently Sutiono *et al*. (2021) have utilized sequence-based identification strategy to design promiscuous enzymes-based cell-free system that successfully metabolize biomass derived L-Arabinose and D-Xylose to 1,4-butanediol (BDO) at rate of 3 g/L/h with an overall productivity of >95 %. This *in-vitro* system was extended further to yield α -ketoglutarate (2-KG) from biomass-derived pentoses by employing O_2 that serve as co-substrate for cofactor regeneration and synthesize 2-KG at rate of 4.2 g/L/h with an overall productivity of 99 % (Sutiono *et al*., 2021). Zhang *et al*. (2017), have designed cell-free biosynthetic pathway for conversion of ethanol into C_4 platform-chemicals including 2-butanol, acetoin and 2,3-butanediol. This purified enzyme-based cell-free system incorporate five heterologous enzymes (formolase, diol-dehydratase, ethanol-dehydrogenase, NADH-oxidase and 2,3-butanediol-dehydrogenase) to convert bioethanol into industrially valuable C_4 chemicals. Under optimized conditions, maximum of 88.28 %, 88.78 % and 27.25 % titer of 2,3-BDO, acetoin and 2-butanol were obtained respectively from 100 mM ethanol (Zhang *et al*., 2017). Moreover, glycerol that constitute about 10 % of biodiesel production as a byproduct; is a valuable, non-toxic and biodegradable green-solvent.

This biomass-derived glycerol is a promising substrate for biocatalysis and to produce industrially important products (Bergquist *et al*., 2020). Li *et al*. (2018) have designed cell-free biosynthetic pathway for metabolic conversion of glycerol into L-Lactate. This cell-free synthetic pathway have incorporated four thermostable-enzymes (dihydroxy-acid-dehydratase, L-Lactate dehydrogenase, alditol-oxidase, and catalase) along-with Formate-dehydrogenase that serve as NADH regeneration system to produce 34.4 mM L-Lactate from 50 g/L glycerol over 72-h (Li *et al*., 2018). Meng *et al*. (2018) have designed cell-free biosynthetic-pathway incorporating 3-enzymes for metabolic conversion of biomass-derived cello-dextrin into energy-rich phosphorylated-oligosaccharides e.g., glucose-6-P. Authors reported that this cell-free system successfully achieved much higher titers of inositol from cellodextrose without any toxicity effect that mainly limit its production in *E. coli* expression system (Meng *et al*., 2018). Some other cell-free systems reported for manufacturing of industrially valuable building-block chemicals and value-added products produced from biomass derived monomers are listed in Table 1.

Recently, Grubbe *et al*. (2020) reported cell-free synthesis of styrene. Styrene is a valuable petroleum-derived product that serve as building-block for manufacturing of different plastics e.g., foamed-packaging material, disposable silverware. This petroleum-derived styrene significantly contributes to global warming as emit over 100-million tons of green-house gases annually (Zheng and Shu, 2019). Thus, engineered *E. coli* strains was demonstrated as a potential substitute for bioconversion

Table 1: Industrially important plat-form chemicals and value-added products from biomass derived monomers.

Substrate	Product	Yield	Biosynthetic pathway enzymes	Industrial applications	References
Glycerol	D-fagomine	70 g/L/h	3	Anti-diabatic activity	Hartley *et al.* (2019)
Sucrose	Glucaric acid	35 mM (70 %)	7	Therapeutic agent, intermediate in slow release of fertilizers, detergent, degradable polymer	Su *et al.* (2019)
Glucose	Lactate	48.2 mM (>90 %)	5	Pathway intermediate	Xie *et al.* (2018)
Chitin	Pyruvate	0.62 mM	12	Precursor for alcohols, amino acid and other building-blocks.	Honda *et al.* (2017)
mannose	Lactic acid	4.4 mM	4	Used in medical, textile food industry, monomer for biodegradable poly-lactic-acid (PLA) manufacturing.	Kopp *et al.* (2019)
Glycerol	Di-hydroxy-acetone phosphate	88 %, 4.1 µM/s	4	Intermediate or precursor for di-hydroxy acetone synthesis	Hartley *et al.* (2017)
Tyrosine	Raspberry ketone	55 mg/L (33.7 %)	5	Natural additive in food and sport industry	Moore *et al.* (2017)
Glucose	n-butanol	82 %	16	Promising next-generation biofuel	Krutsakorn *et al.* (2013)
Starch	Myo-inositol	2.9 g/L/h (>90 %)	4	Used in the pharmaceutical, cosmetic and food and feed industries	You *et al.* (2017)
Pyruvate	2,3-BDO	11.3 g /L/h	3	Used in inks, perfumes, explosives, polymers, paint, flavorings & pharmaceuticals manufacturing	Kay and Jewett (2015)
D-Xylose	Myoinositol	96.6 %	11	Used in the pharmaceutical, cosmetic and food and feed industries	Cheng *et al.* (2019)

of glucose into-styrene through shikimate-pathway. However, cellular toxicity and prerequisite for product removal limit *in-vivo* synthesis of styrene (Liu *et al.*, 2018; Lee *et al.*, 2019). Grubbe *et al.* (2020) have established cell-free biosynthetic pathway for styrene biosynthesis from L- phenylalanine. Recombinant *E. coli* strain was developed to express heterologous enzymatic pathway incorporating "ferulic-acid decarboxylase 1 (FDC1)" (*saccharomyces cerevisiae*) and phenylalanine-ammonia lyase 2 (PAL2) (*Arabidopsis thaliana*) responsible for bioconversion of L-phenylalanine to styrene then simply heat-treatment release lysate of engineered *E. coli* strain which is then employed for *in-vitro* styrene synthesis. This cell-free system was optimized to produce maximum of 40mM styrene, nearly more than 2-fold higher productivity than reported in engineered *in-vivo* system (Grubbe *et al.*, 2020).

Biofuels

Ever-increasing energy-consumption and environmental concerns associated with the exploitation of fossil-fuels leads to rising demand for sustainable and cost-effective production of biofuels. Global biofuel market is estimated to comprised of about 85 % bioethanol and 15 % biodiesel (Gao *et al.*, 2015). Although, several cellular systems have been metabolically engineered to synthesize extended range of biofuels, but development of living cells-based competitive platform for commercial production of biofuels is still challenging (Chubukov *et al.*, 2016; Chen and Liao, 2016; Chae *et al.*, 2017) particularly due to low product-yield (0.2 % v/v), feedback inhibition of substrate, toxicity effects that disrupt life-sustaining processes and lack of cost-effective downstream processing. To overcome these limitations biotechnology research, move towards the development of cell-free systems for biofuel production (Zhang *et al.*, 2011; Zhang, 2011). For example, yeast's extract-based cell-free system was reported for ethanol production from glucose without any prerequisite for pathway prototyping and strain engineering (Khattak *et al.*, 2014). Yeast cells cultivated in waste beer fermentation-broth (WBFB) were acquired, *in-vitro* grown, lysed, buffered, and blended with glucose to produce ethanol at rate of approximately 4 g/L/h. Strikingly, authors reported that concentrations of NAD^+ (0.11 mMol) and ATP (1.8 mMol) in yeast's-cell extract were sufficiently high to maintain metabolic reactions even without adding supplements (Khattak *et al.*, 2014).

Recently, Cui *et al.* (2020) have established extract-based cell-free system to identify factors limiting ethanol yield in *Clostridium thermocellum.* Study reported 25 mM ethanol yield from 15 mM biomass-derived cellobiose at rate of approximately 0.5 mM/h in cell-free environment. Two different approaches (exogenous enzyme addition to cell-free extract and feeding various substrates) are used to determine enzymes responsible for limiting metabolic-flux. NADH recycling was found to be rate limiting factor and major improvement was reported by addition of yeast "Alcohol-dehydrogenase"(ADH) to extract (Cui *et al.*,2020).

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Table 2: Recent studies of using Biomass waste as a potential feedstock to produce biofuels (Bio-diesel, Bio-oil) (Modified from Quevedo-Amador *et al*., 2023).

Waste	Treatment/ Process	Operating conditions	Yield or conversion, %	References
Goat bone	Thermal	Methanol/oil ratio: 11/1, 2% catalyst, 3h, 60 ˚C	92 (bio-diesel)	Mamo and Mekonnen (2019)
Garlic peel	Thermochemical	Methanol/oil ratio: 10/1, 8% catalyst, 3.5 h, 60 ˚C	96 (bio-diesel)	Wei *et al*. (2022)
Banana pseudostem (Dwarf Cavendish)	Thermal	60 ˚C, 2 h, methanol/oil ratio: 9.35/1, 4.7% of catalyst	98 (bio-diesel)	Daimary *et al*. (2023)
Banana peel	Thermal	Methanol/oi ratio: 6/1, 5 ˚C, 1.5 h, 2% of catalyst	98 (bio-diesel)	Husin *et al*. (2023)
Karanja seed shell	Thermal	Methanol/oil ratio: 10/1, 2% catalyst, 1 h, 65 ˚C	96 (bio-diesel)	Prajapati *et al*. (2023)
Rice husk	Thermochemica	Methanol/oil ratio: 16/1, 9.9% catalyst, 4.8 h, 74.8 ˚C	98 (bio-diesel)	Saidi *et al*. (2023)
Corncob	Catalytic pyrolysis	3 g biomass, 500 ˚C, 5 mL/min, 40 min, HZSM-5/ activated carbon ratio: 2/1, biomass /catalyst ratio: 1/1	44 (bio-oil)	Duan *et al*. (2022)
Pistacia lentiscus L seeds	Pyrolysis	20 g biomass, 475 ˚C, heating rate of 25 ˚C/min, particle size: 0.3–0.6 mm	64 (bio-oil)	Farissi *et al*. (2022)
Date seed and plastic waste	Co-pyrolysis	100 g date seed, 70 g plastic, 500 ˚C, heating rate of 50 ˚C/min, 100 mL/min N2, 12.5 MPa, 1200 rpm	59 (bio-oil)	Inayat *et al*. (2022)
Food and plastics waste	Co-pyrolysis	Batch Reactor, 200 g biomass, 400 ˚C, 1 h, food waste/plastic constant ratio: 2/1	Fish bone+plastic: 16 Chicken bone +plastic: 20 Rice +plastic: 29 (bio-oil)	Lim *et al*. (2022)
Eucalyptus wood	Catalytic fast pyrolysis	500 ˚C, 1.5 h, 11 L/min, fuidized bed reactor: 160 g/h, biomass/catalyst mass ratio: 0.4	11 (bio-oil)	Promsampao *et al*. (2022)
Linseed residue	Pyrolysis	50 g biomass, 500 ˚C, 1.5 h, 200 cm3/min, heating rate: 20 ˚C/min	79 (bio-oil)	Bahadorian *et al*. (2023)
Reutealis trisperma oil	Pyrolysis	Non-catalytic, reactor semibatch, 450 ˚C, 190 min Catalytic, dolomite/ Reutealis trisperma oil mass ratio: 1/10	68 (bio-oil) 77	Buyang *et al*. (2023)
Sawdust and rice husk	Co-pyrolysis	Fluidized bed reactor, 500 ˚C, 20 L/min, blending ratio of sawdust/rice husk: 50/50	52 (bio-oil)	Fadhilah *et al*. (2023)
Biomass of water hyacinth	Hydrothermal liquefaction	Impregnation of biomass of water hyacinth with CuCl2 0.2 MHydrothermal liquefaction: Cu-impregnated biomass of water hyacinth/water ratio of 1/9, 270 ˚C, 0.5 h	83 (bio-oil)	Gao *et al*. (2023)
Municipal (household) and horticultural wastes	Co-pyrolysis	50 g biomass, waste blends (0–100 wt%), 550 ˚C, 1 min, 200 mL/min N2, 0–15% of catalyst	58 (bio-oil)	Ghorbannezhad *et al*. (2023)
Corncob	Hydrothermal liquefaction	0.25 h, 300 ˚C, 10 mL/min	10 (bio-oil)	Martins-Vieira *et al*. (2023)
Hydnocarpus de-oiled seed cake, waste electrical and electronic plastic	Microwave co-pyrolysis	Hydnocarpus de-oiled seed cake /Waste electrical and electronic plastic weight ratio: 50/50 (g/g), 500 ˚C, 10 g catalyst, heating rate of 30 ˚C/ min, microwave power of 1100 W	26 (bio-oil)	Muniyappan *et al*. (2023)
Cassava residue	Pyrolysis	500 ˚C, 1–2 h, 0.5–3.5 L/min N2, fuidized bed reactor: 100 g/h	54 (bio-oil)	Rueangsan *et al*. (2023)
Lychee	Pyrolysis	350 ˚C, 125 min, heating rate of 120 ˚C/min, 110 mL/min Ar	38 (bio-oil)	Singh *et al*. (2023)
Corn straw and hair waste	Pyrolysis/ Co-pyrolysis	5 g biomass, corn straw/hair waste blend ratio: 25/75% (w/w), 450 ˚C, 20 min, 80 mL/min	Corn straw: 10 Hair waste: 48 Corn straw/hair waste: 46 (bio-oil)	Xiong *et al*. (2023)

Besides ethanol, isobuatnol is considered be a promising next-generation biofuel. Isobuatnol display superior features as compared to ethanol as a fuel *e.g.,* high energy-content, less hygroscopic, less-volatile, comparable to gasoline, supported by current infrastructure. Recently, Sherkhanov *et al.* (2020) have established purified enzyme-based cell-free system that employed 16-enzymes-incorporated biosynthetic-pathway for conversion of glucose into isobutanol. Through continuous product removal from bioreactor this system provides maximum yield of 4 g/L/h and 95 % productivity over approximately 5 days. Authors reported that this production rate is higher even from the highly developed ethanol-fermentation (Sherkhanov *et al.*, 2020). Wong *et al.* (2019) reported conversion of ketoisovaleric acid into isobutanol through immobilized-enzymes based cell-free system. This system utilized ketoacid-decarboxylase immobilized on maltose-binding protein along with Formate-dehydrogenase and alcohol-dehydrogenase to metabolically convert ketoisovaleric-acid to isobutanol at concentration of 2 g/L with overall productivity of 43 % (Wong *et al.*, 2019). Grimaldi *et al.* (2016). have also established immobilized enzyme-based cell-free system for isobutanol production from ketoisovaleric acid. Two pathway enzymes "Alcohol dehydrogenase" (ADH) and "ketoacid-decarboxylase" (KdcA) covalently immobilized on methacrylate matrix and one enzyme "Formate-dehydrogenase" (FDH) in reaction mixture effectively convert ketoisovaleric-acid into isobutanol (55 % conversion efficiency) at concentration of 0.135 mol/mol. Study reported that this cell-free system achieved 8-20 times higher isobutanol yield than highest reported isobutanol titer in *S. cerevisiae* (Grimaldi *et al.*, 2016).

Moreover, as compared to capacity of living systems cell-free approaches hold much-more potential for biohydrogen production. In natural system, during metabolism each glucose molecule generate just 4 H_2 molecules (Chou *et al.*, 2008). Although theoretically each glucose molecule should yield 12 H_2 molecules. Rollin *et al.* (2015) reported purified enzyme-based cell-free system for biohydrogen production. First, corn-stover was pretreated (acid/ enzymatic hydrolysis) to generate xylose and glucose which were subsequently converted to hydrogen through cell-free biosynthetic pathway incorporating more than10 enzymes. Study reported that improved enzyme-availability, substrate concentration and kinetic modeling leads to 67-times increase in yield with overall productivity of 54 mmol H_2 L/h (Rollin *et al.*, 2015). Some recent studies, reporting the use of bio-waste from various sources as a potential feedstock for biofuel production are enlisted in Table 2.

Biosurfactants

Biosurfactants (BS) are amphoteric molecules synthesized extracellularly by wide range of bacteria, yeast and fungi (Santos *et al.*,2016). Members of Bacillus genus are recognized as best producers of lipopeptide-biosurfactants (Jacques, 2011). In contrast with synthetic surfactants, BS offer various advantages such as high-biodegradability, biocompatibility, low-toxicity, low-irritancy, digestibility along with diverse chemical structures and functions. Despite these benefits, commercial production of BS is low because of high production-cost (Banat *et al.*, 2014). To address this challenge cell-free system might prove be an effective alternative for cost-effective large-scale production of BS. Satpute *et al.* (2018) have reported cell-free synthesis of BS from "*Lactobacillus acidophilus* NCIM 2903" and examined its antiadhesive, antibiofilm properties by employing Microfluidic approach. This *in-vitro* produced biosurfactant displayed antibacterial activity at titer of 625 µg/ml against *P. aeruginosa, E. coli, S. aureus* and *B. subtilis*. Moreover, antiadhesive and antibiofilm action was also demonstrated on Polydimethylsiloxane (PDMS) surface and catheter as intro-synthesized BS strongly inhibited biofilm formation by *S. aureus and E. coli* (Satpute *et al.*, 2018).

Bioplastic

The large-scale manufacturing and use of oil-derived plastic has resulted in irreversible ecological damage (Suaria *et al*, 2016). Despite, serious environmental concerns petrochemical-derived plastic is yet in high-demand mainly due to versatility and cost-effective production. Even current recycling practices seems to be ineffective to affects future de-novo plastic manufacturing (Geyer *et al.*, 2017). Furthermore, introduction of sustainable substitutes to petroleum-derived plastic faced considerable technological and societal challenges. However, advances in synthetic-biology and metabolic engineering offers a way for sustainable commercial production of biopolymer "polyhydroxyalkanoates" (PHA), that is a promising biodegradable alternative to oil-derived plastic (Chen *et al.*, 2017; Dietrich *et al.*, 2017). Extensive research has been conducted to construct effective-microbial PHA's production system through metabolite-recycling (Beckers *et al.*, 2016), pathway engineering (Tao *et al.*, 2017) and the use of industrially viable cost-effective feedstock (Nielsen *et al.*, 2017). However, microbial production of PHAs is currently more costly than petroleum-derived plastic. Thus, highly competent production-systems are required for sustainable commercial production of PHAs (Kelwick *et al.*, 2018).

Cell-free biosynthesis serve as a highly promising platform for designing biosynthetic pathways for cost-effective and sustainable PHAs production. For example, Kelwick *et al.* (2018) have established numerous *E. coli* extract-based cell-free systems for prototyping PHAs synthesis operons and for identifying enzymes responsible for metabolite recycling. *In-vitro* transcription-translation prototyping and gas-chromatography, mass-spectrometry

Links Researchers

quantification of *in-vitro* synthesized 3-hydroxybutyrate (3HB) demonstrated variations in activities of native *ΔPhaC_C319A* (1.18 6 0.39 mM), *C101 Δ PhaC_C319A* (2.65 6 1.27 mM) and *C104 ΔPhaC_C319A* (4.62 6 1.31 mM) *PhaCAB* operon. *C104* was reported to be the most active operon as synthesize higher concentration of PHAs as compared to native *PhaCAB* operon in both *in-vivo* as well as *in-vitro* analyses (Kelwick *et al*., 2018). Study reported that addition of optimal quantity of whey-permeate this cell-free reaction increase *in-vitro GFP mut3b* synthesis by approximately 50 %. Thus, data recommended that cell-free strategies help-out *in-vivo* workflows to demonstrate additional promising approaches for optimizing PHAs production (Kelwick *et al*., 2018).

Scale-up

Despite the obvious advantages of cell-free technologies practical implementation of these systems from lab-scale to industrial manufacturing scale still require considerable efforts to develop cost-effective strategies for synthesis and purification of components essential for bottom-up reconstitution of cell-free system (Claassens *et al*., 2019). Some promising considerations necessary in the designing of these system at commercial level to proceed towards sustainable production of biomolecules and value-added products are listed here.

Effective energy-regeneration system

A prerequisite to improve efficacy of cell-free biomanufacturing is the development of efficient energy-regeneration modules because ATP and NAD(P)H are required to derive biosynthetic reaction under *in-vitro* conditions (Silverman *et al*., 2020; Moon *et al*., 2019). Typically, cell-free systems rely on natural catabolic-pathways for energy regeneration (Bowie *et al*., 2020; Lin *et al*., 2020). Furthermore, addition of artificial vesicles encompassing ATP-synthase and cytochromes or purified *E. coli* membrane-vesicles that are metabolically active in cell-free environment can improve ATP supply for biosynthesis (Lin *et al*., 2019; Otrin *et al*., 2017). However, use of simple carbon substrate mainly limits ATP regeneration in cell-free system through oxidative-phosphorylation. To overcome this limitation, electrical energy can be employed along-with these sources to power biosynthetic pathways in open environment after being converted to chemical energy. For example, electrically active microbes such as *Geobacter sulfurreducens, Shewanella oneidensis* can be utilized as external energy-source to supply energy directly to biological energy-carriers (Fang *et al*., 2020). Such electrically derived *in-vitro* systems could comprised of hybrid reaction mixture incorporating defined content of lysate from electrically active microbes. Moreover, other promising sustainable energy source include solar-energy harnessed by employing plant-derived thylakoids or artificial vesicles comprising ATP-synthase and bacteriorhodopsin (Berhanu *et al*., 2019; Lee *et al*., 2018). Implementation of such energy-regeneration systems could greatly enhance economic viability and efficacy of cell-free biomanufacturing.

Sustainable carbon source

Currently, majority of cell-free approaches utilize glucose as carbon-source. However, there exist great potential to utilize cheaper, renewable biomass as carbon sources to proceed towards cost-effective *in-vitro* biochemical transformations (Rasor *et al*., 2021). Biopolymers such-as whey permeate, starch, cellulose after hydrolysis to monomers could be employed as feedstock as reported in both *in-vitro* and *in-vivo* systems (Mano *et al*., 2020; Tian *et al*., 2019; Yi *et al*., 2018). For example, study reported that addition of whey-permeate to *E. coli* MG1655 extract increase nearly 50 % protein synthesis in cell-free system without supplementing additional enzyme and has also been successfully utilized to enhance *in-vitro* manufacturing of 3-hydroxybutyrate (Kelwich *et al*., 2018). Moreover, single-carbon feedstock (CO_2, methanol, CH_4, formate) represents another potential substrate-group that has not been widely investigated as carbon source for biomanufacturing in an open-system (Cotton *et al*., 2020). CO_2-fixation system is particularly promising as it exhibits potential to manufacture sustainable products from readily available, cheap carbon sources (Liew *et al*., 2016).

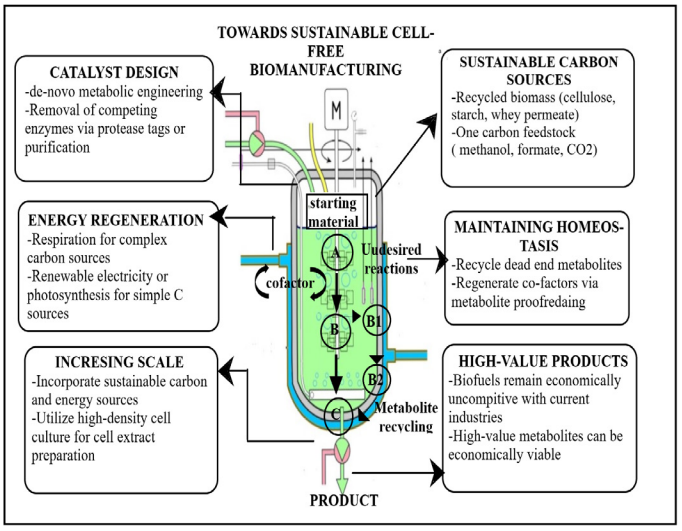

Figure 2: Key considerations for sustainable cell-free biomanufacturing (modified from: Rasor *et al*., 2021).

Recently established purified enzyme-based cell-free system "The CETCH cycle" utilize first synthetic CO_2 fixation-pathway (Miller *et al*., 2020). These economically viable, renewable-feedstock could significantly reduce feedstock-cost; thus, possess potential to enhance efficiency of commercial biomanufacturing in cell-free systems in future (Rasor *et al*., 2021).

Biocatalyst engineering and recycling

One principal factor required to be considered for scale-up of enzyme-based systems is enzyme production

Links Researchers

and purification cost. In case of extract-based systems scale-up efforts are much easier and cost-effective as simply involve cells harvesting and lysing and use cell-lysate for *in-vitro* biomanufacturing (Rollin *et al.*, 2018). While for purified enzyme-based approach economic viability of system largely depends upon host employed for enzyme production, expression level, enzymes purification method and stability. Higher enzyme-stability is vital parameter for commercial implementation of these enzyme-based biosynthetic pathways. As enzyme stability significantly reduce purification cost and allow enzyme recovery simply by heating the extract (Ninh *et al.*, 2015). Furthermore, semi-rational and rational biocatalyst engineering strategies are particularly important as greatly increase biocatalyst's robustness (Longwell *et al.*, 2017; Sheldon and Pereira, 2017). Two promising approaches for biocatalyst recycling include in-situ product recovery and immobilization method (Rollin *et al.*, 2018).

In-situ recovery approach permit separation of limited number of products as precipitate or in gas-phase. By employing concentrated substrate as feedstock, it is possible to operate cell-free biosynthesis at steady-state without losing components of aqueous-phase (Opgenorth *et al.*, 2017). While second in-situ product recovery strategy use membranes for enzymes retention while permit product to be removed from reactor (Rollin *et al.*, 2018). However, enzymes immobilization strategy involves enzymes impregnation on surfaces like hydrogels, membranes or appropriate matrixes (Jochems *et al.*, 2011; Blanchette *et al.*, 2016) or polymer capsules. Besides, these advancements still extensive efforts are required for scale-up of purified enzyme-based cell-free technology for sustainable biomanufacturing of extended range of products. In addition to these key considerations for scale-up of cell-free technologies some important considerations are shown in Figure 2, like increasing scale, high-value product manufacturing.

Challenges and future prospects

Although cell-free systems appear to be exciting technology but also pose several challenges that should be addressed to establish these systems as competitive production platform. Main challenges include high-cost associated with co-factors, energy supplements that are essential for *in-vitro* activation of biological machinery after extracting it from cells. Besides cost-factor, standardization of these *in-vitro* biosynthetic pathways is particularly problematic as different research-labs have developed complementary and competing systems by employing different extract preparation protocol, variable genetic strains of different organisms and acquired different result (Burgenson *et al.*, 2018). Bio-machinery content can also exhibit variations even within same strain and its activity can also influenced by employing different fermentation media and fermenters. Moreover, *in-vitro* manufacturing can be operated in batch (Zawada, 2011), microfluidics (Timmy *et al.*, 2016; Georgi *et al.*, 2016), fed-batch (Salehi *et al.*, 2016) and continuous mode (Li *et al.*, 2017; Stech *et al.*, 2014); although, these varied operational modes offer design-flexibility, but extensive research is required to optimize each product. However, current scenario suggests that development of innovative extract preparation protocols and utilization of renewable energy and carbon source can greatly help in advancement of these cell-free technologies as a competitive platform for commercial manufacturing that will probably replace currently well-established industrial fermentation in future.

Declarations

Funding

The study received no external funding.

Ethical statement

All applicable institutional, national and international guidelines for the care and use of animals were followed.

Statement of conflict of interest

The authors have declared no conflict of interest.

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