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Strategies and tools for the biotechnological valorization of glycerol to 1, 3-propanediol: Challenges, recent advancements and future outlook

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Abstract

Global efforts towards decarbonization, environmental sustainability, and a growing impetus for exploiting renewable resources such as biomass have spurred the growth and usage of bio-based chemicals and fuels. In light of such developments, the biodiesel industry will likely flourish, as the transport sector is taking several initiatives to attain carbon-neutral mobility. However, this industry would inevitably generate glycerol as an abundant waste by-product. Despite being a renewable organic carbon source and assimilated by several prokaryotes, presently realizing glycerol-based biorefinery is a distant reality. Among several platform chemicals such as ethanol, lactic acid, succinic acid, 2, 3-butanediol etc. 1, 3-propanediol (1, 3-PDO) is the only chemical naturally produced by fermentation with glycerol as a native substrate. The recent commercialization of glycerol-based 1, 3-PDO by Metabolic Explorer, France, has revived research interests in developing alternate cost-competitive, scalable and marketable bioprocesses. The current review outlines natural glycerol assimilating and 1, 3-PDO-producing microbes, their metabolic pathways, and associated genes. Later, technical barriers are carefully examined, such as the direct use of industrial glycerol as input material and genetic and metabolic issues related to microbes alleviating their industrial use. Biotechnological interventions exploited in the past five years, which can substantially circumvent these challenges, such as microbial bioprospecting, mutagenesis, metabolic, evolutionary and bioprocess engineering, including their combinations, are discussed in detail. The concluding section sheds light on some of the emerging and most promising breakthroughs which have resulted in evolving new, efficient, and robust microbial cell factories and/or bioprocesses for glycerol-based 1, 3-PDO production.

Keywords: glycerol, 1,3 - propanediol, genetic interventions, bioprospecting, process intensification, adaptive laboratory evolution, mutagenesis, fermentation

1. Introduction

In the last decade, the world has witnessed a burgeoning demand to produce bio-based chemicals using renewable and sustainable routes (Lee et al., 2019; Yadav et al., 2020). Further, rising CO_2 emissions, the primary driver for climate change, have seeded the idea of "decarbonization" and opened new frontiers for "biomass". Biomass, an abundant, cheap, and inexhaustible source of organic carbon, is a precursor for manufacturing several chemical building blocks and commercially important compounds (Chandel et al., 2020). Since the transport sector largely relies on fossil fuels, the augmented role of biomass-derived biofuels in decarbonizing this sector is anticipated to be pivotal (Oke et al., 2022). The most relevant example is the increasing global demand for biodiesel, projected to rise at a compound annual growth rate (CAGR) of 10% by 2030, generating revenues of 73.05 billion USD (Research and Markets, 2022). However, it will generate vast volumes of glycerol, as every 10 tonnes of biodiesel produced by transesterification (chemical or enzymatic) of triglycerides with alcohol invariably yields 1 tonne of crude glycerol (CG) as a by-product (Gerardy et al., 2020; Chilakamarry et al., 2021). Promoting waste minimization, implementing circular bio-economy, and increasing importance of clean, techno-commercially viable and eco-friendly technologies has rekindled research interests in the biotechnological valorization of glycerol, especially of industrial origin (Ripoll and Betancor, 2021).

Chemically, glycerol is a highly functionalized molecule that undergoes oxidation, reduction, halogenations, etherification, and esterification and can be transformed into a spectrum of products, including chemicals and fuels (da Silva Ruy et al., 2020). Besides being a well-known platform chemical, it has the most reduced form of carbon; hence, its transformation gives high product conversion yields. Many known microbes can naturally assimilate glycerol as a sole carbon and energy source, even at high concentrations. Therefore, the bioprocesses that can transform this inexpensive and fermentable substrate into valorized products are gaining popularity. The development of such processes can lead to better glycerol utilization and, at the same time, enhance the revenues of the biodiesel industry through value creation from this waste byproduct (Ripoll and Betancor, 2021; Asopa et al., 2022). Recent reviews on pure glycerol (PG) and CG show their practicability to act as versatile carbon sources, producing commercial products like ethanol, *n*-butanol, lactic acid (LA), 3-hydroxypropionic acid (3-HP), 2,3-butanediol (2,3-BDO), 1, 3-propanediol (1, 3-PDO), 1,2-propanediol (1, 2-PDO), succinic acid (SA), citric acid etc. (Westbrook et al., 2019; Crosse et al., 2020). One such exciting chemical is 1, 3-PDO, where glycerol is a native substrate. Unlike most biochemicals, it cannot be produced naturally from glucose. 1,3-PDO, also known as trimethylene glycol, is a promising bulk chemical, and the presence of two hydroxyl groups makes it a flexible and highly reactive intermediate. It is used in myriad applications, such as producing polyesters (polytrimethylene terephthalate), polyurethane, cosmetics, personal care and cleaning products etc. The global market of 1, 3-PDO was 375.1 million USD in 2021, and nearly 60% of the total revenue was contributed by bio-based PDO (Grandview Research, 2022). The anticipated market trend (799.3 million USD by 2030) suggests a growing interest in the renewable and sustainable production of 1, 3-PDO using environmentally benign fermentative routes (Grandview Research, 2022).

Earlier, 1, 3-PDO was commercially manufactured by the petrochemical route. Two companies, Shell and Degussa-DuPont, had a monopoly over its production. If the former company employed 'ethylene oxide' as the starting feedstock, the latter used acrolein, a costly and carcinogenic material. In the Shell route, ethylene oxide reacts with carbon monoxide to produce β-hydroxyl aldehyde or 3-hydroxypropionaldehyde (3-HPA), which is reduced to 1, 3-PDO. While in the Degussa-Dupont route, water addition of acrolein leads to the formation of 3-HPA, which is later converted to 1, 3-PDO via catalytic hydrogenation (Fokum et al., 2021a). However, each of these processes had its disadvantages. The "Shell" route counters the

principles of green chemistry as it involves employing a catalyst, used methyl tert-butyl ether as a solvent, and petroleum-based starting materials. Another practical disadvantage was that PDO generated by the hydrocarbonylation route contained almost ten times the level of impurities than PDO derived from fermentation. Likewise, the "Degussa-Dupont" employs high pressure, high temperature, and catalyst, making the process costly.

However, Dupont, in the year 2003, developed a commercial bio-based 1,3 PDO fermentation process in collaboration with Genencor and Tate & Lyle group, emerging as the biggest 1,3 PDO producer globally (Zhou et al., 2023). Until 2021, three global players were producing commercial bio-based 1, 3-PDO: US-based Dupont Industry in collaboration with Tate & Lyle Group, who shared their technical know-how with a Chinese company named Glory Biomaterials, and Shenghong Group Holdings Ltd., China (Biorefineries blog, 2021; Market Watch 2022). Commercial bio-based 1, 3- PDO primarily exploits corn-based glucose as its feedstock. It is also a hard fact that, currently, most microbial fermentations exploit glucose to produce a variety of bio-based chemicals. The main reasons include preferential glucose uptake, easier assimilation, efficient biotransformation, and a simple and straightforward scale-up process. However, in 2021, France-based METEX NØØVISTA, which is a joint venture of Metabolic Explorer and Société de Projets Industriels, began their first commercial bio-based 1, 3-PDO production using glycerol as the starting substrate (Biorefineries blog, 2021; Metabolic Explorer press release 2021).
Considering the optimistic future of the biodiesel industry and the recent commercialization of the glycerol platform for 1, 3-PDO production has motivated and accelerated the research pursuits in this area. The present review discusses the latest (last five years) developments, where microbial bioprospecting was done in the hope of obtaining potential but natural 1, 3-PDO producers and different biotechnological strategies were adopted to enhance efficient biotransformation of glycerol to 1,3-PDO, showcasing promising leads, that could be translated into alternate industrially-deployable technologies. Before discussing them in detail, a background of natural, predominant glycerol assimilating and 1, 3-PDO producers, the key metabolic pathways involved, and genes regulating those pathways is given, and technical hurdles for commercially exploiting microbial routes are summarized. The review concludes with key takeaways from the emerging trends in glycerol valorization to 1, 3-PDO via the microbial route, along with the future outlook in the said area.

2. Brief history and overview of glycerol fermentation to 1, 3-PDO

Only prokaryotes have the natural ability to produce 1, 3-PDO from glycerol, and it is one of the oldest known fermentation products. The first evidence of glycerol biotransformation to 1, 3-PDO goes back to 1881 when Freund attempted to biosynthesize *n*-butanol from soap lyes (Werkman and Gillen, 1932). Though he did not thrust on microbial characterization, the spore-forming microbe was later identified as *Clostridium pasteurianum*, as exclusively reviewed by Zhu et al. (2021). Later, in 1928, Braak isolated a bacterium from the canal that produced 1, 3-PDO from glycerol in alkaline conditions. It exhibited characteristics similar to the "coli-aerogens" group and was named "*Bacterium freundii*". However, three years later, Werkman and Gillen (1932) carried out the exhaustive biochemical characterization of this bacterium, which showed some metabolic features specific to *Escherichia*, while some belonging to *Aerobacte*r. This organism with intermediate characters was allocated a new genus, *Citrobacter.* It is a Gram-negative, non-sporulating, rod-shaped bacterium that grew well on citrate agar and reduced nitrates. The organism was re-designated as "*Citrobacter freundii*". Thereafter, several naturally 1, 3- PDO producing microbes were isolated and reported, predominantly belonging to the *Clostridiaceae* and E*nterobacteriaceae* families. According to the two recent reviews by Sun et al. (2018) and Zhu et al. (2021), 1, 3-PDO natural producers from glycerol can be broadly categorized into two types: the abundant and

widely studied bacteria belonging to the genus *Klebsiella, Clostridium, Citrobacter, Enterobacter, and Lactobacillus*; and the new but rare bacteria belonging to the genus *Lactococcus, Halanaerobium, Hafnia, Shimwellia, Klyvera, Pantoea*, and *Trichococcus*.

Glycerol is an excellent carbon source for microbial systems and, like glucose, can be transformed into a wide range of products. However, the more reduced nature of glycerol compared to traditional carbohydrates make its oxidation challenging under anaerobic conditions without exogenous electron acceptors. Therefore, microbes (e.g., *Citrobacter, Clostridium, Klebsiella*, and *Lactobacillus*) that can metabolize glycerol efficiently under anaerobic conditions have evolved a two-step reductive pathway with the formation of a reduced product to serve as an electron sink (Figure 1). The pathway leads to the PDO synthesis, which the cell uses to achieve redox balance without external electron acceptors. The first step of the pathway involves the dehydration of glycerol to 3-hydroxypropionaldehyde or 3-hydroxypropanal (3-HPA) by glycerol dehydratase (GDHt), followed by the subsequent reduction of 3-HPA to PDO by 1, 3-propanediol oxidoreductase (PDOR), rate-limiting step of the pathway. The conversion of glycerol to 3-HPA can also be catalyzed by diol dehydratase, which is known to transform 1, 2-PDO into propanal.

GDHt consists of two subunits encoded by the genes *dhaB1* and *dhaB2*, or *dhaB*. However, some organisms show variations in the number of subunits (Russmayer et al., 2019). The enzyme requires vitamin B_{12} as a cofactor in many microorganisms, including *Klebsiella* and the *Citrobacter* genus. The cofactor often gets inactivated during the catalytic cycle, and the reactivation is done by glycerol dehydratase reactivase (GdrAB). GDHt also exists in a form independent of vitamin B_{12}, which is very sensitive to oxygen and has been found in some strains from the *Clostridium* genus and *Ilyobacter polytropus* (Kumar et al., 2013; Kumar and Park, 2018; Nasir et al., 2020). 3-HPA is a toxic metabolite that must be acted upon to avoid its adverse effects. PDOR (DhaT), the pathway's second enzyme, reduces 3-HPA to non-toxic PDO and safeguards by maintaining low levels of cytotoxic 3-HPA. The dhaT gene encodes PDOR and mediates the electron transfer from NADH to 3-HPA. Some other oxidoreductases catalyze this reaction with NADPH as a cofactor, such as hypothetical oxidoreductase (HOR) and YqhD in *Klebsiella pneumoniae* and *E. coli*, respectively. As NADPH is heavily involved in anabolic reactions, its participation can negatively impact anabolism, and further, its regeneration, like NADH, is not straightforward. At higher PDO levels, the backward reaction from 3-HPA to PDO becomes significant in the case of DhaT, as it catalyzes a reversible reaction. At the same time, it is negligible with NADPH-dependent enzymes such as YqhD, which catalyzes the irreversible reaction. 3-HPA, the immediate precursor of 1,3-PDO, can also be oxidized to 3-hydroxypropionic acid (3-HP) using NAD+ as an electron acceptor and has been obtained as a byproduct during 1,3-PDO synthesis in some microorganisms such as *Klebsiella pneumoniae*, *Lactobacillus reuteri*, *Lactobacillus collinoides* etc. (Kumar et al., 2013) as shown in Figure 2.

The oxidative metabolism of glycerol provides all the ingredients for cell mass formation, including energy in the form of ATP and other cofactors. In the oxidative route, glycerol is metabolized to dihydroxyacetone phosphate (DHAP) by the respiratory (aerobic/anaerobic) and/or fermentative pathways (Fig 1). The genes for respiratory and fermentative metabolism of glycerol are contained in the *glp* and *dha* regulons, respectively. The respiratory pathway begins with glycerol phosphorylation to glycerol-3-phosphate which is catalyzed by glycerol kinase (GlpK), followed by its oxidation to DHAP. The latter reaction is catalyzed by glycerol-3-phosphate dehydrogenase (aerobic GlpD or anaerobic GlpABC). However, during fermentation (microaerobic/ anaerobic conditions), glycerol is dehydrogenated to form dihydroxyacetone (DHA) in the presence of an enzyme called glycerol dehydrogenase (DhaD), with simultaneous reduction of NAD+ to NADH. *K. pneumoniae* has a putative form of it which is encoded by the *gldA* gene. In the next step,

ATP/PEP-dependent dihydroxyacetone kinase (DhaK) catalyzes the phosphorylation of DHA to produce DHAP. This oxidative pathway is characteristic of *Clostridium*, *Klebsiella,* and *Citrobacter* genera. The DHAP from either route is later funneled downstream in central carbon metabolism. DHAP is channelized to produce phosphoenolpyruvate, pyruvate, and acetyl-CoA, which can be transformed into various products, including succinic acid, lactic acid, 2,3-BDO, acetic acid, ethanol, formic acid, etc. (Fig. 1). These byproducts reduce PDO yield through carbon loss, exert toxic effects on cell growth, cause multiple product inhibitions, and complicate downstream processing (DSP) of 1,3-PDO. Further, many products, such as ethanol, lactic acid, and 2, 3-BDO, compete with PDO production for NADH and reduce the yield.

In 2003, Chen et al. carried out a detailed stoichiometric analysis to understand how different metabolite production and the extent of anaerobiosis affected the molar yields of 1, 3-PDO from glycerol in *Klebsiella pneumonia*. They reported that under complete anaerobic or microaerobic conditions, if no H_2 was produced and the sole byproducts were either acetate or ethanol, the maximum theoretical yield of 1,3-PDO was 0.72 and 0.50 mole/mole$_{glycerol,}$ respectively. However, H_2 production negatively impacted the 1, 3-PDO molar yields, which reduced to 0.64 and 0.11 mole/mole$_{glycerol}$, with acetate and ethanol as byproducts. Likewise, they also found that the maximum theoretical yield of 1, 3-PDO could touch 0.85 mol/mol if all the acetyl CoA was funneled in the TCA cycle without forming ethanol, acetate, and hydrogen without oxygen consumption (Chen et al., 2003). This scenario is only possible when acetyl CoA was formed from pyruvate via oxidative decarboxylation catalyzed by pyruvate dehydrogenase complex (PDH) instead of pyruvate formate lyase activity. They experimentally proved that biomass accumulation, ATP generation, and 1,3-PDO yields did not rely solely on the molar fraction of $NADH_2$ oxidized completely by molecular O_2 in the citric acid cycle but also on the fate of acetyl CoA entering entirely into the TCA cycle or co-producing different byproducts. Furthermore, the inactivation or activation of several enzymes in the presence of oxygen and the kind of reaction they catalyze (irreversible or reversible) further complicate the process. Considering the metabolic pattern of microbes and identifying maximum theoretical yields of 1,3-PDO, persistent research efforts are being made to manipulate multiple genes which tightly regulate glycerol metabolism. Some common genes frequently exploited for strain engineering work to enhance titer, yield and productivity (TYP) matrices of 1, 3-PDO are mentioned in Table 1, as exclusively reviewed (Chen and Liu, 2016; Jiang et al., 2016; Zhu et al., 2022). Generally, these genes code for proteins and enzymes and are categorized into four groups. The first are those involved in glycerol transport and metabolism; the second are those who decide the fate of pyruvate and acetyl-Coenzyme A, including byproducts formed after their assimilation; the third are those which replenish various redox cofactors; and the last code for transporters promoting the excretion of 1, 3-PDO. Despite having a long history of fermentative PDO production, well-elucidated metabolic pathways, and extensive knowledge of enzymes and genes encoding them, there are several technical hurdles, making the glycerol bioconversion to 1, 3-PDO economically challenging at the commercial level, as summarized below.

3. Challenges associated with glycerol usage and its bioconversion to 1, 3-PDO

3.1. Direct usage of CG as starting feedstock for microbial fermentation

In the last decade, research focus has been reoriented towards valorizing CG instead of PG, the former being a bio-based, renewable, cheap and readily available waste carbon stream generated from the biodiesel industry (Crosse et al., 2020). Nevertheless, at the same time, this biodiesel-derived glycerol cannot be directly upgraded to value chemicals through the biotechnological route, as it contains several impurities such as soap, methanol, mono-, di- and triglycerides, catalyst (alkali or acid catalyst), ash, water, etc. (Abd Manaf et al., 2019; Laura et al., 2020). These impurities, in general, impede the viability and growth of microbes, affecting glycerol bioconversion into desirable products. However, its severity level is

governed by the chosen microbial strain and targeted product. CG is generally subjected to refining or pretreatment to overcome the inhibitory effect of its inherent contaminants. For instance, Tan et al. (2018) demonstrated the importance of pretreating CG and assessed the performance of locally isolated *Clostridium butyricum* JKT37 on pure, crude, pretreated crude glycerol (PG, CG, PCG). CG was subjected to acidification for pretreatment, followed by microfiltration and neutralization. Though the 1, 3-PDO titers were more or less similar, the productivities were 2.06, 1.33, and 2.16 g/L.h with PG, CG, and PCG, respectively. Alternately microbial strains are acclimatized to grow in CG in the presence of these impurities, thereby creating vigorous microbial factories, mainly through adaptive laboratory evolution (ALE), which will be discussed in detail in the upcoming section.

3.2. Partitioning of glycerol, formation of byproducts and slow/less active TCA cycle

The carbon flux is partitioned between oxidative and reductive pathways at the glycerol node. 1, 3-PDO is a product of the reductive pathway, while oxidative metabolism primarily results in biomass generation and provides ATP and reducing equivalents for PDO synthesis. In the oxidative pathway, glycerol carbon is further distributed between the respiratory (aerobic/anaerobic) and fermentative (microaerobic or anaerobic) pathways, which often operate simultaneously and reduce PDO yield. When oxygen is available, a higher affinity of GlpK for glycerol than GDHt allows more carbon flux through the oxidative route. However, the glycerol flows through the fermentative route, yielding more NADH supply in a reaction mediated by DhaD. Hence, it is anticipated that deletion of the gene encoding for GlpK can minimize the glycerol flow through the respiratory pathway of the oxidative route, furnishing more NADH availability (Chen and Liu, 2016).

Most of the byproducts are generated through the PEP/pyruvate pathway during the oxidative metabolism of glycerol. However, their pathway elimination creates an imbalance between the glycolytic pathway and the TCA cycle. The latter route is inactive under oxygen-limited and anaerobic conditions favoring PDO accumulation. This imbalance gives rise to the excretion of pyruvate and other metabolites such as DHA, DHAP, glyceraldehyde-3-phosphate, methylglyoxal etc., which are very toxic for the cell (Westbrook et al., 2019; Chen and Liu, 2016). For instance, lactic acid and 2, 3-BDO are the primary products of oxidative glycerol metabolism by *K. pneumoniae*. Kumar et al. (2016) engineered *K. pneumoniae* to eliminate lactic acid and 2, 3-BDO formation, but this triggered heavy carbon-metabolic traffic at the pyruvate node and did not improve PDO production. Pyruvate is a toxic metabolite due to its low pKa (2.5), and its accumulation can give rise to the piling up of glycolytic pathway intermediates, which are more toxic than pyruvate. Their study indicated that sometimes knocking out genes that encode oxidative products seems straightforward; however, it sometimes yields an undesirable and a different impact. Therefore, genetic interventions demand rational engineering of pathways downstream pyruvate as this node is more flexible than the glycerol node without losing sight of maximizing 1,3 PDO titers (Chen and Liu, 2016). Besides getting rid of by-products, such manipulations should leave no adverse impact on microbial growth and simultaneously maintain the redox-cofactor balance.

3.3. Inherent issues with microbial GDHt activity and lack of industrially-proven strains

The first reaction catalyzed by GDHt limits the overall reaction rate of the reductive pathway since, in many microbes; this enzyme is dependent on vitamin B_{12}. As an exception, non-dependence on vitamin B_{12} by GDHt gives an edge to *Clostridium*, and optimum PDO production requires ≥ 60 g L^{-1} initial glycerol concentration (Russmayer et al., 2019). However, commercial scale-up of *Clostridium* spp. is difficult as it demands complete anaerobiosis. Further, state-of-the-art reveals that most of the genetic modifications have been performed by Gram-negative bacteria, as their cell wall is thinner than Gram-positive to conduct

electroporation, and well-established genetic tools are available to manipulate them, which is a limitation in the latter group, including the genus *Clostridium*. Varying vitamin B_{12} levels directly affect glycerol metabolism in microbial systems where GDHt depends on this cofactor, impeding PDO synthesis. For instance, though GDHt of *Klebsiella* is vitamin B_{12} dependent, it not only grows well on glycerol under both aerobic and anaerobic conditions, but at the same time, it is a natural producer of coenzyme B_{12} (Kumar and Park, 2018). Despite being a good candidate for 1,3-PDO biosynthesis, due to its pathogenic or virulent nature, *Klebsiella* requires a Biosafety Level 2 (BSL-2) facility, making its industrial scale-up challenging. Unlike *Klebsiella*, the genus *Citrobacter* and *Lactobacillus* belong to BSL-1 category, the latter being generally regarded as safe (GRAS), but both require an external source that can supplement vitamin B_{12} during fermentation. If, in the case of *Citrobacter*, accumulation of 3-HPA limits 1, 3-PDO production, the *Lactobacillus* genus requires enriched media for fermentation, making the process economically disadvantageous (Russmayer et al., 2019). Thus, industrially-proven microbial strains are lacking in the present scenario, which can biotransform glycerol to 1, 3-PDO.

Considering the essential involvement of vitamin B_{12} in GDHt, it is undeniable that this expensive cofactor-containing enzyme requires reactivation after each catalytic cycle; therefore, continuous supply of this coenzyme is imperative for the conversion of glycerol to PDO and achieving a prolonged period of high productivity. One effective solution is cloning or amplifying the entire metabolic pathway for vitamin B_{12} biosynthesis in native and non-native producers. However, it will be very challenging as the biosynthesis of vitamin B_{12} is an expensive process that involves nearly 30 genes (Balabanova et al., 2021). The over-expression of the *cob* gene(s) can consume a lot of energy and reduce PDO yields, by affecting the distribution of carbon at the glycerol node. Therefore, the production of this vitamin should be carefully optimized. Alternately, researchers can explore Vitamin B_{12}-producing microbes as a suitable host for creating a synthetic pathway for glycerol assimilation and its bioconversion to 1, 3-PDO. Likewise, to convert naturally producing 1, 3-PDO strains from just laboratory strains to industrially competent strains, their adaptability towards substrate and product concentrations must be enhanced using various biotechnological tools.

3.4. Accumulation of 3-HPA

The accumulation of 3-HPA is regarded as one of the most severe limitations of 1, 3-PDO biosynthesis. 3-HPA, an immediate precursor to PDO, is a toxic metabolite to the host cells. Its accumulation at even 15-30 mM concentration triggers irreversible metabolic activity and cellular growth cessation, restricting the microbiological production of biochemicals from glycerol (Matsakas et al., 2018). As per the extensive review by Sun et al. (2022), the aldehydic group of 3-HPA reacts with the free sulfhydryl groups of several small proteins and enzymes, such as glutathione reductase, causing oxidative damage to bacterial systems. Likewise, its dimeric form competitively inhibits the ribonucleotide reductase enzyme, which is responsible for DNA synthesis in bacteria, thus contributing to its antimicrobial properties. In addition, PDO buildup can contribute to the accumulation of 3-HPA as PDOR can catalyze the reverse reaction (PDO→3-HPA) under high PDO concentrations.

The accumulation of 3-HPA arises due to an imbalance between the activities of GDHt and PDOR, leading to reduced PDO yields. The synchronization between these enzymes is another challenge. One of the innovative approaches to avoid 3-HPA accumulation is balancing its production and consumption rates. This balance can be accomplished by either reducing GDHt activity or enhancing the activity of PDOR. Reducing GDHt would reduce the overall 1, 3-PDO production; hence, enhancing the activity of PDOR is more desirable. Alternately, the deleterious effects of 3-HPA can be alleviated up to a certain extent by the

action of NADPH-dependent oxidoreductases such as YqhD (from *E. coli*) and HOR (hypothetical oxidoreductase) (from *K. pneumoniae*), which reduce 3-HPA into non-toxic PDO (Russmayer et al., 2019). HOR or YqhD enzymes have activity towards 3-HPA, better resistance towards oxygen, and display much lower reversibility can be expressed to overcome this problem (Przystałowska et al., 2015; Kumar and Park, 2018). Lower reversibility means less toxic 3-HPA is accumulated at higher PDO concentrations. The reduction of toxic 3-HPA can be beneficial. However, the diversion of 3-HPA towards PDO, along with the draining of valuable NADPH, which is required for the biosynthesis of cellular constituents, can diminish biomass and, eventually, PDO yield.

3.5. Product inhibition and tolerance

The tolerance of microorganisms includes the inhibition or toxic effects of not only end products but also substrate, as well as impurities on cell growth and metabolism. Generally, these metabolites belong to the family of short-chain carboxylic acids and alcohols. Thus, the product output of any microbial fermentation is governed by the capacity of the microbe to tolerate the threshold concentration of the targeted metabolite without critically affecting its metabolic activity. It is often exacerbated by the co-formation of other unwanted byproducts, resulting in multi-product inhibition. As a result, microbial fermentations often display a decline in productivity over a prolonged duration. For instance, Kumar et al. (2013) conducted toxicity experiments with *K. pneumoniae* by exogenously adding PDO at different levels. They found that the initial presence of PDO unfavorably affected the growth and production capabilities of *K. pneumoniae*. Cell growth and its metabolism completely arrested, when the PDO concentrations reached 300-400 mM. Similarly, Yang et al. (2019) conducted a 1, 3-PDO tolerance test on *C. butyricum* XYB11 by growing its exponential culture in a medium containing 60-100 g L^{-1} of 1, 3-PDO. A concentration of 65 g L^{-1} 1, 3-PDO adversely affected microbial growth.

In the case of 1, 3-PDO production, byproducts from the oxidative pathway, especially organic acids, invariably negatively affect cell growth, metabolism, and 1, 3-PDO production. Random mutations, genetic interventions, or the ALE approach commonly address these issues. Besides glycerol bioconversion to 1, 3-PDO, its downstream processing (DSP) is expensive, as its biological production is mandatorily hetero-fermentative. Byproducts such as acetate, lactate, butyrate, succinate, 2, 3-BDO, microbial cells, and residual media components interfere during selective extraction, posing difficulties during its recovery and purification. However, commenting on this crucial aspect is beyond the scope of the current review.

4. Tools and strategies for enhanced glycerol bioconversion to 1, 3-PDO

The forthcoming section discusses various strategies and tools effectively exploited in the past five years to enhance 1, 3-PDO titers from glycerol as the sole or predominant carbon substrate, aiming to create a sustainable glycerol-based biorefinery. It encompasses some of the most popular and widely used biotechnological tools, such as genetic interventions, mutagenesis, adaptive laboratory evolution (ALE), microbial bioprospecting, process intensification (PI) during fermentation, and their combinations for enhanced 1, 3-PDO production from glycerol.

4.1. Genetic interventions to enhance 1, 3-PDO formation from glycerol

This subsection discusses recent advances in genetic engineering, in which experiments were rationally designed, and specific gene/genes were targeted for over-expression or deletion to maximize 1, 3-PDO titers. Table 2 outlines the gene manipulation approaches exercised by various research groups globally in the past five years to improve TYP matrices of 1, 3-PDO from glycerol. In most published reports, multiple

gene expressions and deletions were done to attain high glycerol bioconversion. However, three isolated research draw particular attention, in which two achieved molar yields >0.75 mol mol^{-1} and the third attained high titers and productivity.

For instance, Lee et al. (2018) first deleted the "wabG" gene from *K. pneumoniae* KCTC 2242, responsible for the strain's pathogenicity to increase acceptability of this strain for scale-up. Later, the group disrupted the *ldhA* and *pflb* genes sequentially, eliminating lactate and acetate formation. As a result, the 2, 3-BDO titers increased. However, when the *budA* gene was deleted, it significantly reduced glycerol consumption, biomass production, and 1, 3-PDO titers. In the next stage, when the two crucial genes of the oxidative glycerol pathway, namely *glpK* and *dhaK,* were deleted, the impact of the latter gene on 1, 3-PDO production was detrimental, as its product catalyzed the dehydrogenation of glycerol to produce DHA, supplying $NADH_2$ equivalent for uninterrupted 1, 3-PDO synthesis. But when the strain was co-fed with mannitol, it restored cell growth and fulfilled energy needs, while glycerol flux was diverted mainly towards 1, 3-PDO, thereby increasing molar yield. Additionally to reduce the co-feeding of mannitol, the expression levels of mannitol-specific transporter MtlA were reduced targeting the 5' untranslated region (UTR) sequence of its gene. Popular and precise gene editing tool namely clustered regularly interspaced short palindromic repeats (CRISPR) -associated CAS9 endonuclease was used for gene alteration. As a result mannitol utilization reduced, without hampering biomass and 1, 3-PDO production. The final KMY strain with transcriptionally optimized *dha* operon produced 20.59 g L^{-1} 1, 3-PDO with 0.76 of molar yield. This is indeed the highest claimed molar yield in *Klebsiella* reported to date.

Likewise, Zhou et al. (2019) wisely and rationally chose *Pseudomonas denitrificans* ATCC 13867 as the host to create a synthetic pathway for glycerol valorization to 1, 3-PDO, a natural producer of vitamin B12 under aerobic conditions. In the first stage, they heterologously expressed all the genes related to GDHt (*dhaB1, dhaB2, gdrA, and gdrB*) from *K. pneumoniae* under a synthetic constitutive promoter. Later, they simultaneously created two recombinant strains, one in which the *dhaT* gene from *Klebsiella* and the *yqhD* gene from *E.coli* were over-expressed individually. Testing the two recombinant strains under aerobic conditions showed that 3-hydropropionic acid (3-HP) was inevitably produced along with 1, 3-PDO. Owing to the attribute of 1,3-PDOR encoded by the *dhaT* gene to carry a reversible reaction, it was anticipated that aeration would favor the production of 3-HPA, which in turn was oxidized to 3-HP, leading to its higher accumulation. Although 1, the 3-PDOR activity of the enzyme encoded by the *yqhD* gene drove an irreversible reaction, lower 3-HP concentrations were observed. However, it also resulted in relatively lower 1, 3-PDO titers. Therefore, the strain over-expressing the *dhaT* gene was further selected for gene manipulation. To identify specific enzymes favoring the conversion of 3-HPA to 3-HP in *Pseudomonas*, 17 genes encoding for aldehyde dehydrogenase activities were identified. Four genes were shortlisted and targeted for deletion due to high transcription levels under fully aerobic conditions in the presence/absence of 100 mM glycerol. Deletion of genes *aldH*13 and *aldH*16 not only raised the molar yield of 1, 3-PDO to >0.8 but reduced the molar yield of 3-HP significantly. However, aerobic conditions simultaneously created a scenario in which there was a notable increase in NADH dehydrogenase activity of the electron transport chain (ETC), causing oxidation of $NADH_2$, thus limiting its availability for 1, 3-PDO biosynthesis. Further inactivating the gene *nuoA,* which encodes for NADH dehydrogenase Type I, and knocking out the *pta-ackA* gene, which eliminated acetate formation without pyruvate accumulation, they attained a maximum of 0.92 molar yields for 1, 3-PDO. When the final strain was tested for fed-batch bioreactor cultivation under the aerobic mode, the maximum concentration, molar yield, and productivity obtained were 33.4 g L^{-1}, 0.89, and 0.656 g/L.h, respectively.

In the last but not the least important study, the glycerol flux of *Klebsiella* towards lactate, succinate, and ethanol production was reduced by knocking out the *ldhA*, *frdA,* and *adhE* genes (Wang et al., 2021a). Despite deleting the *frdA* gene, three main byproducts were formed besides 1,3-PDO: acetate, succinate, and 2,3-BDO. Further, the detrimental effect of acetate on cell metabolism was reduced by rewiring its entire metabolic pathway towards polyhydroxybutyrate (PHB) biosynthesis. To facilitate PHB formation, the "*acs*" gene from *Acetobacter pastoris*, encoding for Acetyl CoA synthase, was over-expressed, which catalyzes acetyl CoA formation. It promoted acetate consumption, cell growth, and biomass accumulation. Introducing the entire PHB biosynthetic pathway diverted the Acetyl-CoA towards its formation. As a result, succinate became the predominant byproduct. Later deletion of the "*aceA*" gene, which encodes for isocitrate lyase, down-regulated succinic acid production, as this enzyme facilitates the breakdown of isocitrate of glyoxylate and succinate. The final Kpr-6 strain showed an accumulation of 91.2 g L^{-1} 1, 3-PDO and produced 2.56 g L^{-1} PHB, accounting for 34.2% cell dry weight. Moreover, this gene manipulation eased the DSP of 1, 3-PDO from the fermentation broth. This strain seems industrially attractive owing to the lesser requirement of yeast extract (1g L^{-1}) during fermentation, with productivity as high as 3.06 g /L.h and a molar yield of 0.59, besides higher titers. However, a rigorous assessment of safety norms and risk levels needs to be conducted at a pilot scale to understand the feasibility of commercial production since the organism in the current study is genetically modified (GM) and belongs to the BSL-2 category of microbes.

To date, researchers attempted to manipulate only the genes of the glycerol metabolic pathway for attaining high TYP matrices of 1, 3-PDO. However, bold experimentation was done in the last two years, involving the detailed investigation of various transport proteins facilitating glycerol uptake and promoting 1, 3-PDO excretion. Teng et al. (2022) chose an endogenous gene from *Klebsiella* and an exogenous gene from *E. coli* encoding for glycerol uptake facilitator proteins for over-expression in the study conducted with *Klebsiella*. Further, the role of seven genes encoding for resistance-nodulation-cell division (RND) type efflux systems was evaluated. These systems are characteristic of Gram-negative bacteria and facilitate the efflux of a spectrum of chemicals. Compared to the wild strain, over-expression of endogenous *glpF* gene, encoding for protein facilitating the glycerol uptake, and the heterologous *MexF* gene from *Pseudomonas aeruginosa* promoting 1, 3-PDO efflux, enhanced its final titers. During fed-batch cultivation, the recombinant *Klebsiella* in which *glpF* and *MexF* gene were over-expressed individually accumulated 65.1 and 74 g L^{-1} 1, 3-PDO, respectively compared to 55.6 g L^{-1} in parent, and the acetate formation also observed notably (Teng et al., 2022).

The outcome of most of the targeted gene manipulation studies is known to a large extent. However, state-of-the-art reveals that during experimental designing, cell homeostasis was successfully restored by additional metabolic rewiring at the genetic level when gene alterations created a redox imbalance.

4.2. Mutagenesis

Unlike genetic manipulation, physical or chemical mutagenesis is generally random in nature. However, despite being a labor-intensive technique, this route is still considered for selecting highly evolved strains owing to its low cost. There are only two isolated examples in the past five years wherein mutagenesis was used for enhancing glycerol bioconversion to 1, 3-PDO. The first example is *Clostridium buytricum*, which was sequentially subjected to nitrosoguanidine (NTG) followed by atmospheric and room temperature plasma (ARTP) treatment. During both the mutagenesis, high 1, 3-PDO tolerance and faster growth were the selection criteria. As a result, the tolerance limit of 1, 3-PDO was enhanced from 60 to 85 g L^{-1}. Later, batch fermentation was optimized using the OVAT and RSM approaches. The maximum titer, yield, and

productivity of 1, 3-PDO reported by the mutant strain (YP855) were 37.2 g L^{-1}, 0.51 g g^{-1}, 1.71 g/L.h, respectively. The mutant produced a 29.5% higher titers of 1, 3-PDO than the wild parent strain (Yang et al., 2019). In yet another study, electron beam irradiation was used to obtain mutants of *Lactobacillus reuteri*, with selection criteria being higher resistance towards 40 and 20 g L^{-1} lactic acid and acetic acid, respectively (Ju et al., 2021). During fed-batch cultivation on PG, the superiority of the mutant (JH83) was fairly visible over its parent strain (CH53). The 1, 3-PDO concentration and productivity of *L. reuteri* JH83 were higher by 34.7% (69.2 to 93.2 g L^{-1}) and 34.4% (0.96 to 1.29 g/L.h), respectively, compared to *L. reuteri* CH53. The mutant's performance on CG remained unchanged. The high 1, 3 PDO titers with GRAS organism like *Lactobacillus,* without co-feeding of any sugar as alternate carbon source during fermentation, makes this study conspicuous.

4.3. Adaptive Laboratory Evolution (ALE)

During the biotechnological valorization of real-time carbonaceous waste streams, ALE is the most powerful and competitive technique to enhance the robustness of microbial cell factories. In a recent review, Sandberg et al. (2019) listed most ALE studies into five categories, primarily targeting increased tolerance (towards substrate and product), followed by substrate utilization, increased product yields or titers and growth rate optimization. Nutrient and environmental stress, or their combination, is the most common way to induce natural selection and obtain highly evolved strains. Some of the examples wherein ALE was adopted for enhanced bioconversion of glycerol to 1, 3-PDO are shown in Table 3. As observed in Table 3, the most visible outcome of the ALE experiments was obtaining a stable adapted glycerol utilizing strain which showed higher 1, 3-PDO productivity than its parent strain. In general, strain adaptation at higher substrate concentrations and repeated sub-culturing led to the screening of fast-growing strains, which tend to exhibit a shorter lag phase than parents with a better glycerol uptake rate. A pertinent example to quote is the performance displayed by *C. pasteurianum* obtained after continuous ALE in CG (Table 3). The adapted strain showed no lag phase during the fed-batch experiment, resulting in titer and productivity of >80 g L^{-1} and >4 g/L.h, respectively, with CG as substrate in a 1000L fermenter (Zhang et al., 2022). By carrying fermentation under non-sterile conditions and without maintaining complete anaerobiosis, the authors proved that the adapted strain was a strong candidate for commercial-scale demonstration. In contrast to rational engineering and directed modification of specific enzymes, adaptive laboratory evolution (ALE) has an edge by allowing non-intuitive productive mutations to occur in a variety of genes in parallel. These mutations resulting from ALE can activate latent metabolic pathways, increase substrate/ product tolerance, and improve bacterial fitness. With the growing interest in transcriptional and translational approaches, researchers try to decipher changes happening at the molecular level (gene or protein), which plays a pivotal role in rewiring the entire metabolic pathway of the microbes during evolutionary engineering (Mavrommati et al., 2022). For instance, proteomic analysis (using Tandem Mass Tag technology) of adapted *Klebsiella* x546 strain revealed several genes belonging to unrelated pathways (glycolytic, amino acid synthesis, pyrimidine synthesis) etc. were upregulated facilitating the growth and reproduction of the bacterium, besides the reductive pathway of glycerol metabolism. During fermentation, betaine addition counteracted the high osmotic pressure exerted by Na_2CO_3 (neutralizing agent), reducing the fermentation time by 40%. The highest 1, 3-PDO titer reported was 74.44 g L^{-1}, with productivity being 3.1g/L.h (Wang et al., 2021b).

Nearly all the examples cited in this sub-section confirm that after ALE, the natural glycerol assimilating strains are capable of producing industrial relevant titers of 1, 3-PDO.

4.4. Microbial bioprospecting

Although several microbial strains produce 1, 3-PDO by glycerol biotransformation, bio-prospecting is still prevalent. Nature is endowed with rich microbial diversity. Each microbe stands distinctly unique based on its ability to switch metabolic patterns based on environmental conditions, survive by adapting to changes, and thrive in diverse habitats. Depending on their ability to assimilate different types of carbon, nitrogen, sulfur, and phosphorus compounds, each microbial community develops its niche. Thus, each ecosystem is a rich reservoir of microbes harboring diverse industrial potential. Table 4 shows the current state of the art, where glycerol assimilating microbes/ consortiums were isolated from different potential sites, selectively enriched, and evaluated for 1, 3-PDO production studies.

For instance, Ma et al. (2019) used biodiesel-contaminated waste soil to isolate *Klebsiella pneumonia* 2e. They characterized the strain by 16S rDNA ribotyping and compared its performance on PG and CG. Further, they carried out whole genome sequencing to decipher genes involved in glycerol fermentation to 1,3-PDO, the difference with respect to other *Klebsiella* strains, and how their expression changed in response to shifting from PG to CG. Since the state of the art reveals that *Clostridium* is one of the potential 1 3-PDO producers, and since its GDHt is vitamin B12 independent, most of the researchers in the recent past have targeted the isolation of new *Clostridium* species. For instance, Yun et al. (2018) and Gupta et al. (2022) exposed the site samples to heat shock treatment, eliminating most mesophilic bacteria and favouring spore germination of only thermophilic/ thermotolerant bacteria. Further, providing anaerobic conditions and using Reinforced Clostridium Medium (RCM) selectively stimulated the growth of only *Clostridium* species. Some researchers chose sites where anaerobic conditions prevail (Zhou et al., 2018, Wang et al., 2019; Lan et al., 2021), while others chose aerobic sites for isolation, attempted selective enrichment of microbes on glycerol-based medium under aerobic/anaerobic conditions, and later assessed them for 1, 3-PDO production (Ma et al., 2019; Garg et al., 2020; Jiang et al., 2021).

Though pure cultures are widely approved for industrial scale-up, in the case of microbial valorization of glycerol to 1, 3-PDO, acceptance of microbial consortiums is also trending, as reviewed extensively by Sun et al., (2018). Some potential advantages include not requiring strict maintenance of aseptic conditions, better synergism between the microbial strains towards enhanced 1, 3-PDO formation, increased tolerance towards CG, etc. The molecular characterization of microbial consortia shown in Table 3 confirms that they predominantly contain bacteria from two ubiquitous families: Enterobacteriaceae and *Clostridiaceae*. For instance, microbial community analysis of the microbial consortium C2-2M during long-term continuous fermentation (311h) under excess glycerol revealed that 99% of bacteria belonged to the *Clostridiaceae* family (Zhou et al., 2018). Likewise, the microbial consortium CJD-S diversity showed that 86.25% and 13.75% of bacteria belonged to the families: *Enterobacteriaceae* and *Enterococcaceae*, respectively (Jiang et al., 2021). The consortium interestingly co-produced 45.86 g L^{-1} lactic acid besides 41.47 g L^{-1} 1, 3-PDO accumulation from CG in a non-sterile fed-batch process, displaying high organic acid tolerance.

Thus, site selection is essential to isolate suitable organisms from various ecological habitats during microbial bioprospecting. Further, with the advent of the metagenomic approach, even the uncultured and less explored microbes capable of glycerol assimilation and its bioconversion to 1, 3-PDO can be reported.

4.5. Process intensification during fermentation

During fermentation, process intensification (PI) plays a decisive role in maximizing the TYP of any product. Improving rate kinetics, maximizing homogeneity, addressing the limitations of transport phenomena (mass

and heat), and smart integration of process parameters are the four primary targets of PI (Noorman et al., 2018). Since glycerol bioconversion to 1, 3-PDO essentially involves an oxidoreductive pathway, pH-stat fermentation is a must, as the accumulation of organic acids tends to reduce the fermentation medium's pH and arrest the growth of the microbes. Further, maximizing 1, 3-PDO titers demand maintenance of either a micro-aerobic or anaerobic environment. Moreover, high glycerol concentrations generally exert an inhibitory effect on cell growth (due to high osmotic pressure) and its uptake, thus impeding the attainment of high TYP matrices of the product. To address this issue, continuous or fed-batch operation is preferred over batch, and optimum fermentation conditions must be deciphered, favoring high TYP. For instance, dilution rate or hydraulic retention time (HRT) is crucial if the researchers opt for a continuous process. In the case of a fed batch, feed concentration and time of addition, including cell maintenance, are vital. Table 5a shows trending PI strategies used exclusively during pure glycerol fermentation to enhance the concentration and productivity of 1, 3-PDO, citing salient features of each process.

For instance, Sun et al. (2019), before optimizing fermenter conditions, identified the composition of the microbial consortium DUT-08. The biochemical characterization of stable consortium, obtained after 35 repeatedly sub-culturing, revealed that the consortium mainly contained *Clostridium* (>85%), followed by *Escherichia* (>12%) and *Klebsiella* (>0.1%). On deciphering the mechanistic action, they found that *Escherichia* primarily depleted the oxygen content, facilitating *Clostridium's* growth and 1, 3-PDO production. Likewise, the tiny population of *Klebsiella* not only reduced O_2 levels but also contributed towards 1, 3-PDO production. Thus, the commensalism and synergism exhibited by microbes helped to attain high 1, 3-PDO titers. N_2 purging was minimal, and maintenance of strictly anaerobic conditions was not needed, despite *Clostridium* being predominant. In the same year, the feasibility of using soybean cake hydrolysate (SCH) as an N_2 source was evaluated for two strains of *Citrobacter freundii.* Under batch conditions, the effect of glycerol concentration and the free amino nitrogen (FAN) present in SCH was studied. Owing to higher susceptibility towards organic acids, *C. freundii* FMCC-8 accumulated less PDO (43.6 g L^{-1}) during fed-batch cultivation than *C. freundii* VK-19 (55.6 g L^{-1}). Later, the potentiality of both strains was assessed on CG as well (Maina et al., 2019). In another study, Wang et al. (2020) deciphered the right age and size of the inoculum of *Clostridium butyricum* DL07, to bypass the lag phase. Further, they found a linear relationship between the NaOH required to restore pH and glycerol consumption, which helped them develop a fully automated process for hassle-free 1, 3-PDO biosynthesis. During sequential fed-batch process, consistent 1, 3-PDO titers and productivities over eight cycles were demonstrated (Table 5a).

Compared to PG, more studies were conducted with CG in the past five years, as shown in Table 5b. For instance, Oh et al. (2018) created two *Klebsiella* mutants and optimized conditions (size of the inoculum, concentration of the N_2 source, cell recycling ratio, pH) during CG fermentation. As a result, a successful repeated fed-batch fermentation process was developed, which gave reproducible PDO titers and saved fermentation time. Among the two mutants, deleting an extra gene encoding for acetolactate synthase, responsible for 2,3 - BDO synthesis besides *ldh*, reduced 1, 3-PDO titers. Lower PDO titer resulted from the non-production of BDO, which regulates intracellular acidification and balances NADH/NAD+ within the cell (Lee et al., 2021). Likewise, Martins et al. (2020) adopted an efficient screening method wherein 16 variables were evaluated in the first stage of the Plackett-Burman design to obtain a low-cost, simple medium for 1, 3-PDO production from glycerol using *C. butyricum* NCIMB 8082. In the second stage, 11 variables were again screened, which reduced the media components to 7. The final media contained 1.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.3 g $MgSO_4.7H_2O$, 0.02g $CaSO_4.2H_2O$, 20 mg $FeSO_4.7H_2O$, and 1.0 g yeast extract (in 1 L distilled water), besides CG with 82.5% purity. Thus, they tactfully eliminated non-significant components of fermentation media. Later in a two-step anaerobic fermentation process, the batch mode

produced 28.38 g L^{-1} of 1, 3-PDO in merely 16.5h. When the fermentation was shifted to the fed batch, the maximum titer attained in 19.75 h was 73.07 g L^{-1}. Further, they also assessed the product cost using this simple media, which came out to be 31.46 USD/kg 1, 3-PDO. To further reduce the cost, when the same group used only three media components, namely KH_2PO_4, corn steep liquor (CSL) and CG, the batch process with the same organism produced 28.26 g L^{-1}, 3-PDO (Liberato et al., 2022).

Recently, Pan et al. (2019), developed a two-stage process for converting CG to 1, 3-PDO and PHB, which has not been cited in Table 5b, but its results are worth mentioning. Initially, the highly alkaline CG obtained from the biodiesel industry was reduced to a pH of 3.0, and most of its impurities were extracted using hexane. In the first phase, this pretreated CG was subjected to anaerobic digestion using a microbial consortium obtained from mangrove sediments. Glycerol was fed intermittently to circumvent the issue of substrate inhibition. In 5 days, ~499 g of glycerol was fed to the fermenter, of which 384.92 g were consumed, leading to an accumulation of 159.39 g of 1, 3-PDO with lactate, acetate, and butyrate as significant byproducts. Microbial community analysis showed that >75% of the population comprised *Clostridiales,* followed by *Enterobacteriales*. In the second stage, the residual glycerol, lactate, acetate, and butyrate produced in the first stage were exploited for PHB formation using a co-culture containing *Corynebacterium hydrocarbooxydans* ATCC 21767 and *Bacillus megaterium* DSM 90. This is a perfect example of biotransforming glycerol to two valuable products using PI during fermentation.
Even with the examples mentioned in Table 4, where new microbes and the microbial consortium were bio-prospected, the fermentation strategy was optimized to maximize 1, 3-PDO production. Researchers used either one variable at a time (OVAT) or response surface methodology (RSM), or a combinatorial approach to establish the PDO-producing capabilities of these natural producers (Zabed et al., 2019; Garg et al., 2020; Lan et al., 2021; Gupta et al., 2022). For instance, Garg et al. (2020) developed a dye-based method to monitor shake flask production of 1, 3-PDO using newly isolated *Citrobacter freundii* and optimized batch fermentation using the OVAT approach. Likewise, if Zhou et al. (2018) emphasized developing a continuous process from CG for their microbial consortium, Wang et al. (2020) focused on augmenting 1,3-PDO titer and productivity using a fed-batch process where *C. butyricum* DL07 was evaluated on both PG and CG. Thus, process intensification during fermentation is integral to any biotechnological process, providing a favorable environment for the microbes and maximizing product output.

4.6. Combinatorial approach

Besides the biotechnological tools mentioned in the preceding sections, the researchers are adopting combinatorial approaches, wherein each strategy significantly contributed to attaining high TYP matrices of the product. Some of these approaches are briefly discussed below:

4.6.1. Genetic engineering followed by co-culturing

A pertinent example of this category is the recent study, wherein the co-production of two chemical building blocks from glycerol, namely 3-HP and 1, 3-PDO, was targeted (Zhang et al., 2021c). A modular co-cultivation engineering strategy was developed for 3-HP and 1, 3-PDO -producing *Lactobacillus reuteri* and recombinant *E. coli* to accomplish high glycerol bio-conversion. The recombinant *E. coli* was created for 1, 3-PDO biosynthesis by over-expressing two genes, *aldH* or *gabD4* gene from *Cupriavidus necator*, encoding for NAD^+-dependent aldehyde dehydrogenase or PDOR and PudQ gene from *L. reuteri* encoding for $NADPH^+$ dependent PDOR. Further, for balancing the 1, 3-PDO pathway, the 5'-UTR region of *gabD4* gene was engineered. Later, the co-culturing strategy was optimized to attain high glycerol bioconversion and product titers amidst maintaining cell viability. The final devised method included initiating

biotransformation with 10 g L^{-1} of cells of dry weight basis and adding 20 g L^{-1} glycerol. Thereafter, 20 g L^{-1} glycerol and 3 g L^{-1} cells (on a dry weight basis) were fed every 2h. Thus, at the end of 51h, 214.39 g L^{-1}(125.93 g L^{-1} 3-HP and 88.46 g L^{-1} 1, 3-PDO) co-products were formed from 240 g L^{-1} glycerol. This approach improved glycerol consumption from 30 to 240 g L^{-1} and enhanced co-product titers from 25.11 to 214.39 g L^{-1} (Zhang et al., 2021c).

Likewise, in an earlier study, Yun et al. (2018) first over-expressed the *dhaT* gene from newly isolated *Clostridium buytricum* YJH-09 in *E. coli*. Later, by co-biotransforming *C. butyricum* YJH-09 whole cells with *BL21-dhaT*, the effects of substrate concentration, NADH level, and strain mass ratio were fine-tuned to attain high 1,3-PDO titers. The optimized conditions include 12-hour co-biotransformation, where 10 g of *C. butyricum* YJH-09 cells were mixed with 10g of *BL21-dhaT* cells, with initial glycerol concentration being 50 g L^{-1} and exogenous doping of 0.5 mM NADH was required. As a result, the mixed culture biosynthesized 25.88 g L^{-1}1, 3-PDO in 42 h, with the maximum yield occurring at 30 h.

4.6.2. Combined approach of genetic engineering and adaptive evolution

A recent US patent granted to Metabolic Explorer is an appropriate case where the said strategy was used (Tourrasse and Raynaud, 2021). In the said invention, extra copies of the vitamin B_{12} independent *dha* operon from *Clostridium buytricum* were introduced in *Clostridium acetobutylicum*, followed by adaption on high concentrations of industrial glycerol. When the adapted strain was evaluated under anaerobic continuous chemostat conditions with a feed medium containing 106 g L^{-1} glycerol and a dilution rate being 0.071 h^{-1}, it produced 51.5 g L^{-1}1,3-PDO at a yield and volumetric productivity of 0.49 g/g and 3.66 g/L.h, respectively with butyric acid as the major byproduct. The microbial consortium comprising of adapted strain, *Clostridium sphenoides*, and *C. sporogenes* under nearly identical conditions produced 52.9 g L^{-1} 1, 3-PDO with an insignificant impact on yield and productivity.

In their newest study, Yun et al. (2022) adopted two parallel approaches to enhance 1, 3-PDO titers in *Clostridium butyricum* YJH-09. Later, the best mutants obtained from both strategies were subjected to protoplast fusion to get the final strain. In the first approach, after identifying the maximum glycerol tolerance limit of the strain, *Clostridium* was subjected to ARTP mutagenesis for 150s, and screening was conducted on plates containing 140 g L^{-1}glycerol. Further subculturings were done by increasing glycerol titers to 150 and 160 g L^{-1}. Among all the mutants, AJH-35 and AJH-38 were shortlisted based on high biomass formation. In parallel to ARTP, *Clostridium butyricum* YJH-09 was subjected to ALE involving a continuous and automated microbial microdroplet culture (MMC) system. In this automated set-up, the concentration of 1, 3-PDO was gradually increased from 20 to 100 g L^{-1} in 30 days with five adaptation stages. Two potential candidates, MJH-49 and MJH-54, were shortlisted based on high biomass formation. Later, the mutants obtained from ARTP and ALE, were subjected to protoplast fusion. Final strain selection was made based on high biomass accumulation on medium containing 100 g L^{-1} 1 , 3-PDO and 5 g L^{-1} butyric acid. During batch fermentation, mutant strain *C. butyricum* GJH-418 displayed an optical density (OD_{600}) of 10.04 and produced 60.12, 17.45 and 7.65 g $L^{-1}$of 1, 3-PDO, butyric acid and acetic acid, respectively, from 120 g L^{-1} initial glycerol. Under identical conditions, the wild strain produced 12.32, 2.12, 1.12 g L^{-1} 1, 3-PDO, butyric acid, and acetic acid, respectively, with $OD_{600}$of merely 1.45.

4.6.3. Improved glycerol bioconversion using metabolic and bioprocess engineering

Among the latest developments, mentioning a recombinant *E. coli* strain which co-produced 3-HP and 1, 3-PDO from glycerol with a molar yield of 0.85 is noteworthy, as it rationally used both genetic and

bioprocess engineering to obtain 140 g L^{-1}product titers (Zhang et al., 2023). In this study, *E. coli* W3110 (DE3) was chosen as the host organism, where the entire *dhA* operon was constructed. Further, *gabD4* and *ydhD* genes were over-expressed, encoding for two aldehyde dehydrogenases. These enzymes selectively promoted the accumulation of 3-HP and 1, 3-PDO. Both genes' 5' UTR regions were optimized, and cofactor supply was balanced by the over-expression of the membrane-bound transhydrogenase (*PntAB*) gene and inactivating soluble transhydrogenase (*SthA*) gene. Thereafter, the pathway was rebalanced by choosing suitable plasmids with varying strengths of replicons for gene expression.

Further, disrupting *ptca-ackA* and the glycerol repressor gene followed by modulation of glycerol flux resulted in a recombinant strain EC10S7G. During the bioprocess development, the suitability of replacing yeast extract (YE) with CSL and the effect of a pH-controlled environment were evaluated. Fortification with 0.25% CSL was a compatible replacement for supplementing with 0.1% costly YE. They further concluded that a two-stage pH-controlled fermentation was most appropriate, in which a neutral pH in stage I promoted cell growth and provided a conducive environment for the over-expression of genes coding for enzymes responsible for 3-HP and 1, 3-PDO formation. Once the said task was accomplished, the pH was raised from 7 to 8, accelerating the metabolite production. During fed-batch cultivation, the recombinant strain, after 66 h, accumulated 77.34 and 63.16 g L^{-1} of 3-HP and 1,3-PDO as co-products and a productivity of 2.13 g/L.h. However, the authors identified that using IPTG, antibiotics, and an exogenous vitamin B_{12} source were prominent barriers towards making the product cost-competitive. But, their commitment towards improvising the process is appreciated.

Most of the strategies and tools discussed above have been primarily targeted to address two bottlenecks that hinder exploiting the glycerol platform for the biotechnological production of 1, 3-PDO. The first is the reduction of byproducts without creating a redox imbalance so that maximum glycerol flux is diverted towards 1, 3-PDO production. The second aspect is overcoming the inherent issues of microbes, such as substrate and product inhibition and the ability to grow on real-time substrates.

5. Author's perspective and future outlook

Since Metabolic Explorer France has already proven the commercial viability of exploiting the glycerol platform for 1, 3-PDO production, more technologies are anticipated to flourish, harnessing this feedstock either in its pure form or its industrial version (mostly in the biodiesel industry). Microbial bioprospecting and bioprocess development studies from this review doubtlessly indicate that *Clostridium* spp. is a promising and competent workhorse for the biotechnological production of 1, 3-PDO-using a glycerol platform. One strain *C. butyricum* DL07 draws significant attention owing to its remarkable 1, 3-PDO titers and productivity, isolated from activated sludge of anaerobic digestor (Wang et al., 2020). During fed-batch fermentation, the strain produced 104.8 and 94.2 g L^{-1} 1, 3-PDO from PG and CG, with productivity being 3.38 and 3.04 g/L.h, respectively. The titer and productivity obtained by this strain seem industrially competitive, considering glucose-derived 1, 3-PDO (135 g L^{-1}; 3.5 g/L.h) from recombinant *E .coli* as the benchmark (Zhu et al., 2021). Two years later, the same group developed a novel integrated bioprocess to reduce the CO_2 emissions produced from the oxidative branch of glycerol, enhance the relative production of H_2, 1,3-PDO titer and productivity of C. *butyricum* DL07 (Wang et al., 2022b). When they replaced 5M NaOH with 5M $Ca(OH)_2$ as a pH regulator, the latter proved to be a better candidate for *in-situ* capturing of CO_2 and simultaneously produced stable micro-nano (300 nm to 20μm) calcite ($CaCO_3$) particles, which find numerous applications in varied sectors. With CG as starting feedstock, under the fed-batch mode, the maximum 1, 3-PDO titer and productivity attained were 88.1 g L^{-1} and 5.54 g/L.h, respectively. Moreover, there was a notable improvement in the relative concentration of H_2 to CO_2. Such a remarkable

performance by wild and natural 1, 3-PDO producing microbial strains prove that consistent and rigorous efforts are being made across the globe to develop alternate technologies for production of glycerol-derived 1, 3-PDO.

PI studies discussed in this review article indicate an upsurge in using CG as a starting feedstock, speculating that industrial fermentations will be governed by CG shortly. However, from quality control viewpoint, conducting detailed compositional analysis of CG as primary input material will remain vital, as it prominently affectsthe microbial growth and fermentation. Though the pretreatment of industrial glycerol is an alternative way to eliminate various impurities, it adds an extra step and cost to the overall process. Likewise, examples cited in Table 3 prove that ALE can become a vital tool for acquiring sturdy bacterial strains where industrial glycerol is the starting feedstock, as high 1, 3-PDO titers and productivity are reported after adopting ALE approach.

The recent metabolic engineering approaches reveal that 1, 3-PDO yield could be maximized by pushing maximal glycerol carbon towards the reductive pathway with minimal flux through the oxidative pathway. To achieve the maximum theoretical yield, out of every eight moles, seven should be used for PDO production, while only one should pass through the oxidative route to generate seven NADH via the glycolytic pathway and TCA cycle as well as a sufficient number of ATP molecules. This requires very tight control of glycerol flow, which can be achieved with amplification of the reductive pathway and rationale engineering of the oxidative route. Further in case of microaerobic or aerobic bacteria, the fine tuning of oxygen levels is highly important, as it governs the activity of several enzymes in oxidative and reductive branch, indirectly governing the glycerol flux for 1,3-PDO biosynthesis. In this regard, the exceptional performance of non-native, aerobic and Vitamin B_{12} producing *Pseudomonas denitrificans* ATCC 13867 is worth mentioning (Zhou et al., 2019). In the said study, the authors achieved a maximum (1,3-PDO) molar yield of 0.92 after its rationale genetic engineering. This study will surely stimulate the researchers to explore both aerobic and anaerobic microbial strains which can biosynthesize Vitamin B_{12} and create a synthetic pathway in such way that maximum glycerol flux is diverted for 1, 3-PDO production.

Alternately, many researchers have brilliantly used heterofermentative production of 1, 3-PDO production from glycerol to their advantage by focusing towards co-product biosynthesis as well. The co-production of commercially interesting products like lactic acid or 3-HP at industrially relevant titers along with 1,3-PDO has been successfully demonstrated as per latest trending research (Jiang et al., 2021; Zhang et al., 2021c; Zhang et al., 2023). Further, researchers have prudently employed novel approaches to reduce the toxicity levels of organic acids (acetic acid, lactic acid, butyrate) by diverting them towards biosynthesis of non-toxic and valorized products (such as PHB and esters of acetate and butyrate), that can be easily separated and purified from the aqueous fraction post-fermentation (Pan et al., 2019; Wang et al., 2021a; Zhang et al., 2022).

Earlier researchers relied largely on altering the gene transcription, but lately precise control over mRNA translation is becoming increasingly becoming popular.For instance, engineering of 5'UTR region is an uptrend (Lee et al., 2018; Zhang et al., 2021c; Zhang et al., 2023). This is the regulatory region of DNA and in mRNA it is found upstream from initiation codon, containing the ribosomal binding site (RBS), thereby controlling the translational efficiency and hence the protein expression. Use of advance bioinformatics tools such as translational rate calculators can predict the initiation rate of mRNA's translation, thereby providing useful data on how to modulate the translation of mRNA. Depending on whether the protein expression has to be reduced or enhanced, the 5'UTR sequence of interested genes are being optimized. Likewise, CRISPR/Cas-derived genome editors have revolutionized the area of molecular biology as they

introduce variety of mutations in the genome including insertions, deletions, or scar-less single-nucleotide substitutions simultaneously, without leaving disagreeable and off-target impacts. However, this tool requires designing of highly specific and efficient single guide RNA (sgRNA) so that it targets only desired DNA sequences. Based on mechanism, CRISPR/Cas technology is classified into three categories: (a) gene editing wherein deletions and insertions can be done and their fate (precise or random) depends on whether the DNA repair is homology-directed or not (b) interference wherein catalytically inactive Cas9 (dCas9)-sgRNA complex controls protein expression either by interfering during initiation or elongation of transcription (c) activation in which dCAs9 is fused with transcriptional activator, guided to the target by sgRNA, enhancing transcriptional efficiency (Tian et al., 2017). This high-precision and accurate gene editing tool was for the first time used for over-expressing *dhaT* gene and simultaneously attenuating *ldhA* and *budC* genes in *Klebsiella* as demonstrated by Wang et al. (2022a) to enhance 1,3 PDO titers. The study showcases the versatile applications of CRISPR-Cas9 tool such as therapeutics, agriculture, biorefining etc. Likewise, recent optimization by Li et al., (2020) to genetically manipulate *C. diolis* strain have opened newer avenues for this Gram-positive bacterium, including some industrially important and natural 1, 3-PDO producers such as *Lactobacillus*.

Moreover, researchers have also realized that enhanced glycerol conversion yields would rise to a certain degree by adopting a single approach and will soon attain a plateau. But the combinatorial approach can lead to an exponential rise in achieving high TYP matrices for 1, 3-PDO. A bold attempt to experiment with the microbial consortium for glycerol bioconversion reveals that during long-term fermentation, the composition of the microbes becomes more or less consistent (Zhou et al., 2018; Sun et al., 2019; Jiang et al., 2021). Hence, they are equally competent as pure cultures for glycerol bioconversion to 1, 3-PDO but also require necessary validation at a pre-commercial level.

Like, lignocellulosic bioprocessing industry, exploitation of glycerol platform for 1,3 PDO biosynthesis also demands techno-economic analysis (TEA), as it is one of the governing factors considering commercial aspects. In the past five year, there is only one isolated study that attempted to decipher the cost of glycerol-derived 1,3-PDO (Martins et al., 2020). In the future, researchers need to conduct detailed TEA, as it helps in identifying the major and significant cost contributors to the process which require immediate attention, so that overall cost of the process can be reduced. Likewise, the minimum hot and cold utility requirements for promising processes need to be spotted and methodologies like pinch analysis should be adopted to minimize energy requirements. Similarly, life-cycle assessment (LCA) should be performed, so as to decipher process lacunas which disfavor process environmental sustainability and simultaneously establish the benefits offered by processes offering glycerol-derived 1, 3-PDO.

6. Concluding remarks

Recent advancements indicate that a sturdy and infallible framework must be created for commercial exploitation of glycerol platform for 1, 3-PDO production. Green biotechnological processes such as bio-based 1,3 PDO production from glycerol can be converted into a profitable business proposition only when a technology is developed by attuning metabolic, evolutionary, and bioprocess engineering strategies after selecting a commercially scalable microbial strain. Presently, there are some promising microbial strains capable of matching the TYP matrices equivalent to commercial glucose-based 1, 3-PDO and the growth prospects of biodiesel industry also look bright. Hence, it is the right time to implement the concept of circular economy for harnessing glycerol platform, which advocates the concept of sustainability, waste minimization and value-chain creation. Critical and timely interventions from industry are highly desirable so

that research innovations in the area of in glycerol bio-valorization to 1, 3-PDO can be successfully translated into technologies.

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Authors' contributions

Deepti Agrawal: conceptualization, writing-original draft, supervision; Mridul Budakoti: writing-original draft, reviewing & editing; Vinod Kumar: conceptualization, reviewing & editing. All the authors read and approved the final manuscript.

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Figure Captions

Figure1: Key metabolic pathways for glycerol valorization to 1,3 - propanediol in various bacteria

Figure 2: Reductive pathway for glycerol metabolism in prokaryotes.

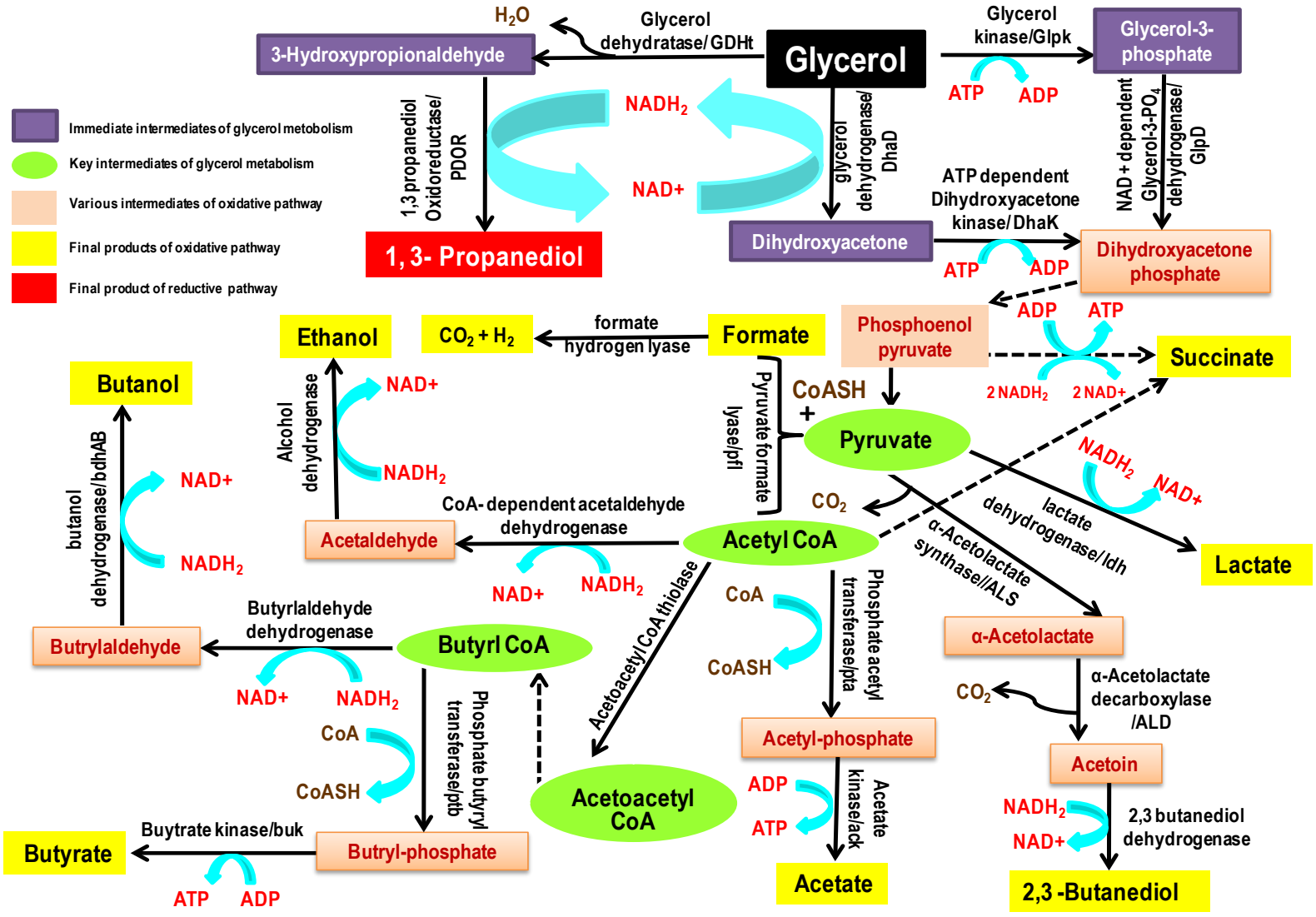
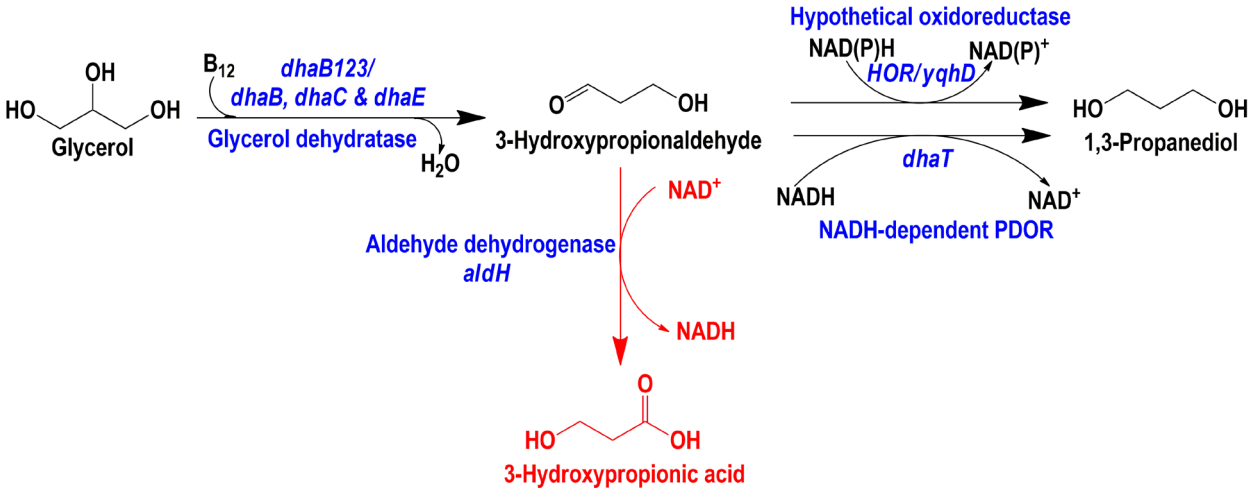


Figure1: Key metabolic pathways for glycerol valorization to 1,3 - propanediol in various bacteria



$C_3H_8O_3$ (Glycerol) + NAD(P)H + $H^+ \rightarrow$ $C_3H_8O_2$ (1,3-Propanediol) + $NAD(P)^+$ + H_2O

Figure 2: Reductive pathway for glycerol metabolism in prokaryotes

Table 1: Detailed account of genes and proteins/ enzymes associated with oxidative and reductive metabolism of glycerol

Genes	Proteins/ enzymes encoding for	Protein/ Enzyme functioning	Reference
dhaB1	Glycerol dehydratase	Catalyzes glycerol dehydration to 3-hydroxypropionaldehyde (3-HPA)	Jiang et al., 2016
dhaB2	Activator protein for glycerol dehydratase	Promotes activity of glycerol dehydratase	
dhaA/ dhaT / pudQ	1,3 - propanediol oxidoreductase	Catalyzes reduction of 3-HPA to 1,3- PDO	
*gdrA*and *gdrB*	Glycerol dehydratase reactivase	Facilitates replacing inactive with catalytically competent cobalamin in GDHt	
yqhD	NADH dependent aldehyde dehydrogenase in E. coli (isoenzyme of PDOR)	Owing to its broad substrate specificity, it reduces 3-HPA to 1, 3-PDO	Chen and Liu et al., 2016
gabD4	NAD^+ dependent aldehyde dehydrogenase in *Cupriavidus necator*	Owing to its broad substrate specificity, it reduces 3-HPA to 1, 3-PDO	Zhang et al., 2023
glpF	Glycerol facilitator aquaglyceroporin	The protein catalyses trans-membrane diffusion of glycerol	Sato et al., 2021
dhaR	Transcriptional activator protein for dha operon	Activates the expression of genes present in *dha* operon	Lee et al., 2018
dhaD/ gldA	Glycerol dehydrogenase	Catalyzes dehydrogenation of glycerol to form dihydroxyacetone (DHA)	Jiang et al., 2016
dhaKLM / dhaK	Dihydroxyacetone kinase (DHAK)	Catalyzes phosphorylation of DHA to dihydroxyacetone phosphate (DHAP)	
glpK	Glycerol kinase	Catalyzes phosphorylation of glycerol	Lee et al., 2018
glpD	Glycerol-3-phosphate dehydrogenase	Catalyzes formation of dihydroxyacetone phosphate (DHAP)	Jiang et al., 2016
arcA	Transcriptional regulator of TCA cycle	Represses expression of genes involved in TCA cycle under oxygen limited conditions	Lee et al., 2019
pflB	Pyruvate formate lyase or formate acetyltransferase	In a bi-substrate reaction, pyruvate reacts with Coenzyme A to form formate and Acetyl CoA	Oh et al., 2018
ldhA	Lactate dehydrogenase	Involved in formation of lactate from pyruvate	
Als/ budB	Acetolactate synthase	Catalyzes α-acetolactate formation from pyruvate with CO_2 as a by-product	
Adc/ budA	Acetolactate decarboxylase	Decarboyxlates α-acetolactate to form acetoin	Zhu et al., 2022
budC	2,3 Butanediol dehydrogenase	Catalyzes dehydrogenation of acetoin to form 2,3-butanediol (2,3 - BDO)	Yang et al., 2018; Zhu et al., 2022
adhH	Acetaldehyde dehydrogenase	Catalyzes acetaldehyde formation from pyruvate	
adhE	Ethanol dehydrogenase	Catalyzes ethanol formation from acetaldehyde	
poxB	Pyruvate oxidase or dehydrogenase	Catalyzes oxidative decarboxylation of pyruvate to acetate and CO_2	Lin et al., 2016; Zhu et al., 2022
pta *ackA*	Phosphotrans-acetylase Acetate kinase	Catalyzes acetate formation from Acetyl Coenzyme A	
frdABCD	Fumarate reductase	Catalyzes succinate formation from fumarate	Zhu et al., 2022
aceA	Isocitrate lyase	This cytosolic enzyme catalyzes the splitting of isocitrate to form succinate and glyoxylate	Wang et al., 2021a

Table 2: Genetic interventions for enhanced 1, 3-PDO production from glycerol by various microbes

Microbe used	Salient Features of the study	Mode of fermentation	1, 3-PDO T	1, 3-PDO P	1, 3-PDO MY	Reference
Klebsiella pneumoniae	• Strain with *ΔldhA*, *ΔpflB*, *ΔbudA, ΔglpK, ΔdhaD* constructed • Transcriptional factors encoding for *dha* operon optimized • Mannitol co-fed with glycerol & no 2, 3- BDO formed	Batch	20.59	0.86	0.76	Lee et al., 2018
E. coli JA11	• In *E. coli* JA03, heterologous $NADP^+$ dependent glycerol-3-phosphate dehydrogenase pathway constructed and fine-tuned. Glucose added for cell growth. • PEP dependent glucose transport disrupted but ATP dependent transport over-expressed	Batch	13.47	0.224	0.64	Yang et al., 2018
Pseudomonas denitrificans	• Host chosen, as it produced Vitamin B_{12} under aerobic conditions • Genes from *Klebsiella pneumonia dhaB1*, *dhaB2, dhaT, gdrA, gdrB* heterologously over-expressed under constitutive synthetic promoter • Strain with *ΔaldH13, ΔnuoA, Δptc-ackA* constructed	Fed-batch	33.4	0.656	0.89	Zhou et al., 2019
E. coli	• *dhaB1* and *dhaB2* over-expressed in *E.coli* Rosetta (DE3) strain • *E. coli* BL21-*dha*T co-cultured with recombinant *E. coli* Rosetta (DE3) in the ratio of 1.5:1 and glucose co-fed along with glycerol in the ratio of 1:8	Batch	41.65	0.69	0.67	Yun et al., 2021
Vibrio natriegens	• Strain with *Δldh*, *ΔadhE*, *Δpfl, Δpta-ackA, ΔaldA/ aldB, ΔfrdABCD* constructed • Genes encoding for transcriptional regulators: arcA and glpR deleted • Cofactor engineering done to enhance NADPH supply	Fed-batch	56.2	2.36	0.61	Zhang et al., 2021a
	• *glpD* gene expressed in plasmid to enhance its stability		69.5	2.89		Zhang et al., 2021b
K. pneumoniae	• Strain with *Δ ldhA*, *ΔadhE*, *Δ frdA,ΔaceA* • *"acs"* gene heterologously over-expressed from *Acetobacter pastoris* under constitutive promoter and PHB pathway introduced	Fed-batch	91.2	3.06	0.59	Wang et al., 2021a
K. pneumoniae	• Using CRISPR-dCas9 system, *dhaT* gene over-expressed under constitutive promoter P32 and genes coding for lactate and 2, 3-BDO formation attenuated	Batch	57.85	1.44	-	Wang et al., 2022a
Citrobacter braakii	• Pathways for lactate and formateformation disrupted • Corn steep liquor (CSL) used as sole nitrogen source during fermentation	Fed-batch	60	-	-	Alawi et al., 2022
Clostridium diolis	• *Clostridium diolis* DSM 15410 and shuttle plasmid pXY1-PCA_C102 chosen • Plasmid DNA was pre-methylated to prevent its degradation • Electroporation protocol & appropriate medium for transformant selection devised and *aldH* gene over-expressed	Batch	13.2	0.137	-	Li et al., 2020

Note: Genes encoding for enzymes *ldh*- lactate dehydrogenase; *pfl*- pyruvate formate lyase; *budA- α-acetonelactate decarboxylase; glpK*- glycerol kinase; *dha* operon- genes encoding for 1, 3-PDO biosynthesis; *dhaD*- glycerol dehydrogenase; *dhaT*-1,3 - propanediol oxidoreductase;*dhaB1*- glycerol dehydratase; *dhaB2*- glycerol dehydratase activation factor*; gdrA and gdrB* - glycerol dehydratase reactivase; *aldH13*- aldehyde dehydrogenase catalyzing formation of 3-hydroxypropanoic acid from 3 hydroxypropianaldehyde*;* nuoA-NADH dehydrogenase Type I of electron transport chain (ETC); *pta-ackA*- phosphate acetyltransferase *and* acetate kinase; glpR-transcriptional repressor controlling glycerol utilization; aldA/aldB- aldehyde dehydrogenase resulting in formation of 3-hydroxypropanoic acid; *frdABCD*- fumarate reductase; arcA- transcriptional repressor for genes of ETC and depressor for genes for TCA cycle favouring NADPH accumulation; *adhE*-alcohol dehydrogenase*; aceA*- isocitrate lyase; *acs*- Acetyl-CoA- synthetase; Δ– deletion; *aldH*; aldehyde dehydrogenase; T-titer in g L^{-1}; MY- molar yield; P- productivity in g/L.h.

Table 3: State of the art where ALE was adopted for enhanced bioconversion of glycerol to 1, 3 PDO

Microbe obtained	Selection criteria	Strategy adopted and salient features of the study	Outcome of the study	Reference
Clostridium butyricum 7th generation	Faster growth and acid tolerance	• Two-stage adaptation strategy used • In stage I, wild strain adapted up to 110 g L^{-1} glycerol • In stage II glycerol adapted strain screened for butyric acid (20 g L^{-1}) tolerance • 50 sub-culturing done for obtaining stable adapted strain	• Compared to wild strain, the fermentation time for adapted strain reduced from 36 to 20h. • The productivity of 1,3-PDO increased from 0.97 to 2.14 g/L.h. • In a 5 and 50 L fermenter, 66.23 and 61.7 gL^{-1} 1,3-PDO was produced in 48 & 28h respectively, under fed-batch mode	Zhang et al., 2019
Klebsiella pneumoniae x546	High substrate tolerance	• ALE conducted with *K. pneumonia* ATCC 15380 • Gradual adaptation at 120g/L glycerol, beginning with 40 g/L and 20g/L rise at each stage. • Acclimatized strain at 120g/L glycerol re-domesticated at 20g/L conc. • During fermentation betaine counteracted the high osmotic pressure created by Na_2CO_3 as neutralizing agent	• In 7.5L fermenter, the adapted strain showed 1.5-fold higher 1,3-PDO titers compared to parent • Betaine addition reduced the fermentation time by ~40% • Best titer attained was 74.44 g L^{-1} 1, 3-PDO in 24h • Tandem mass tags provided clear evidence that several genes involved in 1, 3-PDO biosynthesis were up-regulated in adapted strain.	Wang et al., 2021b
Clostridium pasteurianum	Tolerance towards high substrate and CG toxicity	• Continuous ALE with increasing CG (30 to 120 g L^{-1}), using automated method and real-time measurement of optical density • Fermentation performed under non-sterile conditions without N_2 purging & yeast extract supplementation. • Acetate and butyrate produced as by-products converted to esters to ease downstream processing	• Adapted strain showed no lag phase • In a 1 KL fermenter, under fed-batch mode, 81.21 g L^{-1} 1, 3-PDO produced from CG • The 1, 3-PDO yield and productivity of the adapted strain was 0.49 g g^{-1} and 4.27g/L.h, respectively.	Zhang et al., 2022

Table 4: Glycerol assimilating and 1, 3-PDO producing microbes/microbial consortium obtained by bioprospecting

Microbe or Microbial consortium	Sitechosen for bioprospecting	Feed type	Mode of fermentation	1, 3-PDO			Reference
				T	P	Y	
Clostridium butyricum YJH-09	Pond Soil	PG	Batch	11.72	-	-	Yun et al., 2018
Lactobacillus reuteri FXZ014	Feces of infant		Batch	9.94		0.55	Zabed et al., 2019
Klebsiella pneumoniae 2e	Biodiesel-derived waste contaminated soil		Batch	12.16	1.01	0.586	Ma et al., 2019
Citrobacter freundii IIPDR3	Solvent Storage Site		Batch	9.85	0.82	0.54	Garg et al., 2020
Clostridium butyricum DL07	Active sludge from anaerobic digester		Fed-batch	104.8	3.38	0.54	Wang et al., 2020
Clostridium butyricum SCUT343-4	Deep soil of Daling Mountain		Batch	45.35	0.47	0.41	Lan et al., 2021
Microbial consortium C2-2M	Anaerobic Sludge	CG	Continuous	57.86	5.55	-	Zhou et al., 2018
Klebsiella pneumonia 2e	Biodiesel-derived waste contaminated soil		Batch	10.28	1.01	0.53	Ma et al., 2019
Microbial consortium LS30	Activated sludge from sewage treatment plant		Batch	13.22	0.85	-	Wang et al., 2019
Clostridium butyricum DL07	Active sludge from anaerobic digester		Fed-batch	94.2	3.04	0.52	Wang et al., 2020
Clostridium butyricum L4	Biogas reactor leachate		Fed-batch	70.1	0.46	0.54	Gupta et al., 2022
Microbial consortium CJD-S	Intertidal sludge from sea		Fed-batch	41.47	1.15	0.28	Jiang et al., 2021

Note: PG- Pure glycerol; CG; Crude Glycerol; T-titer in g/L; Y- yield (g/g glycerol); P- productivity in g/L.h.

Table 5a: Process intensification during fermentation using pure glycerol (PG) as carbon feedstock

Microbe used	Salient Features of the study	Fermentation type	1, 3-PDO Conc (g/L)	1, 3-PDO Productivity (g/L/h)	Reference
Microbial consortium DUT-08	• Effect of N_2 purging studied • N_2 purging 1h before and 2h after inoculation enhanced 46% 1, 3-PDO yields	Batch	61.49	2.46	Sun et al., 2019
Citrobacter freundii VK-19	• Feasibility of using soybean cake hydrolysates as N_2 source assessed for 1, 3-PDO production • Initial glycerol concentration: 50g/L	Fed-batch	55.6	0.99	Maina et al., 2019
Citrobacter freundii FMCC-8	• Feasibility of using soybean cake hydrolysates as N_2 source assessed for 1, 3-PDO production • Initial glycerol concentration: 20g/L	Fed-batch	43.6	1.01	
Clostridium butyricum DL07	• Cells in exponential phase harvested from first bioreactor and transferred to second reactor. • To avoid lag phase, new cycle initiated by adding 2% biomass from 10h old culture • Total eight cycles demonstrated. • CSL used as cheap N_2 source	Sequential Fed-batch	85€	3.1€	Wang et al., 2020
C. beijerinckii CCIC 22954	• Fermentation parameters optimized using OVAT approach • Optimal conditions: Glycerol concentration-30 g/L; Temperature-37°C; Initial pH-7.5; Size of the inoculum-4%	Batch	14.3	0.30	Fokum et al., 2021b
Citrobacter freundii AD119	• Fermentation parameters optimized using OVAT and 2-stage statistical approach • Among all the components, mineral salts namely $CoCl_2$ and $MgSO_4$ were most critical. • Yeast extract emerged as the best N_2 source, which could be partially replaced by $(NH_4)_2SO_4$	Fed-batch	41.7	0.868	Drożdżyńska et al., 2023

Note: CSL- Corn Steep Liquor; € denotes the average values of eight cycles

Table 5b: Process intensification during fermentation using crude glycerol (CG) as carbon feedstock

Microbe used	Salient Features of the study	Fermentation type	1, 3-PDO		Reference
			Conc (g/L)	Productivity (g/L/h)	
Mixed microbial consortia (MMC)	• *Clostridium* relatively abundant (>34%) • HRT, pH and FC optimized using statistical approach • Optimized conditions: pH- 6.4; HRT- 17.6h and FC- 49.3 g/L	Continuous and Non-sterile	20.7	3.44	Varrone et al., 2018
Recombinant *Klebsiella pneumonia* (Δ{ldhA, als})	• Fresh medium replaced in the ratio of 1:10 after every 24h • 1% CSL used in fermentation medium and pH maintained at 6.5	Repeated Fed-batch	54.95¥	2.29¥	Oh et al., 2018
Recombinant *K. pneumonia* (ΔldhA)	• Fresh medium replaced in the ratio of 1:10 after every 24h • 1% CSL used in fermentation medium and pH stat conditions maintained at 6.0	Repeated Fed-batch	79.96β	3.33 β	Oh et al., 2018
Citrobacter freundii VK-19	• Feasibility of using CG SCH assessed for 1, 3-PDO production • Initial glycerol concentration: 50g/L	Fed-batch	47.2	0.73	Maina et al., 2019
Citrobacter freundii FMCC-8	• Feasibility of using CG and SCH assessed for 1, 3-PDO production • Initial glycerol concentration: 20g/L	Fed-batch	35	0.53	Maina et al., 2019
Lactobacillus reuteri CH53	• Un-aerated conditions and agitation at 100 RPM favored high 1, 3-PDO biosynthesis • CSL, as N_2 source up-regulated the GDHt and 1, 3-PDO R enzyme activities	Fed-batch	68.32	1.27	Ju et al., 2020
C. butyricum NCIMB 8082	• 2-stage Plackett- Burman design used to identify critical media components of fermentation including pH • Two step batch followed by fed-batch anaerobic fermentation performed	Combination of batch and fed-batch	42.7	2.63	Martins et al., 2020
Klebsiella pneumonia BLh-1	• Bacteria entrapped in the permeable support of polyvinyl alcohol • Compared to PB, FB bioreactor configuration gave better yields. • FC at 65g/L fed at DR of 0.33 h^{-1}, without pH control gave best results	Continuous	13.6	4.48	Damasceno et al., 2022
C. butyricum NCIMB 8082	• Fermentation parameters optimized using statistical approach • KH_2PO_4 medium containing CG and CSL was used during fermentation	Batch	28.26	1.71	Liberato et al., 2022

Note: ¥ denotes average of four cycles;βdenotes average of five cycles; (Δ{ldhA, als}) deletion of lactate dehydrogenase and acetolactate synthase gene encoding for lactate and 2,3 - BDO production respectively; ΔldhA- lactate dehydrogenase deficient mutant; GDHt- Glycerol dehydratase; 1, 3-PDO R- 1,3 - propanediol oxidoreductase; FC- Feed concentration; HRT-Hydraulic retention time; CSL- Corn Steep Liquor; PB- Packed Bed; FB- Fluidized bed; DR- Dilution rate; CG- Crude Glycerol; SCH- Soybean cake hydrolysate