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1 **Strategies and tools for the biotechnological valorization of glycerol to 1, 3-propanediol:**
2 **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

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

84 However, Dupont, in the year 2003, developed a commercial bio-based 1,3 PDO fermentation process in
85 collaboration with Genencor and Tate & Lyle group, emerging as the biggest 1,3 PDO producer globally
86 (Zhou et al., 2023). Until 2021, three global players were producing commercial bio-based 1, 3-PDO: US-
87 based Dupont Industry in collaboration with Tate & Lyle Group, who shared their technical know-how with a
88 Chinese company named Glory Biomaterials, and Shenghong Group Holdings Ltd., China (Biorefineries
89 blog, 2021; Market Watch 2022). Commercial bio-based 1, 3- PDO primarily exploits corn-based glucose
90 as its feedstock. It is also a hard fact that, currently, most microbial fermentations exploit glucose to
91 produce a variety of bio-based chemicals. The main reasons include preferential glucose uptake, easier
92 assimilation, efficient biotransformation, and a simple and straightforward scale-up process. However, in
93 2021, France-based METEX NØØVISTA, which is a joint venture of Metabolic Explorer and Société de
94 Projets Industriels, began their first commercial bio-based 1, 3-PDO production using glycerol as the
95 starting substrate (Biorefineries blog, 2021; Metabolic Explorer press release 2021).
96 Considering the optimistic future of the biodiesel industry and the recent commercialization of the glycerol
97 platform for 1, 3-PDO production has motivated and accelerated the research pursuits in this area. The
98 present review discusses the latest (last five years) developments, where microbial bioprospecting was
99 done in the hope of obtaining potential but natural 1, 3-PDO producers and different biotechnological
100 strategies were adopted to enhance efficient biotransformation of glycerol to 1,3-PDO, showcasing
101 promising leads, that could be translated into alternate industrially-deployable technologies. Before
102 discussing them in detail, a background of natural, predominant glycerol assimilating and 1, 3-PDO
103 producers, the key metabolic pathways involved, and genes regulating those pathways is given, and
104 technical hurdles for commercially exploiting microbial routes are summarized. The review concludes with
105 key takeaways from the emerging trends in glycerol valorization to 1, 3-PDO via the microbial route, along
106 with the future outlook in the said area.

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

108 Only prokaryotes have the natural ability to produce 1, 3-PDO from glycerol, and it is one of the oldest
109 known fermentation products. The first evidence of glycerol biotransformation to 1, 3-PDO goes back to
110 1881 when Freund attempted to biosynthesize *n*-butanol from soap lyes (Werkman and Gillen, 1932).
111 Though he did not thrust on microbial characterization, the spore-forming microbe was later identified
112 as *Clostridium pasteurianum*, as exclusively reviewed by Zhu et al. (2021). Later, in 1928, Braak isolated a
113 bacterium from the canal that produced 1, 3-PDO from glycerol in alkaline conditions. It exhibited
114 characteristics similar to the "coli-aerogens" group and was named "*Bacterium freundii*". However, three
115 years later, Werkman and Gillen (1932) carried out the exhaustive biochemical characterization of this
116 bacterium, which showed some metabolic features specific to *Escherichia*, while some belonging
117 to *Aerobacte*r. This organism with intermediate characters was allocated a new genus, *Citrobacter.* It is a
118 Gram-negative, non-sporulating, rod-shaped bacterium that grew well on citrate agar and reduced nitrates.
119 The organism was re-designated as "*Citrobacter freundii*". Thereafter, several naturally 1, 3- PDO
120 producing microbes were isolated and reported, predominantly belonging to the *Clostridiaceae* and
121 E*nterobacteriaceae* families. According to the two recent reviews by Sun et al. (2018) and Zhu et al. (2021),
122 1, 3-PDO natural producers from glycerol can be broadly categorized into two types: the abundant and

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

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

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

157 The oxidative metabolism of glycerol provides all the ingredients for cell mass formation, including energy
158 in the form of ATP and other cofactors. In the oxidative route, glycerol is metabolized to dihydroxyacetone
159 phosphate (DHAP) by the respiratory (aerobic/anaerobic) and/or fermentative pathways (Fig 1). The genes
160 for respiratory and fermentative metabolism of glycerol are contained in the *glp* and *dha* regulons,
161 respectively. The respiratory pathway begins with glycerol phosphorylation to glycerol-3-phosphate which is
162 catalyzed by glycerol kinase (GlpK), followed by its oxidation to DHAP. The latter reaction is catalyzed by
163 glycerol-3-phosphate dehydrogenase (aerobic GlpD or anaerobic GlpABC). However, during fermentation
164 (microaerobic/ anaerobic conditions), glycerol is dehydrogenated to form dihydroxyacetone (DHA) in the
165 presence of an enzyme called glycerol dehydrogenase (DhaD), with simultaneous reduction of NAD+ to
166 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

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

220 *3.2. Partitioning of glycerol, formation of byproducts and slow/less active TCA cycle*

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

231 Most of the byproducts are generated through the PEP/pyruvate pathway during the oxidative metabolism
232 of glycerol. However, their pathway elimination creates an imbalance between the glycolytic pathway and
233 the TCA cycle. The latter route is inactive under oxygen-limited and anaerobic conditions favoring PDO
234 accumulation. This imbalance gives rise to the excretion of pyruvate and other metabolites such as DHA,
235 DHAP, glyceraldehyde-3-phosphate, methylglyoxal etc., which are very toxic for the cell (Westbrook et al.,
236 2019; Chen and Liu, 2016). For instance, lactic acid and 2, 3-BDO are the primary products of oxidative
237 glycerol metabolism by *K. pneumoniae*. Kumar et al. (2016) engineered *K. pneumoniae* to eliminate lactic
238 acid and 2, 3-BDO formation, but this triggered heavy carbon-metabolic traffic at the pyruvate node and did
239 not improve PDO production. Pyruvate is a toxic metabolite due to its low pKa (2.5), and its accumulation
240 can give rise to the piling up of glycolytic pathway intermediates, which are more toxic than pyruvate. Their
241 study indicated that sometimes knocking out genes that encode oxidative products seems straightforward;
242 however, it sometimes yields an undesirable and a different impact. Therefore, genetic interventions
243 demand rational engineering of pathways downstream pyruvate as this node is more flexible than the
244 glycerol node without losing sight of maximizing 1,3 PDO titers (Chen and Liu, 2016). Besides getting rid of
245 by-products, such manipulations should leave no adverse impact on microbial growth and simultaneously
246 maintain the redox-cofactor balance.

247 *3.3. Inherent issues with microbial GDHt activity and lack of industrially-proven strains*

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

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

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

280 *3.4. Accumulation of 3-HPA*

281 The accumulation of 3-HPA is regarded as one of the most severe limitations of 1, 3-PDO biosynthesis.
282 3-HPA, an immediate precursor to PDO, is a toxic metabolite to the host cells. Its accumulation at even 15-
283 30 mM concentration triggers irreversible metabolic activity and cellular growth cessation, restricting the
284 microbiological production of biochemicals from glycerol (Matsakas et al., 2018). As per the extensive
285 review by Sun et al. (2022), the aldehydic group of 3-HPA reacts with the free sulfhydryl groups of several
286 small proteins and enzymes, such as glutathione reductase, causing oxidative damage to bacterial
287 systems. Likewise, its dimeric form competitively inhibits the ribonucleotide reductase enzyme, which is
288 responsible for DNA synthesis in bacteria, thus contributing to its antimicrobial properties. In addition, PDO
289 buildup can contribute to the accumulation of 3-HPA as PDOR can catalyze the reverse reaction
290 (PDO→3-HPA) under high PDO concentrations.

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

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

305 *3.5. Product inhibition and tolerance*

306 The tolerance of microorganisms includes the inhibition or toxic effects of not only end products but also
307 substrate, as well as impurities on cell growth and metabolism. Generally, these metabolites belong to the
308 family of short-chain carboxylic acids and alcohols. Thus, the product output of any microbial fermentation
309 is governed by the capacity of the microbe to tolerate the threshold concentration of the targeted metabolite
310 without critically affecting its metabolic activity. It is often exacerbated by the co-formation of other
311 unwanted byproducts, resulting in multi-product inhibition. As a result, microbial fermentations often display
312 a decline in productivity over a prolonged duration. For instance, Kumar et al. (2013) conducted toxicity
313 experiments with *K. pneumoniae* by exogenously adding PDO at different levels. They found that the initial
314 presence of PDO unfavorably affected the growth and production capabilities of *K. pneumoniae*. Cell
315 growth and its metabolism completely arrested, when the PDO concentrations reached 300-400 mM.
316 Similarly, Yang et al. (2019) conducted a 1, 3-PDO tolerance test on *C. butyricum* XYB11 by growing its
317 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
318 adversely affected microbial growth.

319 In the case of 1, 3-PDO production, byproducts from the oxidative pathway, especially organic acids,
320 invariably negatively affect cell growth, metabolism, and 1, 3-PDO production. Random mutations, genetic
321 interventions, or the ALE approach commonly address these issues. Besides glycerol bioconversion to
322 1, 3-PDO, its downstream processing (DSP) is expensive, as its biological production is mandatorily hetero-
323 fermentative. Byproducts such as acetate, lactate, butyrate, succinate, 2, 3-BDO, microbial cells, and
324 residual media components interfere during selective extraction, posing difficulties during its recovery and
325 purification. However, commenting on this crucial aspect is beyond the scope of the current review.

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

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

333 *4.1. Genetic interventions to enhance 1, 3-PDO formation from glycerol*

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

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

341 For instance, Lee et al. (2018) first deleted the "wabG" gene from *K. pneumoniae* KCTC 2242, responsible
342 for the strain's pathogenicity to increase acceptability of this strain for scale-up. Later, the group disrupted
343 the *ldhA* and *pflb* genes sequentially, eliminating lactate and acetate formation. As a result, the 2, 3-BDO
344 titers increased. However, when the *budA* gene was deleted, it significantly reduced glycerol consumption,
345 biomass production, and 1, 3-PDO titers. In the next stage, when the two crucial genes of the oxidative
346 glycerol pathway, namely *glpK* and *dhaK,* were deleted, the impact of the latter gene on 1, 3-PDO
347 production was detrimental, as its product catalyzed the dehydrogenation of glycerol to produce DHA,
348 supplying $NADH_2$ equivalent for uninterrupted 1, 3-PDO synthesis. But when the strain was co-fed with
349 mannitol, it restored cell growth and fulfilled energy needs, while glycerol flux was diverted mainly towards
350 1, 3-PDO, thereby increasing molar yield. Additionally to reduce the co-feeding of mannitol, the expression
351 levels of mannitol-specific transporter MtlA were reduced targeting the 5' untranslated region (UTR)
352 sequence of its gene. Popular and precise gene editing tool namely clustered regularly interspaced short
353 palindromic repeats (CRISPR) -associated CAS9 endonuclease was used for gene alteration. As a result
354 mannitol utilization reduced, without hampering biomass and 1, 3-PDO production. The final KMY strain
355 with transcriptionally optimized *dha* operon produced 20.59 g L^{-1} 1, 3-PDO with 0.76 of molar yield. This is
356 indeed the highest claimed molar yield in *Klebsiella* reported to date.

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

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

399 To date, researchers attempted to manipulate only the genes of the glycerol metabolic pathway for
400 attaining high TYP matrices of 1, 3-PDO. However, bold experimentation was done in the last two years,
401 involving the detailed investigation of various transport proteins facilitating glycerol uptake and promoting 1,
402 3-PDO excretion. Teng et al. (2022) chose an endogenous gene from *Klebsiella* and an exogenous gene
403 from *E. coli* encoding for glycerol uptake facilitator proteins for over-expression in the study conducted
404 with *Klebsiella*. Further, the role of seven genes encoding for resistance-nodulation-cell division (RND) type
405 efflux systems was evaluated. These systems are characteristic of Gram-negative bacteria and facilitate
406 the efflux of a spectrum of chemicals. Compared to the wild strain, over-expression of endogenous
407 *glpF* gene, encoding for protein facilitating the glycerol uptake, and the heterologous *MexF* gene
408 from *Pseudomonas aeruginosa* promoting 1, 3-PDO efflux, enhanced its final titers. During fed-batch
409 cultivation, the recombinant *Klebsiella* in which *glpF* and *MexF* gene were over-expressed individually
410 accumulated 65.1 and 74 g L^{-1} 1, 3-PDO, respectively compared to 55.6 g L^{-1} in parent, and the acetate
411 formation also observed notably (Teng et al., 2022).

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

415 *4.2. Mutagenesis*

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

424 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,
425 respectively. The mutant produced a 29.5% higher titers of 1, 3-PDO than the wild parent strain (Yang et
426 al., 2019). In yet another study, electron beam irradiation was used to obtain mutants of *Lactobacillus*
427 *reuteri*, with selection criteria being higher resistance towards 40 and 20 g L^{-1} lactic acid and acetic acid,
428 respectively (Ju et al., 2021). During fed-batch cultivation on PG, the superiority of the mutant (JH83) was
429 fairly visible over its parent strain (CH53). The 1, 3-PDO concentration and productivity of *L. reuteri* JH83
430 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.*
431 *reuteri* CH53. The mutant's performance on CG remained unchanged. The high 1, 3 PDO titers with GRAS
432 organism like *Lactobacillus,* without co-feeding of any sugar as alternate carbon source during
433 fermentation, makes this study conspicuous.

434 *4.3. Adaptive Laboratory Evolution (ALE)*

435 During the biotechnological valorization of real-time carbonaceous waste streams, ALE is the most
436 powerful and competitive technique to enhance the robustness of microbial cell factories. In a recent
437 review, Sandberg et al. (2019) listed most ALE studies into five categories, primarily targeting increased
438 tolerance (towards substrate and product), followed by substrate utilization, increased product yields or
439 titers and growth rate optimization. Nutrient and environmental stress, or their combination, is the most
440 common way to induce natural selection and obtain highly evolved strains. Some of the examples wherein
441 ALE was adopted for enhanced bioconversion of glycerol to 1, 3-PDO are shown in Table 3. As observed in
442 Table 3, the most visible outcome of the ALE experiments was obtaining a stable adapted glycerol utilizing
443 strain which showed higher 1, 3-PDO productivity than its parent strain. In general, strain adaptation at
444 higher substrate concentrations and repeated sub-culturing led to the screening of fast-growing strains,
445 which tend to exhibit a shorter lag phase than parents with a better glycerol uptake rate. A pertinent
446 example to quote is the performance displayed by *C. pasteurianum* obtained after continuous ALE in CG
447 (Table 3). The adapted strain showed no lag phase during the fed-batch experiment, resulting in titer and
448 productivity of >80 g L^{-1} and >4 g/L.h, respectively, with CG as substrate in a 1000L fermenter (Zhang et
449 al., 2022). By carrying fermentation under non-sterile conditions and without maintaining complete
450 anaerobiosis, the authors proved that the adapted strain was a strong candidate for commercial-scale
451 demonstration. In contrast to rational engineering and directed modification of specific enzymes, adaptive
452 laboratory evolution (ALE) has an edge by allowing non-intuitive productive mutations to occur in a variety
453 of genes in parallel. These mutations resulting from ALE can activate latent metabolic pathways, increase
454 substrate/ product tolerance, and improve bacterial fitness. With the growing interest in transcriptional and
455 translational approaches, researchers try to decipher changes happening at the molecular level (gene or
456 protein), which plays a pivotal role in rewiring the entire metabolic pathway of the microbes during
457 evolutionary engineering (Mavrommati et al., 2022). For instance, proteomic analysis (using Tandem Mass
458 Tag technology) of adapted *Klebsiella* x546 strain revealed several genes belonging to unrelated pathways
459 (glycolytic, amino acid synthesis, pyrimidine synthesis) etc. were upregulated facilitating the growth and
460 reproduction of the bacterium, besides the reductive pathway of glycerol metabolism. During fermentation,
461 betaine addition counteracted the high osmotic pressure exerted by Na_2CO_3 (neutralizing agent), reducing
462 the fermentation time by 40%. The highest 1, 3-PDO titer reported was 74.44 g L^{-1}, with productivity being
463 3.1g/L.h (Wang et al., 2021b).

464 Nearly all the examples cited in this sub-section confirm that after ALE, the natural glycerol assimilating
465 strains are capable of producing industrial relevant titers of 1, 3-PDO.
466

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

509 and heat), and smart integration of process parameters are the four primary targets of PI (Noorman et al., 510 2018). Since glycerol bioconversion to 1, 3-PDO essentially involves an oxidoreductive pathway, pH-stat 511 fermentation is a must, as the accumulation of organic acids tends to reduce the fermentation medium's pH 512 and arrest the growth of the microbes. Further, maximizing 1, 3-PDO titers demand maintenance of either a 513 micro-aerobic or anaerobic environment. Moreover, high glycerol concentrations generally exert an 514 inhibitory effect on cell growth (due to high osmotic pressure) and its uptake, thus impeding the attainment 515 of high TYP matrices of the product. To address this issue, continuous or fed-batch operation is preferred 516 over batch, and optimum fermentation conditions must be deciphered, favoring high TYP. For instance, 517 dilution rate or hydraulic retention time (HRT) is crucial if the researchers opt for a continuous process. In 518 the case of a fed batch, feed concentration and time of addition, including cell maintenance, are vital. Table 519 5a shows trending PI strategies used exclusively during pure glycerol fermentation to enhance the 520 concentration and productivity of 1, 3-PDO, citing salient features of each process.

521 For instance, Sun et al. (2019), before optimizing fermenter conditions, identified the composition of the 522 microbial consortium DUT-08. The biochemical characterization of stable consortium, obtained after 35 523 repeatedly sub-culturing, revealed that the consortium mainly contained *Clostridium* (>85%), followed 524 by *Escherichia* (>12%) and *Klebsiella* (>0.1%). On deciphering the mechanistic action, they found 525 that *Escherichia* primarily depleted the oxygen content, facilitating *Clostridium's* growth and 1, 3-PDO 526 production. Likewise, the tiny population of *Klebsiella* not only reduced O_2 levels but also contributed 527 towards 1, 3-PDO production. Thus, the commensalism and synergism exhibited by microbes helped to 528 attain high 1, 3-PDO titers. N_2 purging was minimal, and maintenance of strictly anaerobic conditions was 529 not needed, despite *Clostridium* being predominant. In the same year, the feasibility of using soybean cake 530 hydrolysate (SCH) as an N_2 source was evaluated for two strains of *Citrobacter freundii.* Under batch 531 conditions, the effect of glycerol concentration and the free amino nitrogen (FAN) present in SCH was 532 studied. Owing to higher susceptibility towards organic acids, *C. freundii* FMCC-8 accumulated less PDO 533 (43.6 g L^{-1}) during fed-batch cultivation than *C. freundii* VK-19 (55.6 g L^{-1}). Later, the potentiality of both 534 strains was assessed on CG as well (Maina et al., 2019). In another study, Wang et al. (2020) deciphered 535 the right age and size of the inoculum of *Clostridium butyricum* DL07, to bypass the lag phase. Further, 536 they found a linear relationship between the NaOH required to restore pH and glycerol consumption, which 537 helped them develop a fully automated process for hassle-free 1, 3-PDO biosynthesis. During sequential 538 fed-batch process, consistent 1, 3-PDO titers and productivities over eight cycles were demonstrated 539 (Table 5a).

540 Compared to PG, more studies were conducted with CG in the past five years, as shown in Table 5b. For 541 instance, Oh et al. (2018) created two *Klebsiella* mutants and optimized conditions (size of the inoculum, 542 concentration of the N_2 source, cell recycling ratio, pH) during CG fermentation. As a result, a successful 543 repeated fed-batch fermentation process was developed, which gave reproducible PDO titers and saved 544 fermentation time. Among the two mutants, deleting an extra gene encoding for acetolactate synthase, 545 responsible for 2,3 - BDO synthesis besides *ldh*, reduced 1, 3-PDO titers. Lower PDO titer resulted from 546 the non-production of BDO, which regulates intracellular acidification and balances NADH/NAD+ within the 547 cell (Lee et al., 2021). Likewise, Martins et al. (2020) adopted an efficient screening method wherein 16 548 variables were evaluated in the first stage of the Plackett-Burman design to obtain a low-cost, simple 549 medium for 1, 3-PDO production from glycerol using *C. butyricum* NCIMB 8082. In the second stage, 11 550 variables were again screened, which reduced the media components to 7. The final media contained 1.0 g 551 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 552 (in 1 L distilled water), besides CG with 82.5% purity. Thus, they tactfully eliminated non-significant 553 components of fermentation media. Later in a two-step anaerobic fermentation process, the batch mode

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

559 Recently, Pan et al. (2019), developed a two-stage process for converting CG to 1, 3-PDO and PHB, which
560 has not been cited in Table 5b, but its results are worth mentioning. Initially, the highly alkaline CG obtained
561 from the biodiesel industry was reduced to a pH of 3.0, and most of its impurities were extracted using
562 hexane. In the first phase, this pretreated CG was subjected to anaerobic digestion using a microbial
563 consortium obtained from mangrove sediments. Glycerol was fed intermittently to circumvent the issue of
564 substrate inhibition. In 5 days, ~499 g of glycerol was fed to the fermenter, of which 384.92 g were
565 consumed, leading to an accumulation of 159.39 g of 1, 3-PDO with lactate, acetate, and butyrate as
566 significant byproducts. Microbial community analysis showed that >75% of the population comprised
567 *Clostridiales,* followed by *Enterobacteriales*. In the second stage, the residual glycerol, lactate, acetate, and
568 butyrate produced in the first stage were exploited for PHB formation using a co-culture containing
569 *Corynebacterium hydrocarbooxydans* ATCC 21767 and *Bacillus megaterium* DSM 90. This is a perfect
570 example of biotransforming glycerol to two valuable products using PI during fermentation.
571 Even with the examples mentioned in Table 4, where new microbes and the microbial consortium were bio-
572 prospected, the fermentation strategy was optimized to maximize 1, 3-PDO production. Researchers used
573 either one variable at a time (OVAT) or response surface methodology (RSM), or a combinatorial approach
574 to establish the PDO-producing capabilities of these natural producers (Zabed et al., 2019; Garg et al.,
575 2020; Lan et al., 2021; Gupta et al., 2022). For instance, Garg et al. (2020) developed a dye-based method
576 to monitor shake flask production of 1, 3-PDO using newly isolated *Citrobacter freundii* and optimized batch
577 fermentation using the OVAT approach. Likewise, if Zhou et al. (2018) emphasized developing a
578 continuous process from CG for their microbial consortium, Wang et al. (2020) focused on augmenting 1,3-
579 PDO titer and productivity using a fed-batch process where *C. butyricum* DL07 was evaluated on both PG
580 and CG. Thus, process intensification during fermentation is integral to any biotechnological process,
581 providing a favorable environment for the microbes and maximizing product output.

582 *4.6. Combinatorial approach*

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

586 *4.6.1. Genetic engineering followed by co-culturing*
587

588 A pertinent example of this category is the recent study, wherein the co-production of two chemical building
589 blocks from glycerol, namely 3-HP and 1, 3-PDO, was targeted (Zhang et al., 2021c). A modular co-
590 cultivation engineering strategy was developed for 3-HP and 1, 3-PDO -producing *Lactobacillus reuteri* and
591 recombinant *E. coli* to accomplish high glycerol bio-conversion. The recombinant *E. coli* was created for 1,
592 3-PDO biosynthesis by over-expressing two genes, *aldH* or *gabD4* gene from *Cupriavidus necator*,
593 encoding for NAD^+-dependent aldehyde dehydrogenase or PDOR and PudQ gene from *L. reuteri* encoding
594 for $NADPH^+$ dependent PDOR. Further, for balancing the 1, 3-PDO pathway, the 5'-UTR region of *gabD4*
595 gene was engineered. Later, the co-culturing strategy was optimized to attain high glycerol bioconversion
596 and product titers amidst maintaining cell viability. The final devised method included initiating

597 biotransformation with 10 g L^{-1} of cells of dry weight basis and adding 20 g L^{-1} glycerol. Thereafter, 20 g L^{-1}
598 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}
599 (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
600 approach improved glycerol consumption from 30 to 240 g L^{-1} and enhanced co-product titers from 25.11 to
601 214.39 g L^{-1} (Zhang et al., 2021c).
602
603 Likewise, in an earlier study, Yun et al. (2018) first over-expressed the *dhaT* gene from newly isolated
604 *Clostridium buytricum* YJH-09 in *E. coli*. Later, by co-biotransforming *C. butyricum* YJH-09 whole cells with
605 *BL21-dhaT*, the effects of substrate concentration, NADH level, and strain mass ratio were fine-tuned to
606 attain high 1,3-PDO titers. The optimized conditions include 12-hour co-biotransformation, where 10 g of C.
607 *butyricum* YJH-09 cells were mixed with 10g of *BL21-dhaT* cells, with initial glycerol concentration being 50
608 g L^{-1} and exogenous doping of 0.5 mM NADH was required. As a result, the mixed culture biosynthesized
609 25.88 g L^{-1}1, 3-PDO in 42 h, with the maximum yield occurring at 30 h.
610
611 *4.6.2. <u>Combined approach of genetic engineering and adaptive evolution</u>*
612
613 A recent US patent granted to Metabolic Explorer is an appropriate case where the said strategy was used
614 (Tourrasse and Raynaud, 2021). In the said invention, extra copies of the vitamin B_{12} independent *dha*
615 operon from *Clostridium buytricum* were introduced in *Clostridium acetobutylicum*, followed by adaption on
616 high concentrations of industrial glycerol. When the adapted strain was evaluated under anaerobic
617 continuous chemostat conditions with a feed medium containing 106 g L^{-1} glycerol and a dilution rate being
618 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,
619 respectively with butyric acid as the major byproduct. The microbial consortium comprising of adapted
620 strain, *Clostridium sphenoides*, and *C. sporogenes* under nearly identical conditions produced 52.9 g L^{-1}
621 1, 3-PDO with an insignificant impact on yield and productivity.
622
623 In their newest study, Yun et al. (2022) adopted two parallel approaches to enhance 1, 3-PDO titers in
624 *Clostridium butyricum* YJH-09. Later, the best mutants obtained from both strategies were subjected to
625 protoplast fusion to get the final strain. In the first approach, after identifying the maximum glycerol
626 tolerance limit of the strain, *Clostridium* was subjected to ARTP mutagenesis for 150s, and screening was
627 conducted on plates containing 140 g L^{-1}glycerol. Further subculturings were done by increasing glycerol
628 titers to 150 and 160 g L^{-1}. Among all the mutants, AJH-35 and AJH-38 were shortlisted based on high
629 biomass formation. In parallel to ARTP, *Clostridium butyricum* YJH-09 was subjected to ALE involving a
630 continuous and automated microbial microdroplet culture (MMC) system. In this automated set-up, the
631 concentration of 1, 3-PDO was gradually increased from 20 to 100 g L^{-1} in 30 days with five adaptation
632 stages. Two potential candidates, MJH-49 and MJH-54, were shortlisted based on high biomass formation.
633 Later, the mutants obtained from ARTP and ALE, were subjected to protoplast fusion. Final strain selection
634 was made based on high biomass accumulation on medium containing 100 g L^{-1} 1 , 3-PDO and 5 g L^{-1}
635 butyric acid. During batch fermentation, mutant strain *C. butyricum* GJH-418 displayed an optical density
636 (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,
637 respectively, from 120 g L^{-1} initial glycerol. Under identical conditions, the wild strain produced 12.32, 2.12,
638 1.12 g L^{-1} 1, 3-PDO, butyric acid, and acetic acid, respectively, with $OD_{600}$of merely 1.45.
639
640 *4.6.3. <u>Improved glycerol bioconversion using metabolic and bioprocess engineering</u>*
641
642 Among the latest developments, mentioning a recombinant *E. coli* strain which co-produced 3-HP and 1, 3-
643 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

688 performance by wild and natural 1, 3-PDO producing microbial strains prove that consistent and rigorous
689 efforts are being made across the globe to develop alternate technologies for production of glycerol-derived
690 1, 3-PDO.

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

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

713 Alternately, many researchers have brilliantly used heterofermentative production of 1, 3-PDO production
714 from glycerol to their advantage by focusing towards co-product biosynthesis as well. The co-production of
715 commercially interesting products like lactic acid or 3-HP at industrially relevant titers along with 1,3-PDO
716 has been successfully demonstrated as per latest trending research (Jiang et al., 2021; Zhang et al.,
717 2021c; Zhang et al., 2023). Further, researchers have prudently employed novel approaches to reduce the
718 toxicity levels of organic acids (acetic acid, lactic acid, butyrate) by diverting them towards biosynthesis of
719 non-toxic and valorized products (such as PHB and esters of acetate and butyrate), that can be easily
720 separated and purified from the aqueous fraction post-fermentation (Pan et al., 2019; Wang et al., 2021a;
721 Zhang et al., 2022).

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

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

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

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

763 **6. Concluding remarks**

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

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

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783

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1049 **Figure Captions**

1050 **Figure1: Key metabolic pathways for glycerol valorization to 1,3 - propanediol in various bacteria**
1051 **Figure 2: Reductive pathway for glycerol metabolism in prokaryotes.**

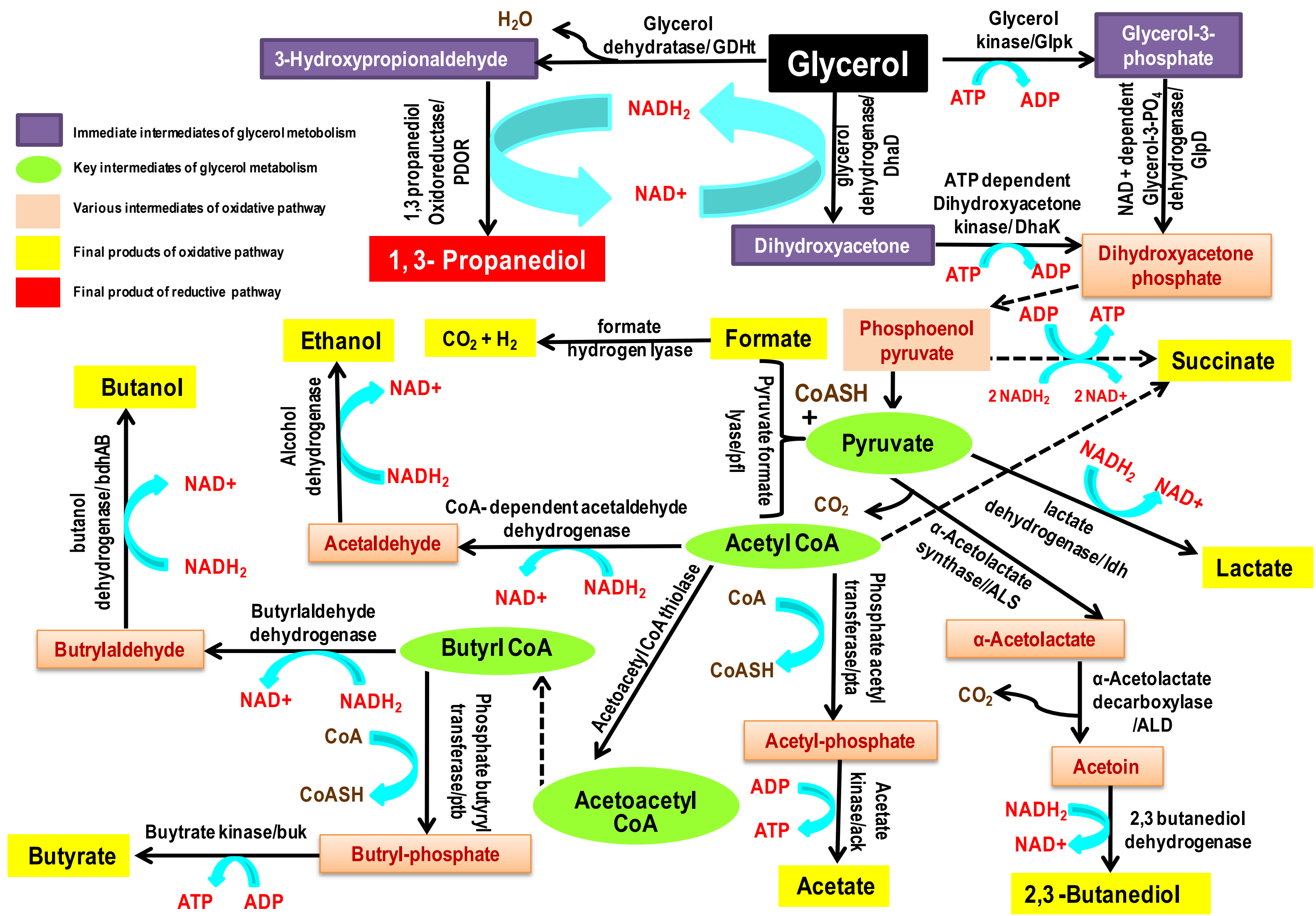
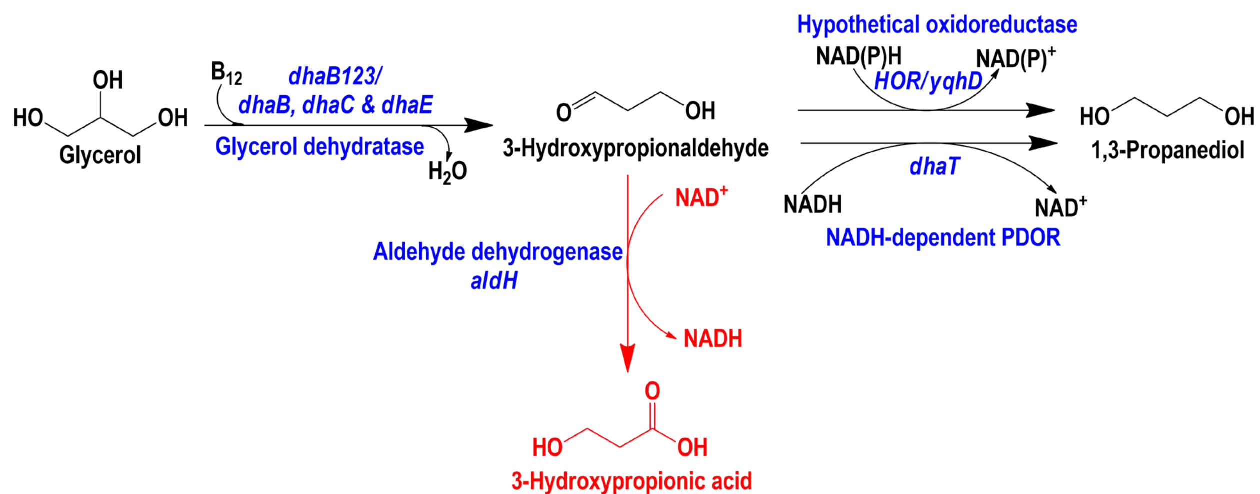
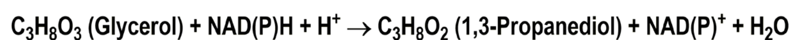


Figure1: Key metabolic pathways for glycerol valorization to 1,3 - propanediol in various bacteria





$C_3H_8O_3$ (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	P	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 T	P	Y	Reference
Clostridium butyricum YJH-09	Pond Soil	PG	Batch	11.72	-	-	Yun et al., 2018
Lactobacillus reuteri FXZ014	Feces of infant	PG	Batch	9.94		0.55	Zabed et al., 2019
Klebsiella pneumoniae 2e	Biodiesel-derived waste contaminated soil	PG	Batch	12.16	1.01	0.586	Ma et al., 2019
Citrobacter freundii IIPDR3	Solvent Storage Site	PG	Batch	9.85	0.82	0.54	Garg et al., 2020
Clostridium butyricum DL07	Active sludge from anaerobic digester	PG	Fed-batch	104.8	3.38	0.54	Wang et al., 2020
Clostridium butyricum SCUT343-4	Deep soil of Daling Mountain	PG	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	CG	Batch	10.28	1.01	0.53	Ma et al., 2019
Microbial consortium LS30	Activated sludge from sewage treatment plant	CG	Batch	13.22	0.85	-	Wang et al., 2019
Clostridium butyricum DL07	Active sludge from anaerobic digester	CG	Fed-batch	94.2	3.04	0.52	Wang et al., 2020
Clostridium butyricum L4	Biogas reactor leachate	CG	Fed-batch	70.1	0.46	0.54	Gupta et al., 2022
Microbial consortium CJD-S	Intertidal sludge from sea	CG	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 Conc (g/L)	1, 3-PDO Productivity (g/L/h)	Reference
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; β denotes 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

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