

An eclectic review on dicarboxylic acid production through yeast cell factories and its industrial prominence

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Abstract

Dicarboxylic acid (DCA) is a multifaceted chemical intermediate, resorted to produce many industrially important products such as adhesives, plasticizers, lubricants, polymers, etc. To bypass the shortcomings of the chemical methods of synthesis of DCA and to reduce fossil fuel footprints bio-based synthesis is gaining attention. In pursuit of an eco-friendly sustainable alternative method of DCA production, microbial cell factories, and renewable organic resources are gaining popularity. Among the plethora of microbial communities, yeast is being favored industrially compared to bacterial fermentation due to its hyperosmotic and low pH tolerance and flexibility for gene manipulations. By application of rapidly evolving genetic manipulation techniques, the bio-based DCA production could be made more precise and economical. To bridge the gap between supply and demand of DCA many strategies are employed to improve the fermentation. This review briefly outlines the advancements in DCA production using yeast cell factories with the exemplification of strain improvement strategies.

Keywords: Bioproduction, Dicarboxylic acid, Genetic manipulation, Green manufacturing, Yeast cell factories.

49 **List of abbreviations**

AA	Adipic acid
CAGR	Compound annual growth rate
DCA	Dicarboxylic acid
FA	Fumaric acid
GA	Glutaric acid
GCA	Glucaric acid
GRAS	Generally recognized as safe
IA	Itaconic acid
MA	Malic acid
MCA	Muconic acid
SA	Succinic acid
TCA Cycle	Tricarboxylic acid cycle
US DoE	United States Department of Energy

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1 Introduction

Rapid growth in the manufacturing sectors such as textile, food, pesticide, pharmaceutical, and cosmetic industries embark on platform chemicals that serve as raw materials for the production of different diversified products. However, the prevalent practice of synthesizing these chemicals through conventional chemical methods using non-renewable resources derived from fossil fuels raises concerns about the long-term sustainability of these industries. There is a need for a paradigm shift in the synthesis of platform chemicals through sustainable, renewable, and greener route options that might mitigate the problem [1]. Among the array of valuable platform chemicals, dicarboxylic acids (DCAs) hold significant importance due to their widespread utilization across various industries. DCAs are a group of organic compounds with two carboxylic acids ($-\text{COOH}$) as the functional group and are classified based on the carbon (C) chain length as given below:

- (i) Short chain DCAs: 2-3 carbons (oxalic acid (C2) and malonic acid (C3));
- (ii) Medium chain DCAs: 4-10 carbons (succinic acid (C4), malic acid (C4), fumaric acid (C4), glutaric acid (C5), itaconic acid (C5), adipic acid (C6), glucaric acid (C6), pimelic acid (C7), suberic acid (C8), azelaic acid (C9), muconic acid (C9) and sebacic acid (C10)); and
- (iii) Long chain DCAs: 11- 18 carbons (dodecanedioic acid (C12), tridecanedioic acid (C13), tetradecanedioic acid (C14), pentadecanedioic acid (C15) and octadecanedioic acid (C18)).

DCAs such as succinic acid (SA), adipic acid (AA), malonic acid, glutaric acid (GA), and α , ω -DCAs find widespread application in industries. Especially the food and polymer sectors are major consumers of DCAs as these monomers polymerize into high molecular weight compounds such as nylon 6,6 polyamide and fumarate polyesters [2]. Fig. S1 illustrates the chemical structure of the medium-chain DCAs. According to the U.S. Department of Energy

(US DoE) report of top value-added chemicals from biomass (2004), SA is identified as one of the top 12 building block chemicals produced from biomass-derived sugar using the biological or chemical method of conversion. Other DCAs listed are fumaric acid (FA), malic acid (MA), 2,5 furan dicarboxylic acid, glucaric acid (GCA), and itaconic acid (IA) [3]. In 2010, an updated list was released by the US DoE in which DCAs such as SA and 2,5 furan dicarboxylic acid are again recognized as the top value-added chemicals [4].

Currently in many industries, the majority of DCAs are synthesized through chemical methods, for example, FA is produced by hydrogenation of MA and maleic anhydride. Both the substrates are heated together at the high temperature of around 100-190°C in an inert atmosphere, during which FA is produced via partial hydration and isomerization, and the chemical reaction is depicted in Fig. S2(b) [5]. Similarly, chemical synthesis of other DCAs such as SA Fig. S2(a) [6], MA Fig. S2(c) [7], GA Fig. S2(d) [8], and AA Fig. S2(e) [9] requires the high energy demanding reaction conditions and catalyst and thus, chemical methods are economically unsustainable and have a negative impact on the environment. Moreover, the chemical synthesis approach necessitates expensive raw materials, posing financial challenges. In response, researchers are actively exploring eco-friendly bio-based methods for DCA production, leveraging cost-effective renewable feedstocks to enhance the economic viability of the production process compared to conventional chemical methods [2]. Zhang et al., [10] explored the potential of biomass-based 5-hydroxymethylfurfural as a substrate for the production of the DCA, 2,5-furan dicarboxylic acid. During the process of production, the stability of potassium ferrate was improved using stabilizers such as phosphate buffer and ferric hydroxide. Further, this produced DCA was utilized as a monomer for the synthesis of the complete bio-based polyester, poly(hexamethylene 2,5-furan dicarboxylate).

The biological production of DCAs has been demonstrated since 1971 with various microbes and diversified substrates [11–16]. Initially, filamentous fungi and bacteria are exploited for

111 DCA production. Though these microflora were reported to produce DCAs as their metabolic
112 intermediate, the downside of using these host systems for DCA production include: (i)
113 difficulty with downstream processing; (ii) low pH intolerance; (iii) osmotic intolerance; (iv)
114 hyphal breakage in filamentous fungi due to shear force in high-density fermentation; (v) low
115 biomass growth and poor DCA production in obligate anaerobes; (vi) high density of
116 filamentous fungi increases the viscosity of fermentation broth and affects mixing and aeration;
117 and (vii) DCA produced by *Escherichia coli* fermentation cannot be used in food industries as
118 it doesn't come under GRAS status [2]. In order to address these limitations and improve the
119 cost-effectiveness of the production process, numerous yeast species are being investigated for
120 their potential to synthesize various DCAs. Some of the potential yeast candidates include
121 *Yarrowia lipolytica* [13] *Saccharomyces cerevisiae* [12], and *Candida* sp. [17]. Certain distinct
122 characteristics of yeast such as the ability to utilize the carbon source from diversified
123 substrates (agro-/industrial wastes); resistance to strain degeneration; capacity to tolerate acidic
124 and hyperosmotic conditions; ease of genetic modification; and compliance with food safety
125 make them suitable producer organism [17]. Though the microbial synthesis of DCA shows
126 promise, progress in production strategies appears limited. As stated earlier, the demand for
127 this top valued DCA seems to be engrossing in future, there is a requirement to adopt various
128 approaches that starts from strain selection to strategizing the improvement in product yield
129 using conventional and non-conventional yeasts. Zhang et al. [2] reviewed on the role of
130 *Saccharomyces cerevisiae* in synthesizing medium-chain DCA leaving behind other potential
131 yeasts. As only sporadic reviews on comprehensive yeast cell factories for DCA production
132 exist, this review article provide a detailed exploration of various non-conventional yeast
133 species such as *Yarrowia lipolytica*, *Pichia kudriavzevii*, *Candida tropicalis*, *Pichia pastoris*,
134 *Wickerhamiella sorbophila* along with *S. cerevisiae* involved in DCA production. Furthermore,
135 it encompasses recent advancements in strain improvement through genetic engineering,

metabolic engineering, and systems biology (OMICS technologies) to enhance industrial DCA production. It also addresses bottlenecks in strain selection and improvement, feedstock selection and processing, fermentation process, and proposing viable solutions. This article also discussed in detail on elucidating the DCA application across diverse industries and the associated hurdles in commercial production. To the best of authors' knowledge, this article presents an eclectic perspective on the yeast cell factories engaged in DCA production, addressing both the challenges and probable solutions.

2 Yeast cell factories in DCA production

2.1 Yeast: A potent microbial cell factory for DCA production

Most of the yeast species can tolerate extreme acidic and alkaline fluctuations and can be a better host organism in the production of DCAs [12]. *S. cerevisiae* is highly exploited for the production of DCA and can utilize a broad range of substrates from simple sugars like glucose to mixed monomeric sugars from lignocellulosic biomass to synthesize DCAs with a higher yield [12, 15, 18–20]. Its high tolerance toward fluctuations in fermentation conditions makes it a suitable organism to produce DCAs. Next to *S. cerevisiae*, the oleaginous yeast *Y. lipolytica*, is being extensively explored for the biotechnological synthesis of DCAs on a large scale [13, 21–23]. This oleaginous yeast genome has been completely sequenced and annotated for different functional characteristics. It is quite easy for researchers to understand and manipulate the organism at genomic and proteomic levels to attain higher productivity. The *Yarrowia lipolytica* genome database [24] is an effective platform for exploring the role of genes in different metabolic pathways. The combination of knowledge and information on the yeast genome has made it amenable to genetic manipulation for the enhanced production of DCAs.

Other yeast species employed in the production of DCAs include *Candida*, in particular, *C. tropicalis*, *C. sorbophila*, *C. maltosa*, *C. cloacae*, *C. guilliermondii*, and *C. viswanathii* [17,

25–29]. Other yeast species investigated for DCA production include *Cryptococcus neoformans* [30], *Schizosaccharomyces pombe* [31], *Starmerella bombicola* [32], *Issatchenkia orientalis* [33], *Pichia occidentalis* [34], *Pichia kudriavzevii* [35], and *P. pastoris* [36]. In line with DCAs, several studies have attempted to exuberate high productivity through metabolic engineering and genetic manipulation strategies. Table 1 shows different genetic engineering strategies for yeast strains for increased DCA production.

2.2 Biobased feedstocks for DCA production

Common substrates used for the biological production of DCAs are glucose, fumarate, glycerol, alkane, fatty acids, and bicarbonate. Glucose is employed as the major substrate to produce DCA. To cut short the expenditure, recent research is focused on exploring cheap renewable lignocellulosic feedstock such as post-harvest agro-residues, industrial organic wastes, and forest residues for DCA production [37]. Some of the cheaper carbohydrate-rich feedstock such as sorghum grits, sugarcane bagasse, corn stover, wood chips, rice straw, waste cooking oil, and vegetable oil were investigated as raw materials for DCA production [38–40]. Apart from glucose, oleaginous yeast utilizes fats and lipids as carbon sources and produces DCAs. Fatty acids and alkanes with different chain lengths are converted to their corresponding DCAs by selective oxidation of the terminal methyl group. The carbon chain of fatty acids undergoes β -oxidation in the mitochondrial matrix and acetyl CoA is released [13]. It enters the tricarboxylic acid cycle (TCA cycle) resulting in the synthesis of DCAs [37]. Fig. 1 illustrates the metabolic pathway in yeast involved in the biosynthesis of DCAs. The two main transmembrane transporters involved in the cellular and mitochondrial membrane movement of DCAs are major facilitator superfamily transporters and mitochondrial tricarboxylic transporters respectively [41]. The type of the resultant DCA directly corresponds to the chain length of fatty acid which is used as substrate.

2.3 Medium chain DCAs and their production strategy using yeast cell factories

2.3.1 Succinic acid

Succinic acid (SA) $[(CH_2)_2(COOH)_2]$ is a four-carbon DCA that has huge applications in polymers and polyester production to cater to the needs of the pharmaceutical, lubrication, and food industries [42]. Bioproduction of SA is favored by an increasing demand from a technological perspective. Polybutylene succinate, a biodegradable polyester resin synthesized from SA is extensively utilized in packing industries. Its current market value is relatively small and it is expected to expand rapidly due to the blooming of biobased compounds derived from succinate plays a crucial role. The company Bioamber in Sarnia, Canada reported a total annual production of 30 tons of SA in 2014 using a pH-tolerant *Pichia kudriavzevii* [43]. The global market value of SA was \$143.19 million in 2020 and it is anticipated to be around \$290.72 million by the end of the year 2031 [44].

In general, SA is synthesized by the conversion of α -ketoglutarate mediated by succinyl CoA synthetase in mitochondria during the TCA cycle. However, there exists a bottleneck where SA is transformed into fumarate which prohibits the efflux from the cell. This led to the minimal yield of SA during cell growth under normal conditions. On the other hand, with the intervention of metabolic engineering, the yield can be maximized by knocking out the succinate dehydrogenase gene responsible for SA to fumarate conversion thereby leading to its accumulation. Gao et al., [45] reported the highest production of SA in a recombinant yeast strain, *Y. lipolytica* PGC01003. This recombinant strain has been developed by deletion of the gene encoding succinate dehydrogenase subunit 5 (*sdh5*). Upon optimization of fermentation media and conditions, 43 g/L SA was produced from crude glycerol. Further, the yield was increased to 160 g/L by fed-batch fermentation strategy in a 2.5 L fermenter with a substrate concentration of 750 g/L of crude glycerol. This engineered *Y. lipolytica* PGC01003 is a promising SA producer. However, cell growth is inhibited by the accumulation of the by-

211 product, acetic acid. Investigators are working towards solutions such as the deletion of native
 212 *ach* gene encoding acetyl-CoA hydrolase and the engineering of phosphoenolpyruvate carboxy
 213 kinase or succinyl-CoA synthetase gene to maximize the productivity and reduce toxicity
 214 caused by acetic acid accumulation [21]. Another recombinant strains *Y. lipolytica* H222-AZ1
 215 and H222-AZ2 were developed by replacing the native succinate dehydrogenase promoter
 216 genes (*sdh2*) with inducible promoter regions of the isocitrate lyase gene (*icl1*) in *Y. lipolytica*
 217 H222-AZ1 and 3- ketoacyl-CoA thiolase gene (*pot1*) in *Y. lipolytica* H222-AZ2. Crude
 218 glycerol is utilized by the yeast as a sole carbon source during fed-batch fermentation under
 219 oxygen-limited conditions and the SA yield was compared. These genetic modifications have
 220 induced SA synthesis in *Y. lipolytica* H222-AZ2 with a maximum titer of 25 g/L of SA in 165
 221 h whereas, *Y. lipolytica* H222-AZ1 with a titer of 9.2 g/L at a similar fermentation period [46].
 222 Other than *Y. lipolytica*, non-oleaginous yeasts were also explored to produce SA. Raab et al.,
 223 [47] reported a gene manipulation strategy involving disruption of the TCA cycle and
 224 maximizing the yield of intermediates viz succinate, and isocitrate by deleting isocitrate
 225 dehydrogenase genes (*idp1* and *idh1*) and succinate dehydrogenase genes (*sdh1* and *sdh2*), in
 226 the engineered *S. cerevisiae* AH22ura3 strain. This strain utilized glucose as the sole carbon
 227 source and produced SA at a maximum titer of 3.62 g/L, which is 4.8-fold higher compared to
 228 the wild strain. Similarly, Yan et al., [48] reported the enhanced production of succinate in
 229 pyruvate decarboxylase-deficient *S. cerevisiae* TAM strain by deleting glycerol-3-phosphate
 230 dehydrogenase gene (*gpd1*) and regulating CO₂, biotin, and urea concentration in the
 231 fermentation medium that attained maximum succinate titer of 12.97 ± 0.42 g/L with a yield of
 232 0.21 mol/mol glucose. In wild *S. cerevisiae* BY4739 strain, SA production is enhanced by (i)
 233 knocking out succinate dehydrogenase genes (*sdh1* and *sdh2*); (ii) introduction of
 234 *Schizosaccharomyces pombe*-derived MA transporter gene (*mae1*) that resulted in an
 235 engineered strain, named as BY4739sdh12. By knocking out *sdh1* and *sdh2* genes, the

conversion of SA to FA has been reduced from 0.72 ± 0.08 to 0.066 ± 0.003 g/L, resulting in a higher SA titer of 1.45 ± 0.11 g/L after 48h of batch fermentation compared to wild strain by utilizing glucose [49].

2.3.2 Fumaric acid

Fumaric acid (FA) is the trans-isomer of butanedioic acid and an important intermediate metabolite of the TCA cycle which finds a huge application in the food industry as a flavoring and buffering agent. The price of FA ranges between \$2.0 and \$2.2 per kg in the United States, making it about 10 percent more expensive than maleic anhydride, a key ingredient in the chemical synthesis of FA [43]. The global market value of FA was valued at \$501.9 million in 2023 and it is expected to reach \$969 million by 2033 [50]. Currently, the annual demand for FA, around 100 tons has been achieved through the chemical conversion of maleic anhydride which seems to be unsustainable both technically and economically. However, biological production of FA is feasible by dehydrogenation of SA by the action of the enzyme succinate dehydrogenase. However, FA is an intermediate in the TCA cycle and to maximize its production rate there is a need for an intervention through gene manipulation strategies. Xu et al., [18] showed enhanced FA production by incorporating *Rhizopus oryzae*-derived fumarase gene (*fum1*) and malate dehydrogenase gene (*mdh*) into the host strain *S. cerevisiae* FMME-001 and upregulating endogenous pyruvate carboxylase gene (*pyc2*) which results in a higher FA titer of 3.18 ± 0.15 g/L by utilized glucose as a carbon source. Xu et al., [51] developed a strain, *S. cerevisiae* CEN.PK2-1C by knocking out the native fumarate hydratase gene (*fum1*) responsible for the conversion of fumarate to malate and introducing *R. oryzae* derived pyruvate carboxylase gene (*pyc*) and obtained the maximum FA titer of 1675 ± 52 mg/L in batch fermentation with glucose as a carbon source. In another similar study using *S. cerevisiae*, enhanced FA titer of 5.64 ± 0.16 g/L was achieved by overexpressing the heterologous pyruvate

carboxylase gene (*pyc*) and increasing biotin concentration by 32 µg/L in the fermentation medium [52].

2.3.3 Malic acid

Malic acid (MA) is a 4-carbon alpha hydroxy DCA that is predominantly used in food and beverage industries for its flavor-enhancing properties. It is used in pharmaceutical industries for the preparation of cough syrups, throat lozenges, and effervescent powder. It has a refreshing property due to which employed in the manufacturing of mouthwash and toothpaste and used in the formulation of skin care products. In 2022, the global MA market is estimated at \$211.7 million and it is expected to increase to meet a CAGR of 5.1% over the next 6 years [53].

Valorization of crude glycerol to produce MA (24.9 g/L) and citric acid (81.11 g/L) using wild-type *Y. lipolytica* strain (ATCC20460) was reported by Qian et al., [13]. However, to achieve the targeted product from a mainstream metabolic pathway, and to efflux it out of the cell there is a requirement for intervention in the reductive TCA cycle by incorporating the relative gene from other organisms. Chen et al., [41] altered the native reductive TCA pathway by combining the pyruvate carboxylase (*pyc*) gene from *Aspergillus flavus* and the malate dehydrogenase (*mdh*) gene from *R. oryzae* in *S. cerevisiae* RWB837 strain that resulted in the increased production of MA using glucose as a sole carbon source with the overall productivity of 0.32 g/L/h. Zelle et al., [54] have developed a potent malate-producing *S. cerevisiae* RWB525 strain by knocking out the pyruvate decarboxylase-encoding gene (*pdh*) responsible for ethanol production and overexpressing the native pyruvate carboxylase gene (*pyc*), malate dehydrogenase gene (*mdh*) and incorporation of malate transporter gene derived from *S. pombe*. This novel strain was adapted to conditions through a series of batch fermentations in a shake flask followed by scaling up in a 1-liter bioreactor to optimize the process conditions such as pH, CO₂ level, calcium concentration, and oxygen availability. A maximum volumetric

production of 59 g/L was achieved in shake flask fermentation buffered with calcium carbonate and under oxygen-limiting conditions with a malate yield of 0.48 ± 0.01 mol /mol glucose was obtained which is a 19% increase as compared to the native strain. Chen et al., [41] developed an engineered *S. cerevisiae* strain W4209 by incorporating three genes responsible for MA production namely pyruvate carboxylase gene (*pyc*) from *Aspergillus flavus*, malate dehydrogenase gene (*mdh*) from *R. oryzae*, and gene for L-malic acid transporter (*mae*) isolated from *S. pombe*. The resultant engineered strain produced L- MA with a maximum yield of 0.30 g/g of glucose and the titer and productivity was reported to be 30.25 g/L and 0.32 g/L/h respectively.

2.3.4 Glutaric acid

Glutaric acid (GA) (Pentanedioic acid) is a linear 5-carbon α , ω -DCA and its two ketone derivatives are α -ketoglutaric acid (α -KGA) and β -ketoglutaric acid. GA is extensively used in chemical, cosmetic, and healthcare industries as a platform chemical in the manufacturing of polymers (nylons, *polyester polyols*, and *polyamides*) and plasticizers (polyesters and polyvinyl chloride). Among the two forms, α -KGA is commercially important as it finds its applications in the medical field due to its antioxidant properties. The global GA market value is estimated at \$5.3 billion in 2022 and it is anticipated to increase by \$8.63 billion in 2033 with a projected growth rate of 5% CAGR [55].

Kamzolova et al., [56] reported that the wild yeast strain, *Y. lipolytica* VKMY-2412 showed a maximum α -KGA titer of 172 g/L α -KGA. Strategically, limiting the cell growth using thiamine and excess supplementation of nitrogen and ethanol (as carbon source) in culture conditions led to further increased yield of SA by 71.7 g/L along with concomitant enhanced GA production. Following this study, Kamzolova and Morgunov [23] utilized rapeseed oil as a carbon source and reported the maximum α -KGA titer of 102 g/L with the same wild yeast strain. Along with α -KGA synthesis, pyruvate was also produced in a larger amount resulting

in a decreased yield of the targeted DCA, α -KGA. The major challenges are to increase the yield and divert the carbon flux toward the targeted DCAs. To address this problem, Yin et al., [57] engineered *Y. lipolytica* WSH-Z06 strain and developed two strains namely *Y. lipolytica* ScPYC1 (containing pyruvate carboxylase gene (*pyc*) of *S. cerevisiae*) and *Y. lipolytica* RoPYC2 (containing pyruvate carboxylase gene (*pyc*) of *R. oryzae*). Overexpression of this gene regulates the pyruvate carboxylation pathway resulting in decreased pyruvate production from 35.2 to 13.5 g/L and an increased α -KGA production to 62.5 g/L in *Y. lipolytica* RoPYC2 and 53.6 g/L in *Y. lipolytica* ScPYC1.

2.3.5 Itaconic acid

Itaconic acid (IA) (2-methylene,1,4-butanedioic acid) is a five-carbon containing unsaturated DCA. It is a diprotic acid with a density of 1.63 g/mL at 20°C. IA contains two carboxyl groups and conjugated double bonds making it easier to polymerize at different degrees. In 1836, Baup [58] identified IA as a byproduct produced during pyrolytic distillation of citric acid. Later in 1931, Kinoshita et al., [59] were the first research group to biologically synthesize IA using the fungus, *Aspergillus itaconicus*. The biological route for the synthesis of IA starts with glycolysis. Citric acid produced through the TCA cycle is converted to aconitate and further, it is decarboxylated into itaconate by the action of the cytosolic enzyme, *cis*-aconitate decarboxylase. Another biosynthetic pathway involves IA synthesis via citramalic acid, from pyruvate.

In later years, many fungal species (*Aspergillus terreus* [60], *A. niger* [61], *S. cerevisiae* [20], *Ustilago maydis* [62], *Y. lipolytica* [22], *Pichia* sp. [35]) were tapped for their potential to synthesize IA by utilizing different carbon sources starting from glucose [63] to waste cooking oil [22]. In the following years, IA gained popularity as it is used extensively as a super-absorbent, coating agent, and anti-scaling agent and also in the production of chemicals and solvents such as itaconic diamide, 3-methyltetrahydrofuran and 2-methyl 1, 4-butanediol. The

global market value of IA is expected to be \$134.19 million by 2030 compared to its current market value of \$97.75 million [64].

Blazeck and his coworkers [20] employed *in silico* methods to identify the advantageous genetic modifications by sequential knockout of three genes resulting in the alteration of the IA synthetic pathway to enhance its yield. They employed three strategies namely (i) high cell density fermentation; (ii) overexpression of *cis*-aconitic acid decarboxylase gene using Glyceraldehyde 3-phosphate dehydrogenase (GPD) promoter; and (iii) deletion of three genes encoding for cytoplasmic trifunctional C1-tetrahydrofolate (THF) synthase (*ade3*), indoleamine 2,3-dioxygenase (*bna2*), and peroxisomal acyl-CoA thioesterase (*tes1*). The resulting engineered *S. cerevisiae* strain yielded 168 mg/L of IA by utilizing glucose as the carbon source. Due to low IA yield, different gene editing tools were targeted towards the manipulation of different genes in the IA synthetic pathway to substantially increase the IA yield. Sun et al., [35] engineered acidic tolerant, non-conventional yeast species *P. kudriavzevii* using the novel gene-editing tool CRISPR-Cas9 and knocked out two genes coding for isocitrate dehydrogenase and bypassed the intermediate *cis*-aconitate towards IA production. The resulting engineered strain produced 1232±64 mg/L IA through fed-batch fermentation even when the final pH dropped to 3.9. Rong et al., [22] have developed an engineered strain of *Y. lipolytica* by cloning the *cis*-aconitic acid decarboxylase gene (converts *cis*-aconitate to itaconate) of *Aspergillus terreus*. They achieved an IA yield of 54.55 g/L using waste cooking oil as a carbon source and it is the highest IA yield reported to date using yeast cell factories.

2.3.6 Adipic acid

Adipic acid (AA), a hexanedioic DCA, is a 1,4-dicarboxy derivative of butane. It is commercially produced in large amounts for the manufacturing of polymers such as nylon and polyurethane [65]. AA is extensively exploited in many industries as a platform chemical and its market value is increasing annually. It is specifically used in the food industry as an additive,

gelling agent, and flavoring compound. It is also used as a platform chemical for the manufacturing of lubricants, skin care products, health care products, and paper additives. In 2022, the worldwide market for AA was \$4.8 billion and it is expected to increase at a CAGR of 3.8% between 2023 and 2030 [66]. A variety of bacterial and fungal species are investigated for the bioproduction of AA and many strategies are employed to enhance its yield [67]. To accelerate the synthesis rate of AA, the engineered strain *S. cerevisiae* ADP2 was developed by Raj et al., [15] by cloning five different genes responsible for enhancing its production. The cloned genes are transketolase gene (*tkl1*), 3- dehydroshikimate dehydratase gene (*dhs-d*), catechol dioxygenase gene (*cat-do*), protocatechuate decarboxylase gene (*pca-d*) and enoate reductase gene (*er*). This engineered strain utilized glucose as a carbon source and produced AA at a maximum titer of 2.59 ± 0.5 mg/L after 144 h of batch fermentation. Fig. 2 illustrates the engineered metabolic pathway in yeast leading to the biosynthesis of AA. Ju et al., [26] developed a mutant strain of *C. tropicalis* KCTC 7212 lacking acyl CoA oxidase encoding genes (*aox4* and *aox5*) resulting in its increased ability to utilize methyl laurate, a substrate for fatty acid methyl ester production and obtained a maximum yield of 0.34 g/L of AA (5.4-fold higher compared to the wild strain). Further upon fed-batch fermentation, the yield was increased to 12.1 g/L by optimizing the fermentation parameters such as agitation rate, media pH, and substrate concentration.

2.3.7 Glucaric acid

Glucaric acid (GCA) is a naturally occurring aldaric acid and its derivatives can be used as an alternative to AA due to the presence of dicarboxylic acid attached to the sixth carbon. Phosphate is a prominent ingredient in liquid detergents and many countries have banned its usage, due to its negative environmental impact. Currently, many industries replaced phosphate with GCA in the production of liquid detergents, liquid dishwashers, and household cleaning liquid. Derivatives of GCA are used as a deicing agent and corrosion inhibitor in many

industries. Its current global market value is \$1.09 million and it is expected to reach \$1.93 million by 2028 with a 9.9% CAGR [68]

Industrial GCA is produced by the oxidation of glucose in the presence of nitric acid [69]. This process is highly exothermic, produces many toxic by-products, and results in low yield which doesn't meet the current global demand. To make the production process more economical, microbial fermentation using the prokaryotic host *E. coli* was employed. Moon et al., [70] introduced three different genes namely the *myo*-inositol-1-phosphate synthase gene (from *S. cerevisiae*), uronate dehydrogenase gene (from *Pseudomonas syringae*), and *myo*-inositol oxygenase gene (from *Mus musculus*), and constructed the GCA biosynthetic pathway in *E. coli*. This engineered strain produced GCA titer of 0.72 g/L. Later Shiue et al., [71] developed a novel *E. coli* strain with increased activity of these genes using synthetic scaffolds and obtained a maximum titer of 4.75 g/L. However, the titer cannot be increased above 5 g/L due to the acid toxicity created by the accumulation of GCA that limits the activity of the enzymes. Due to this unsatisfactory GCA yield with bacterial fermentation, *E. coli* was replaced by *S. cerevisiae* as a host system for the production of GCA.

Gupta et al., [72] developed two engineered *S. cerevisiae* strains, namely the 'At' strain (containing *Arabidopsis thaliana* derived *myo*-inositol oxygenase gene) and the 'Mm' strain (containing *M. musculus*-derived *myo*-inositol oxygenase gene). A maximum titer of 1.6 g/L and 0.94 g/L was obtained using 'At' and 'Mm' strains respectively, when fermented with glucose enriched with *myo*-inositol as substrate and he demonstrated that the availability of *myo*-inositol is the rate-limiting step in GCA production. Liu et al., [36] developed another engineered yeast strain *P. pastoris* GS115 for GCA production by introducing two genes (i) *myo*-inositol oxygenase gene (from *M. musculus*) and (ii) uronate dehydrogenase gene (derived from *Pseudomonas putida* KT2440). This engineered strain accumulated 6.61 ± 0.30 g/L of GCA through fed-batch fermentation with glucose and *myo*-inositol as substrates. Similar

results were obtained from the engineered *S. cerevisiae* (containing the *myo*-inositol oxygenase gene from *A. thaliana*, and uronate dehydrogenase gene from *P. syringae*). This engineered strain was reported to produce a maximum titer of 6 g/L at 216 h of fed-batch fermentation using glucose as a carbon source [19].

2.3.8 Muconic acid

Muconic acid (MCA) (2,4-hexadiene dioic acid), is a six-carbon containing DCA and occurs in three different isomeric forms namely *cis*, *cis*-MCA, *trans*, *trans*-MCA, and *cis*, *trans*-MCA due to the presence of two double bonds in the middle of its structure. It has high potential as the precursor for the synthesis of many value-added chemicals and intermediates such as 3-hexenedioic acid, and terephthalic acid (TPA) for the production of bioplastics and polyesters. The current global market value of MCA is \$102.5 million and it is expected to reach \$138.9 million by 2028 with a 5.9% CAGR [73].

De novo synthesis of MCA starts from the intermediate of the shikimate pathway. The intermediate, 3-dehydroshikimate is converted to MCA by the action of three enzymes namely 3-dehydroquinate dehydratase, protocatechuic acid decarboxylase, and catechol dioxygenase. Draths and Frost [74] synthesized bio-based MCA using mutant *E. coli* that lacked shikimate dehydrogenase showed the increased synthesis of 3-dehydroshikimic acid, and further synthesis was enhanced by the introduced plasmid encoding genes for the enzymes transketolase, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase, and 3-dehydroquinate synthase. MCA yield was found to be elevated under acidic conditions. A quest for a more potent host organism was started, as *E. coli* cannot thrive well under acidic environments and the complicated expensive downstream processes due to high salt addition. *S. cerevisiae* is preferred for MCA production as it is used for the production of many valuable chemicals and also due to its ability to thrive under acidic and hyperosmotic conditions. Weber et al., [75]

implemented *S. cerevisiae* for the first time for MCA production but the yield (0.78 mg/g glucose) was not very promising.

Curran et al., [76] engineered *S. cerevisiae* for MCA production by cloning three different genes namely (i) dehydroshikimate dehydratase encoding gene from *Podospora anserina*, (ii) protocatechuic acid decarboxylase encoding gene from *Enterobacter cloacae*, and (iii) catechol1,2-dioxygenase encoding gene from *C. albicans*. Wang et al., [77] developed the engineered *S. cerevisiae* ST10209 strain by manipulating its shikimate pathway thereby increasing the supply of phosphoenolpyruvate to enhance MCA yield. This strain utilized glucose as a carbon source and produced 22.5 g/L and 20.8 g/L *cis*, *cis*-MCA in 2 L and 10 L fermenters respectively. Through efficient downstream processing, 95.4% pure MCA was recovered. It is the highest-recorded MCA production to date using the engineered yeast strain.

3 DCAs and their commercial applications

DCAs are highly used in a wide spectrum of industries such as food, pharmaceutical, agricultural, textile, automobile, and chemical industries. Fig. 3 shows the commercial importance of DCA and the corresponding global manufacturers. Properties like flexibility, durability, strength, and anti-corrosive nature increase the commercial value of DCAs. Some of the predominant industries that depend on DCAs in terms of raw materials and value addition have been discussed in subsequent sections.

3.1 Food industries

FA is used in food industries for its preservative and flavor-enhancing properties. It is used as a buffering agent to maintain and adjust the acidic and alkaline nature of food. Bio-based FA is approved as a food additive under food safety guidelines. FA is also used in the preparation of animal feed and can reduce up to 70% of methane emissions [43]. MA gives a mild acidic taste to food and is extensively used in the beverage industry to enhance the taste of soft drinks. Food industries are focused on the utilization of organically derived products to minimize the

negative impacts of chemicals on human health [78]. To overcome the drawbacks of using plastics for food packaging, biopolymers are used in the production of food packaging materials. FA is a prominent platform chemical for the production of polyester resin upon polymerization and these resins are used as a plasticizer in food packaging. These packaging materials are not only biodegradable but also do not affect the quality of the stored food material with increased shelf-life. Central furan ring containing DCA, 2,5-furan-dicarboxylic acid is polymerized with monoethylene glycol to produce the durable polyethylene furanoate (PEF). This polymer is used as packaging material in beverage industries [79].

3.2 Pharmaceutical industries

IA is used in the preparation of polymers with super-absorbent properties due to its cross-linking properties. These super-absorbent polymers are utilized in the manufacturing of sanitary pads, diapers, and bed pads [2]. FA is applied in pharmaceutical industries in the preparation of lotion and creams for the treatment of skin conditions like psoriasis. In addition to FA, azelaic acid is highly exploited in pharmaceutical industries for its antimicrobial and anti-inflammatory properties. It is used to treat acne problems and the skin condition called rosacea. It helps to lessen the hyperproduction of fibrous protein, and keratin and thus reduces inflammation and erythema of the skin. FA is used as a precursor in the manufacturing of ferrous fumarate, which is an important ingredient in the preparation of medicines to treat anemia. Derivatives of GCA are therapeutically utilized to reduce obesity by lowering cholesterol levels in the blood and to treat cancer.

3.3 Agricultural industries

DCA plays a vital role in the agricultural industry in the manufacturing of commercially important herbicides [80]. One of the most highly utilized DCAs in this field is IA. It finds its application in ornamental gardens for root dipping and as a seed coating. Sebacic acid is used in the preparation of cold-resistant films (dioctyl sebacate), which are highly used in the

agricultural industry. SA is used as a plant growth stimulator and its succinic acid-2,2-dimethyl-hydrazide (daminozide) derivative is highly exploited as a bioactive compound in the preparation of plant growth regulators [81].

3.4 Chemical industries

Pimelic acid is employed to produce many commercially important intermediates, that are used in the preparation of lubricants, adhesives, heat-conducting oil and plasticizers. Sebacic acid is used in the manufacturing of lubricant oil (hexyl hexaacetate) which can withstand high temperatures. Sebacic acid and FA are used to synthesize high-strength polyamides which is applied in the manufacturing of durable tires and tire cords [82]. SA is utilized as a building block for the manufacture of polyesters such as polyethylene terephthalate, polyethylene, polylactic acid, and polypropylene [83]. Azelaic acid plays an important role in the automotive and electronics industries. It is used as an intermediate for the synthesis of polyesters, plasticizers, polyamides, hydraulic fluids, and lubricants [84]. GA is chemically transformed into dibasic esters and used as a solvent. It is also used as a water refreshing agent. IA is utilized in the manufacturing of detergent as a substitute for sodium tripolyphosphate [85]. IA and 2,5-furan dicarboxylic acid are commercially used in the production of bio-based resin. These resins possess anti-bacterial, anti-corrosive, and thermal stability properties and are employed widely in the manufacturing of floor coatings, adhesives, electrical insulators, composting matrices, and paint brushes [86].

4 Barriers and strategies to enhance DCA production

The primary obstacles and difficulties in bio-based DCA production encompass (i) the accessibility of affordable renewable feedstock, (ii) identifying organisms with optimal productivity, and (iii) fine-tuning fermentative parameters. These challenges are elaborated upon in detail in the following sections.

4.1 Feedstock-based challenges

Since the chemical synthesis of DCA is highly energy-consuming and costly, biological synthesis makes the overall process cost-effective, sustainable, and eco-friendly. Simple sugars and fatty acids are the conventional choice as a carbon source. However, to maintain sustainability, the choice of substrate moves from simple carbon sources to cheap renewable feedstocks such as agro-industrial wastes, lignocellulosic, and municipal wastes [1, 2, 39]. This has the potential to enhance the economic feasibility of large-scale industrial production and facilitate the effective utilization of agricultural residues. To valorize the sugar from these organic and lignocellulosic feedstocks, treatment strategies such as physical, chemical, biological, or combined methods are being employed [87]. Some pretreatment methods especially chemical methods generate toxic breakdown products that could hinder the growth of the organism. These toxic inhibitory products should be neutralized and removed to prevent their negative impact on the fermentation. To withstand the toxic effects, organisms are gradually exposed to increasing concentrations of inhibitory products through the process of adaptive laboratory evolution [88].

4.2 Organism-based challenges

The selection of organisms plays a crucial role and is based on the ultimate application of the produced DCA. For food and pharmaceutical applications, the organism should have GRAS status. Bacteria and filamentous fungi show many downsides (discussed in the Introduction section), and to counteract yeast become a promising alternative. Conventional yeast *S. cerevisiae* has been used in the synthesis of many value-added chemicals for more than two decades. High production titer and yield are the main targets of industrial production. Different microbial strains that are currently utilized or that are planned to be used need continuous genetic/metabolic improvement to attain high production rates to maximize the yield. Since DCAs are not primary metabolites and are produced as intermediates, major attempts are made

to divert the carbon flux towards the production of DCAs. In hands with the conventional genetic manipulation techniques, various system biology strategies such as omics analysis, *in silico* analysis, genome-scale metabolic modeling, and CRISPR Cas9 mediated gene knockout could augment the efficiency and reliability of strain improvement for DCAs bioproduction [89, 90]. Genetic engineering approaches also offer cutting-edge strategies for targeting the following four major areas of concern: i) carbon fluxing; ii) increasing resistance to toxic inhibitory compounds; iii) enhancing the substrate uptake and overcoming feedback inhibition; iv) high DCA titer. The resulting strain may become an efficient DCA producer but this diversion of carbon flux will take a toll on the growth, proliferation, and overall metabolism of the organism that has to be circumvented through metabolic re-wiring.

4.3 Process-based challenges

In addition to strain improvement strategies, modifications in fermentation parameters and upstream and downstream processing play an important role in enhancing the productivity of DCAs. As the fermentation duration increases, the pH of the broth becomes low due to the accumulation of DCA [35]. The selection of osmotic and acidic tolerant strains can counteract this issue. Monitoring of foam formation is an important hurdle in the fermentation process. Excess foaming leads to reactor overflow and contamination [91]. Foaming should be controlled using lesser toxic compounds like mineral oil. Different downstream techniques such as cell disruption, solid-liquid separation, thermal pretreatment, filter-press, nanofiltration, ultrafiltration purification, product stabilization, and formulation are studied extensively to maximize the recovery percent of the product from the fermentation broth [92, 93]. The selection of different downstream process techniques is based on the following parameters such as types of microbial strain, physical and chemical properties of the product (intra or extracellular or both), fermentation process, media composition, and required end-product purity [94].

5 Conclusion

DCA holds a significant role as a high market value intermediate or precursor for the production of various products. To compete with the environmental crisis of fossil fuel-derived chemicals, many industries are focused on the biological method of production of DCAs on a large scale. Biobased production of DCA is a promising alternative to its chemical synthesis strategy and could be cost-effective due to the use of cheap renewable resources as substrate. The use of cellulosic wastes is economical and environmentally amicable and makes as a better substitute for fossil fuel-derived alternatives. An important challenge is the selection of the pretreatment strategy and its optimization to increase the simple sugar yield from these feedstocks and to make the DCA production process more economical. The high growth rate of yeast even in unconventional substrates and acidic conditions makes it the best choice as a host organism for DCA production. To maximize the yield and productivity advanced metabolic engineering techniques are employed aimed at producing efficient engineered yeast strains. On the contrary, these engineered strains should pass through the government food safety guidelines, which is essential in the marketing aspects of DCAs, especially for food and pharmaceutical use. Substantial research work in DCA over decades is inevitable and should focus on the development of novel yeast strains through genetic engineering approaches and optimization of fermentative conditions aimed at maximizing DCA yield to meet the demand.

Author Contribution

Ramalingam Kayalvizhi and Jayacumar Sanjana equally share the first authorship and are involved in the literature collection, drafting of the manuscript, and illustration preparations. Vinod Kumar provided valuable inputs and critical suggestions for manuscript improvement. Samuel Jacob is responsible for the technical inputs and finalization of the manuscript.

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Data Availability

There is no original data associated with this review manuscript and the relevant information provided has been cited appropriately.

Declarations

Ethics Approval and Consent to Participate Not applicable.

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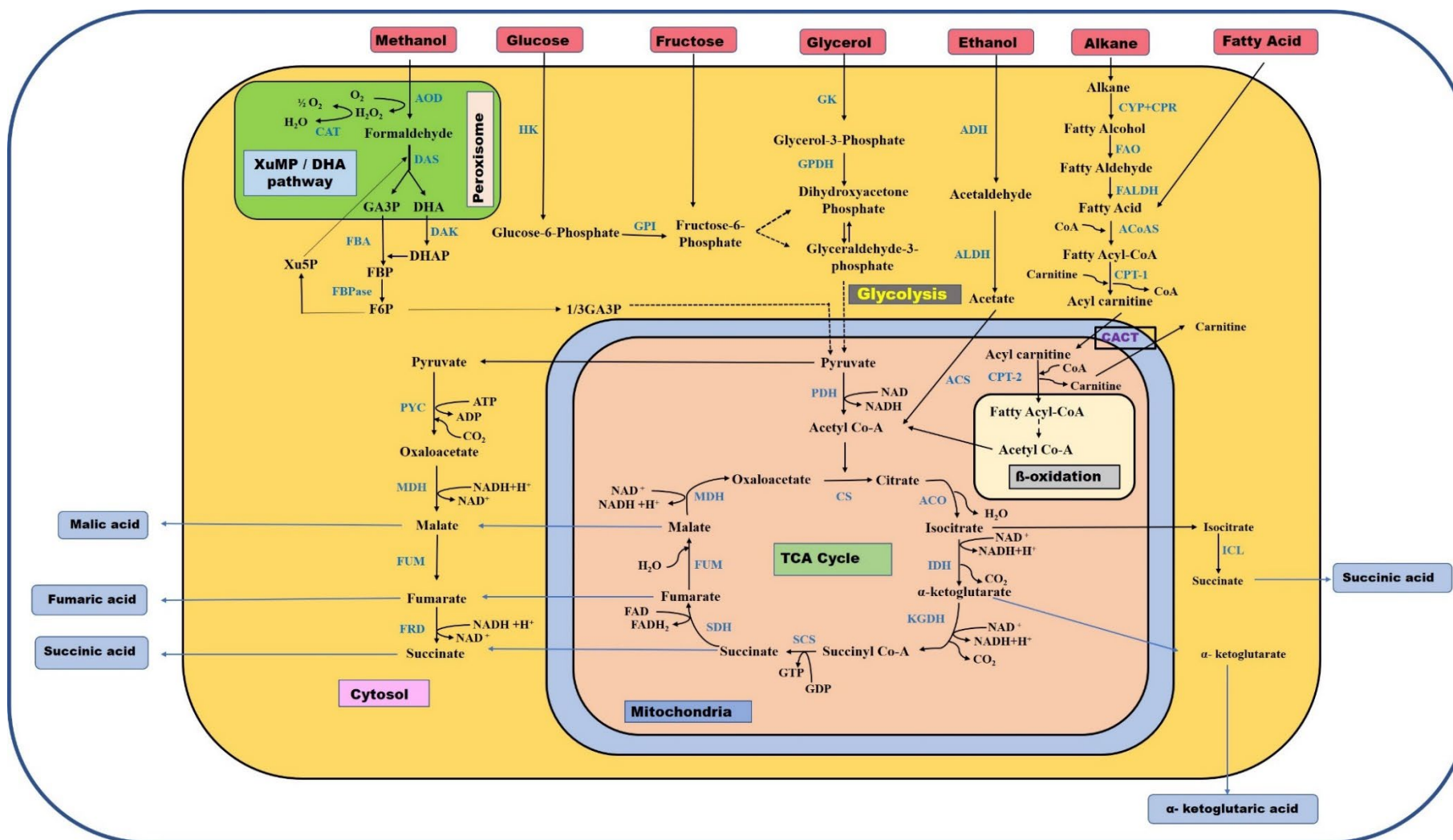


Fig. 1 Schematic representation of biosynthesis pathway of selected DCAs such as succinic acid, α -ketoglutaric acid, malic acid, and fumaric acid

913 Abbreviations for the enzymes and cofactors are as follows: **ACO**- Aconitase; **ACoAS**- Aceyl Co-A Synthase; **ACS**- Acetyl Co-A synthetase; **ADH**-
914 Alcohol dehydrogenase; **ADP**- Adenosine diphosphate; **ALDH**- Aldehyde dehydrogenase; **AOD**- Alcohol oxidase; **ATP**- Adenosine triphosphate;
915 **CACT**- Carnitine acylcarnitine translocase; **CAT**- Catalase; **CPR**- Cytochrome P450 reductase complex; **CPT1**- Carnitine palmitoyltransferase I;
916 **CPT2**- Carnitine palmitoyltransferase II; **CS**- Citrate synthase; **CYP** - Cytochrome P450 monooxygenase; **DAK**- Dihydroxyacetone kinase; **DAS**-
917 Dihydroxyacetone synthase; **DHA**- Dihydroxy acetone; **DHAP**- Dihydroxy acetone phosphate; **F6P** – Fructose-6-phosphate; **FAD** - Flavin Adenine
918 Dinucleotide (oxidized form); **FADH** - Flavin Adenine Dinucleotide (reduced form); **FALDH**- Fatty aldehyde dehydrogenase; **FAO**- Fatty alcohol
919 oxidase; **FBA**- Fructose-1,6-bisphosphate aldolase; **FBP**- Fructose-1,6-bisphosphate; **FBPase**- Fructose-1,6-bisphosphatase; **FRD**- Fumarate
920 reductase; **FUM**- Fumarase; **GA3P**- Glyceraldehyde-3-Phosphate; **GDP**- Guanosine diphosphate; **GK**- Glucokinase; **GPDH**- Glucose-3-phosphate
921 dehydrogenase; **GPI**- Glucose-6-phosphate isomerase; **GTP**- Guanosine triphosphate; **HK**- Hexokinase; **ICL**- Isocitrate lyase; **IDH**- Isocitrate
922 dehydrogenase; **KGDH**- α -ketoglutarate dehydrogenase; **MDH**- Malate dehydrogenase; **NAD⁺** - Nicotinamide adenine dinucleotide (oxidized
923 form); **NADH** - Nicotinamide adenine dinucleotide (reduced form); **PDH**- Pyruvate dehydrogenase; **PYC**- Pyruvate decarboxylase; **SCS**- Succinyl
924 Co-A dehydrogenase; **SDH**- Succinate dehydrogenase; **Xu5P**- Xylulose-5-phosphate; **XuMP** - Xylulose monophosphate.

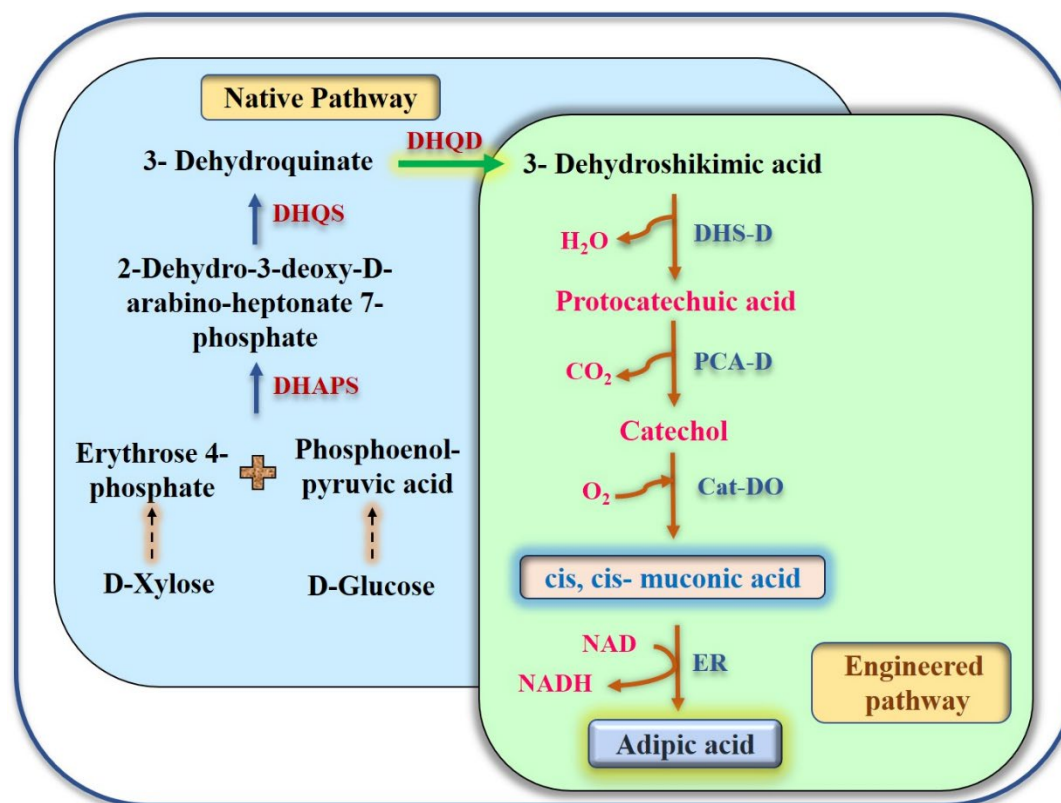


Fig. 2 Biosynthetic pathway of adipic acid in yeast

Abbreviations for the enzymes are as follows: **DHS-D** - 3-Dehydroshikimate dehydratase; **PCA-D** -Protocatechuate decarboxylase; **Cat-DO** - Catechol 1,2-dioxygenase; **ER**-Enoate reductase; **DAHPS**- 2-dehydro-3-deoxy-D-arabino-heptonate 7-phosphate synthase; **DHQS**- 3-Dehydroquinate synthase; **DHQD**- 3- Dehydroquinate dehydratase.

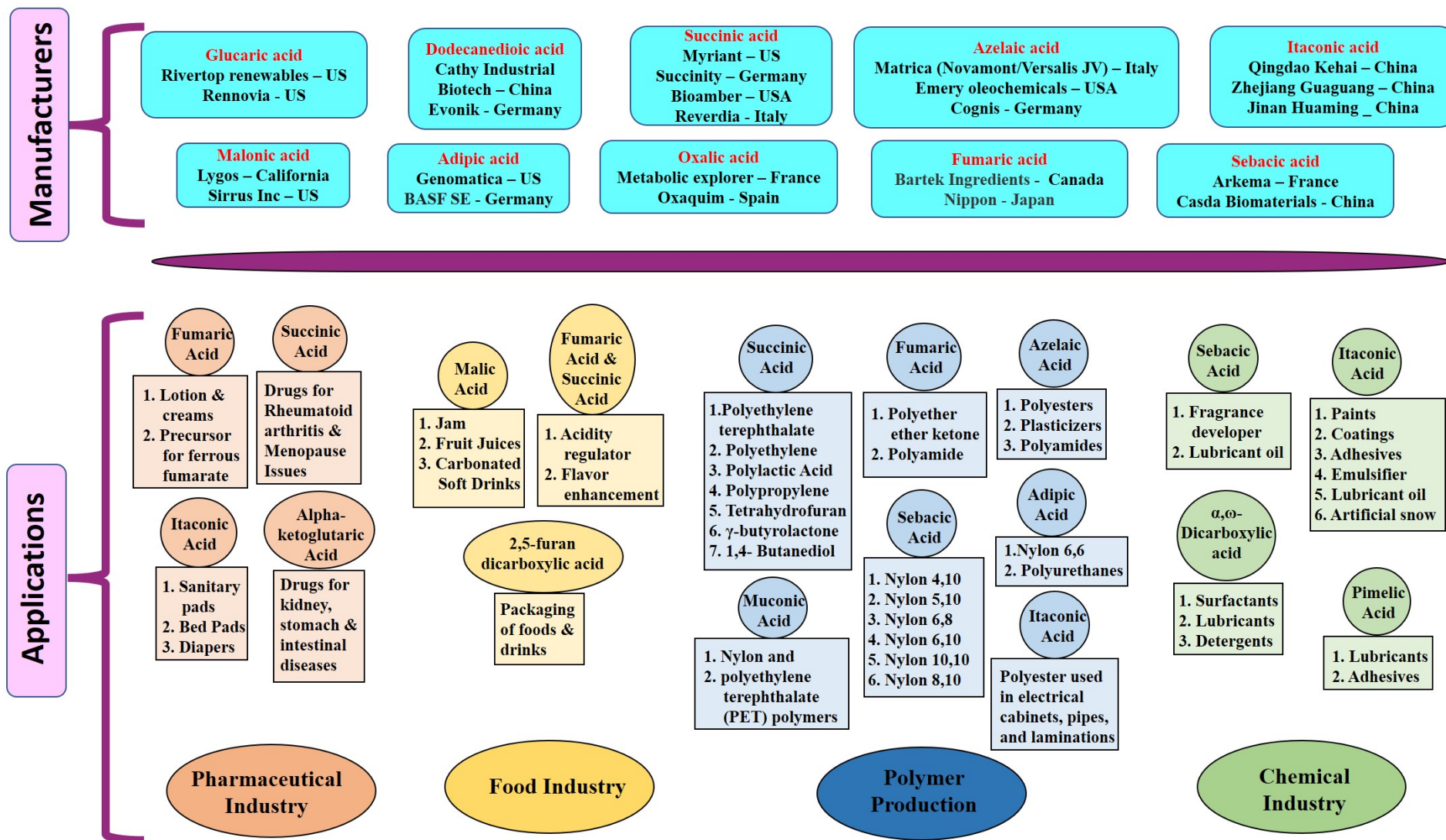


Fig. 3 Selected manufacturing companies and applications of dicarboxylic acids

Table 1 Genetic engineering approaches of selected yeast strains for increased dicarboxylic acids yield

Dicarboxylic acid	Strain	Genetic approach	engineering used	Substrate used	Substrate Conc. (g/L)	Titre (g/L)	Yield (g/g)	Productivity (g/L/h)	References
Succinic acid	<i>Saccharomyces cerevisiae</i> FA1	Deletion of glycerol phosphate dehydrogenase gene (<i>gpd1</i>) and Fumarate hydratase gene (<i>fum1</i>)		Glucose	50	1.96	0.17	4×10 ⁻⁵	[95]
		Multi-copy integration of the native pyruvate carboxylase gene (<i>pyc2</i>)							
L-Malic acid	<i>S. cerevisiae</i> TAM	Cloning of pyruvate carboxylase gene (<i>pyc</i>) from <i>Aspergillus flavus</i> and malate dehydrogenase		Glucose	100	30.25	0.30	0.32	[41]

gene (*mdh*) from *Rhizopus*

oryzae

Removing the

ubiquitination

modification of L-malic

acid transporter (*mae*)

from

Schizosaccharomyces

pombe

<i>Pichia</i>	<i>pastoris</i>	Cloning of pyruvate	Methanol	-	2.79	-	-	[96]
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CM301	carboxylase gene (<i>pyc</i>)
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and malate dehydrogenase

gene (*mdh*) from *R. oryzae*

and C4 dicarboxylate

transporter gene (*mae*)

from *S. pombe*

Adipic acid	<i>S. cerevisiae</i> ADP2 strain	Cloning of transketolase gene (<i>tkl1</i>), 3-Dehydroshikimate dehydratase gene (<i>dhs-d</i>), catechol dioxygenase gene (<i>cat-do</i>), protocatechuate decarboxylase gene (<i>pca-d</i>) and enoate reductase (<i>er</i>)	Glucose	20	2.59 × 10 ⁻³	-	-	[15]
Glucaric acid	<i>S. cerevisiae</i> CEN.PK2-1D	Cloning of <i>myo</i> -inositol-1-phosphate synthase (<i>ino1</i>) from <i>S. cerevisiae</i> , uronate dehydrogenase (<i>udh</i>) from <i>Pseudomonas syringae</i> , phosphatase gene from <i>Escherichia coli</i> , <i>myo</i> -	Glucose	20	1.6	0.033	-	[72]

		inositol oxygenase gene (<i>miox</i>) from <i>Mus musculus</i> and <i>Arabidopsis thaliana</i>							
Glucaric acid	<i>S. cerevisiae</i> BY4741	Co-expression of uronate dehydrogenase (<i>udh</i>) from <i>Pseudomonas syringae</i> and <i>myo</i> -inositol oxygenase gene (<i>miox4</i>) from <i>Arabidopsis thaliana</i>	Glucose	30	6	-	-		[97]
Itaconic acid	<i>S. cerevisiae</i> iMM904	Cloning of <i>cis</i> -aconitic acid decarboxylase gene (<i>cada</i>) and plasma membrane transporter gene (<i>mfsa</i>) from <i>Aspergillus terreus</i>	Glucose	15	0.815	-	-		[98]

Overexpression of native
aconitase gene (*aco*),
pyruvate carboxylase gene
(*pyc*) and citrate synthase
gene (*csc*)

<i>cis, cis</i> -muconic acid	<i>S. cerevisiae</i> MuA10	Overexpression of gene coding for aromatic decarboxylase multifunctional enzyme (<i>aroit</i>) and phenylacrylic acid decarboxylase (<i>pad1</i>)	Glucose	160	2.1	0.013	-	[99]
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Other DCAS

Sebacic acid	<i>C. tropicalis</i> ATCC20962	-	Methyl decanoate	0.35	27	-	0.5	[16]
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Dodecanedioic acid	<i>Wickerhamiella sorbophila</i> UHP4	β -oxidation pathway was blocked completely homologous recombination efficiency at <i>pox1</i> and <i>pox2</i> loci was increased	Methyl laurate and	-	92.5	-	0.83	[14]
Long chain DCAs	<i>C. tropicalis</i> ATCC20962	β -oxidation pathway was blocked by knocking out genes acyl-CoA-oxidase gene (<i>pox</i>)	Chocolate water	100	5.8			[100]



DSPACE

<https://dspace.org/>

An eclectic review on dicarboxylic acid production through yeast cell factories and its industrial prominence

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