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Recent Advances in Itaconic Acid Production from Microbial Cell Factories

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Abstract

Itaconic acid is an unsaturated organic acid with two carboxyls and one methylene group. The presence of these functional groups, along with a conjugated double bond, makes itaconic acid a versatile molecule with a vast number of applications. Itaconic acid can be produced through chemical as well as biological routes. *Aspergillus terreus* is the most prevalent microbial cell factory for the biological production of itaconic acid, reaching titers of >100 g/L. However, it suffers from low yield and volumetric productivities leading to high manufacturing costs. The wider applications of itaconic acid can be enabled with a low-cost production process, which can be achieved with cheaper feedstocks and robust cell factories accumulating itaconic acid efficiently. The current review summarizes the recent advances in the biological production of itaconic acid with a focus on the metabolic engineering of prokaryotic and eukaryotic systems for the overproduction of itaconic acid. It comprehensively describes various microbial cell factories with an insight into the pathway leading to itaconic acid production in natural producers like *A. terreus* and *U. maydis*. It also discusses the metabolic engineering approaches to improve strain performance in terms of high itaconic acid productivity, less by-product generation, and the ability to utilize unconventional cheap substrates. Moreover, the alternative strategies for the development of non-native producers through genome engineering and the hurdles related to itaconic acid production have been elaborated.

Keywords:

Itaconic acid, metabolic engineering, bioprocess engineering, polymer industry.

1. Introduction

In the past few decades, there has been extensive research regarding the use of micro-organisms as cellular factories for the biosynthesis of commercially significant chemicals (Dhamankar and Prather, 2011). These microbial factories provide an economical and easy synthetic route with the ability to grow on renewable feedstocks. They require fewer steps involving lesser chemicals and energy consumption as compared to tedious petrochemical routes. Moreover, microbial biosynthesis is a green alternative route to petroleum-based production of value-added chemicals, which could solve the age-old problem of fossil fuel depletion (Pfaltzgraff and Clark, 2014; Sheldon, 2014). These microbial factories can utilize inexpensive materials viz. renewable feedstocks and organic wastes as substrates in the biorefineries, making the production of biofuels and value-added chemicals more cost-effective and eco-friendly (Pfaltzgraff and Clark, 2014).

Itaconic acid is a biosynthetically derived natural chemical that can be a sustainable and non-toxic substitute for many petroleum-derived monomers to produce commercially significant polymers (Ray et al., 2017; Sriariyanun et al., 2019). Itaconic acid and its derivatives, owing to their easily polymerizable chemical structure with two carboxylic groups and a double bond, have the potential to be used as versatile building blocks for the manufacture of polymeric products like paints, adhesives, plastics, paper, resins, textiles, super-absorbent polymer, etc. (Kumar et al., 2017; Sano et al., 2020). It is industrially produced in biorefineries through microbial fermentation of fungi. *Aspergillus terreus* and *Ustilago maydis* are native producers of itaconic acid and have specialized gene cluster for its biosynthesis (Wierckx et al., 2020).

The demand and production of environment-friendly products through biotechnological routes is tremendously increasing, and itaconic acid is no exception. The itaconic acid market is majorly concentrated in the Asia Pacific, contributing over 54% of the global demand, followed by Europe and North America. A significant portion of the itaconic acid market is based on the production of SBR (Styrene-butadiene rubber) latex, methyl methacrylate, and polyitaconic acid. The global itaconic market was around $75 million in 2015; it reached $95.4 million in 2021 and is estimated to reach ~$108.4 million by 2026 (Market Data Forecast, 2020; Global Market Insights, 2016).

Though the itaconic acid market is attaining significant growth with the growing popularity of sustainable chemicals, usage of itaconic acid in products of day-to-day applications lags much behind petroleum-based products. Further development of the itaconic acid market for wide-scale production and consumption of its products as an economically viable replacement of petroleum-based products requires lowering its production cost to at least $0.5/kg from the current cost of $1.5-2.0/kg (Klement and Büchs, 2013a; Yang et al., 2019). Hence, there is a need to design microbial cell factories with efficient fermentation routes enabling high-level itaconic acid production on low-cost substrates. Such hyper-accumulating itaconic acid cell factories can be designed with the aid of synthetic biology, metabolic, evolutionary, and bioprocess engineering approaches (Bafana and Pandey, 2018).

Metabolic engineering of the microbial cell factory could be a critical step for improving the metabolic flux towards the itaconic acid (Liu et al., 2017). Additionally, the genes responsible for itaconic acid production could be engineered into non-producing strains to incorporate productivity into them (Saha, 2017). Several studies are being carried to target different aspects of metabolic engineering for strain improvement, which includes fine-tuning of gene expression, by-products elimination, sub-cellular compartmentalization, and transport regulation (Li et al., 2021).

The current review comprehensively summarizes ongoing advancements in the bio-based production of itaconic acid. The review provides an insight of various microbial cell factories with a focus on the itaconic acid pathway and metabolic engineering work carried out among natural producers like fungi *A. terreus* and *U. maydis* to improve strain quality in terms of high itaconic acid productivity, less by-product generation, and ability to utilize unconventional cheap substrates. Furthermore, various strategies adopted to generate alternative non-native producers by engineering their genome to overcome the hurdles associated with the wild-type itaconic acid producers are well described. Finally, the review includes the challenges associated with microbial itaconic acid production and future perspectives.

2. Early studies on biotechnological itaconic acid production

The discovery of itaconic acid is dated back to 1836 when Baup discovered it as a compound obtained through thermal decomposition of citric acid (Baup, 1836). Kinoshita first reported the microbial synthesis of itaconic acid in 1931 and the fungal species which produced it was named *Aspergillus itaconicus* (Kinoshita, 1931). In 1939, itaconic acid production was reported in *A. terreus,* and the initially obtained yield was 0.12 g/g (Calam et al., 1939). To date, *A. terreus* is considered the most prominent producer of itaconic acid and is majorly employed for its industrial production (El-imam and Du, 2014; Willke and Vorlop, 2001). In 1945, a high yield strain, *A. terreus* NRRL 1960, was identified by screening several strains in the Northern Regional Research Laboratory (NRRL) of the United States Department of Agriculture. This strain, when employed for itaconic acid production from glucose, generated 42% (0.3 g/g) of the theoretical yield (0.72 g/g) (Cunha da Cruz et al., 2018; Lockwood and Ward, 1945).

In collaboration with Pfizer and Co. Inc., a research group filed the first patent for the 0generation of itaconic acid through aerobic fermentation that could be applicable for commercial production (Kane et al., 1945). Nelson et al. (1952) extended the production process to a semi-pilot plant scale using glucose substrate in 20 L fermentors. Pfeifer et al. (1952) carried out pilot plant experiments in 600 gallons fermentors to produce itaconic acid by fermentation of corn sugar and product recovery in crystalline form. The first plant for industrial synthesis was established in Brooklyn by Pfizer Co. Inc. in 1955 (Okabe et al., 2009). For the next few decades, the interest in itaconic acid declined but was later regained with increasing environmental concerns related to the use of petroleum-based products (Willke and Vorlop, 2001).

Historically, itaconic acid production was based on synthetic chemical routes, which included pyrolysis of citric acid, decarboxylation of aconitic acid through heating, carboxylation of acetylene, oxidation of isoprene, oxidation of mesityl oxide, etc. However, these routes are tedious, non-sustainable, and unfeasible for commercial production with a negative impact on the environment (da Cruz et al., 2017). On the other hand, the biotechnological route was found to be more promising in terms of yield, production rate, sustainability, simplicity, energy requirement, and other economic factors (Kuenz and Krull, 2018; Willke and Vorlop, 2001).

According to Van Dien, bio-based methods for the commercial production of valuable chemicals should be competitive enough to beat conventional fossil-based manufacturing methods. For a bio-based production process to stand firm in the commercial race, it should at least generate a final titer of 50 g/L, 80% of theoretical yield, and productivity of 2 g/L/h (Van Dien, 2013). There have been numerous biotechnological studies based upon bioprocess engineering and genetic engineering of various microbial systems to enhance the production efficiency and economic viability of itaconic acid fermentation (De Carvalho et al., 2018; Kuenz and Krull, 2018). Later sections describe such reports in detail.

3. Gene cluster for itaconic acid biosynthesis in microorganisms

Natural itaconic acid producers have specialized gene cluster which encodes for the enzymes and transporter proteins participating in the biosynthesis and secretion of itaconic acid. This gene cluster serves as the primary target for metabolic engineering to enhance productivity in natural producers. Table 1 shows all the genes and encoded enzymes related to microbial itaconic acid production. The intermediates of the TCA cycle (citric acid and cis/trans-aconitate) are precursors for itaconic acid, and the whole biosynthetic process involves subcellular compartmentalization and shuttling between those compartments at different steps (Steiger et al., 2016b; Wierckx et al., 2020).

The prototype version of the metabolic pathway for converting glucose into itaconic acid via glycolysis and TCA cycle was presented by Bonnarme et al. (1995). Pyruvate is made from glucose through glycolysis in the cytosol and then transported to the mitochondria, where it is transformed to acetyl-CoA by releasing CO_2. In the TCA cycle, acetyl-CoA and oxaloacetate are catalyzed into citrate, which further converts into *cis*-aconitate in the mitochondria. Bentley and Thiessen (1957) proposed that itaconic acid is formed by decarboxylation of *cis*-aconitate, a TCA cycle intermediate, and this theory was supported by Winskill (1983). The enzyme *cis*-aconitase decarboxylase (CAD), i.e., responsible for bioconversion of *cis*-aconitate to itaconic acid in *A. terreus*, is localized in the cytosol (Jaklitsch et al., 1991). Therefore, cis-aconitate has to travel from mitochondrion to cytosol for being acted upon by cis-aconitase. Dwiarti et al. (2002) isolated and characterized this enzyme from *A. terreus* and reported that the higher activity of this enzyme directly relates to higher itaconic acid accumulation. It can also be

introduced into the genome of non-itaconic acid-producing micro-organisms having TCA cycle to incorporate the ability to produce itaconic acid into them (Li et al., 2011). The metabolic pathways followed in *A. terreus* and *U. maydis* are slightly different, but the transport mechanism involved is quite similar (Hosseinpour Tehrani et al., 2019b; Wierckx et al., 2020). Fig.1a shows a diagrammatic representation of the biosynthetic pathway along with the enzymes and transport proteins responsible for itaconic acid synthesis in *A. terreus* and *U. maydis*. Fig.1b shows the chief biochemical reaction which differentiates the biosynthetic pathway of these species.

The key gene of the itaconic acid gene cluster that encodes for CAD enzyme (*cadA*) was identified and characterized from *A. terreus* and cloned in other non-producing microbes (Kanamasa et al., 2008; Li et al., 2011). The *cis*-aconitate needs to be transported from mitochondria to the cytosol, where it is catalyzed into itaconic acid by the action of the CAD enzyme. In the gene cluster, the *mttA* gene, which is located adjacent to the *cadA* gene, is transcriptionally upregulated in itaconic acid-producing conditions in *A. terreus*. This gene encodes for mitochondrial tricarboxylate transporter protein (MttA), which helps in translocating *cis*-aconitate across the mitochondrial membrane (Li et al., 2011; Steiger et al., 2016a). The third gene of the gene cluster, *mfsA* encodes for a membrane permease belonging to the major facilitator superfamily responsible for the secretion of the produced itaconic acid in the extracellular environment. A binuclear zinc finger domain is present next to it, a transcriptional regulator of genes in the cluster (Klement and Büchs, 2013a; Li et al., 2011; Steiger et al., 2016b).

The biosynthetic pathway of itaconic acid in *U. maydis* was elucidated by Geiser et al. (2016c) through genome analysis. In the *U. maydis* gene cluster, there exists a homolog of *mttA* with a 35% sequence similarity called *mtt1*. The *mtt1* gene encodes for the mitochondrial carrier protein, which shuttles *cis*-aconitate to the cytosol. It was found that there was no *cis*-aconitate decarboxylase in the *U. maydis* gene cluster as in the case of *A. terreus,* and instead of direct decarboxylation of *cis*-aconitate, itaconic acid is formed via *trans*-aconitate. The *trans*-aconitate is a more thermodynamically favored isomer of aconitate, and the isomerization is catalyzed by aconitase-Δ-isomerase, encoded by gene *adi1,* in the cytosol. Novel cytosolic enzyme *trans*-aconitate decarboxylase (encoded by *tad1*) is responsible for decarboxylation of *trans*-aconitate

to itaconic acid. Itaconate transporter protein (encoded by *itp1*), belonging to the primary facilitator super family, transports the synthesized itaconic acid across the cell membrane. Also, a gene (*ria1*) regulating the expression of other genes in the cluster was identified. This gene was assumed to be itaconate pathway-specific transcription factor, and overexpression of this gene could be instrumental in enhancing itaconic acid production in related species (Geiser et al., 2016c; Geiser et al., 2018). Adjacent to the genes involved in itaconic acid biosynthesis, *U. maydis* also has two other genes (*cyp3*, *rdo1*), which cause further conversion of itaconic acid to other by-products. *Cyp3* encodes for P450 monooxygenase of the CYP504 family, which converts itaconic acid into 2-hydroxyparaconate, a known by-product of many *Ustilago* sp. Overexpression of this gene decreases itaconic acid production. On the other hand, *rdo1* encodes for ring-cleaving di-oxygenase, which catalyzes the conversion of 2-hydroxyparaconate to itatartarate (Geiser et al., 2016c; Steiger et al., 2016b). Some studies have speculated the presence of homologs of these genes (*cypC*, *rdoA*) in some strains of *A. terreus* (Hosseinpour Tehrani et al., 2019b; Steiger et al., 2016b). The presence of this gene cluster was reported in other itaconic acid-producing *Ustilago* sp. also (Geiser et al., 2016a).

4. Native itaconic acid-producing fungi

4.1 *Aspergillus* strains

Aspergillus is a genus of filamentous fungi which are commonly employed for the industrial production of organic acids like itaconic, gluconic, kojic, and citric acids (Bennett, 2010; L. Yang et al., 2017). The biotechnology of itaconic acid biosynthesis by *Aspergillus* strains, especially *A. terreus,* has been intensely studied and applied at laboratory and industrial scale using various substrates and processing conditions.

Since 1955, itaconic acid is being manufactured through glucose fermentation by *A. terreus* owing to its excellent synthesis ability (Okabe et al., 2009; Willke and Vorlop, 2001). Hevekerl et al. (2014) studied the effect of pH on production efficiency and obtained 146 g/L titer of itaconic acid by pH shift after two days of cultivation. This was found to be 68% more than the previously obtained concentration of 90 g/L without pH optimization, as reported by Kuenz et al. (2012). Krull et al. (2017) developed a process based on well-timed and fine-tuned

pH control that could generate the highest itaconic acid titer 160 g/L using *A. terreus* DSM 23081 strain. Other investigations related to the influence of media composition, nitrogen sources, oxygen supply, fermentation techniques, and recovery process on itaconic acid yield by *A. terreus* have also been conducted (Boruta and Bizukojc, 2017; Karaffa et al., 2015; Kuenz et al., 2012; Kuenz and Krull, 2018; Magalhães et al., 2017; Nemestóthy et al., 2019; Saha et al., 2019).

Glucose is the most efficient substrate in terms of strain growth, yield, and ease to get metabolized by the microbes but lacks cost-effectiveness. Sucrose is another high-yielding but expensive substrate (El-imam and Du, 2014; Kuenz and Krull, 2018). Many studies were conducted to find out inexpensive yet efficient substrates to cut down the fermentation cost. Juy and Lucca (2010) reported itaconic acid production using glycerol as the sole carbon source by *A. terreus* MJL05 and achieved a final titer of 27.6 g/L with further media optimization. Saha and group have attempted fermentation of hexose (galactose, mannose) and pentose (xylose, arabinose) sugars, which are biomass constituents, and screened 100 *A. terreus* strains. These monosaccharides were found to generate a lesser quantity of itaconic acid than glucose, but the obtained yields were quite significant (Saha et al., 2017; Saha and Kennedy, 2018, 2017). Monosaccharides are more promising carbon sources for fermentation as they do not require additional hydrolysis steps, unlike polysaccharides (Yang et al., 2019).

Among polysaccharides, starch is the best substrate as it can be converted into glucose through enzymatic or acidic hydrolysis in the bioreactor. Starch from different sources has been used for itaconic acid production, but using food-based substrates for industrial production of chemicals creates ethical issues and conflicts with the food industry (Bardhan et al., 2015; Sheldon, 2014). Petruccioli et al. (1999) studied fermentation of different raw starchy materials (soft wheat flour, sorghum starch, cassava flour, potato flour, sweet potato, industrial potato flour, and corn starch) by *A. terreus* NRRL 1960. According to a study based upon fermentation of starch-rich industrial wastes (rice, potato, corn), the maximum itaconic yield was obtained with potato starch waste. Further deionization of potato starch led to enhanced yield (Bafana et al., 2019, 2017). In another study, 31 and 28.5 g/L itaconic acid was obtained through fermentation of α-amylase and nitric acid hydrolysate of corn starch, respectively, using *A. terreus* SKR10 (Reddy and Singh, 2002). In a recent study, a newly isolated thermotolerant

strain, *A. terreus* BD was investigated for itaconic acid production from enzymatic hydrolysate of synthetic food waste (potato, rice, noodles). This strain showed excellent itaconic acid productivity at high temperature (45°C), and the resultant itaconic acid titer and yield obtained from starchy food waste (41.1 g/L, 0.27 g/L, respectively) were quite comparable to that of pure glucose (44.7 g/L, 30g/g, respectively) (Narisetty et al., 2021).

The utilization of lignocellulosic biomass as an inexpensive carbon source for microbial fermentation to generate itaconic acid through solid-state or submerged fermentation has been well studied. However, biomass substrate requires intensive pretreatment due to its complex and recalcitrant nature (Yang et al., 2019). *A. terreus* is quite sensitive to the presence of fermentation inhibitors in biomass hydrolysates. A study found that *A. terreus* NRRL 1960 was unable to grow on organosolv beech wood hydrolysate without pretreatment to remove phenolic compounds from lignin and ionic compounds (Tippkötter et al., 2014). Kerssemakers et al. (2020) used cellulose pulp of *Eucalyptus* from the paper industry as feedstock which generated a good amount of itaconic acid due to the absence of lignin and other inhibitory compounds. Other studies involved the usage of agricultural biomass like husk, straw, bran, stalk, corn stover, corn cob, sugarcane bagasse, bamboo residues, etc. for itaconic acid production (Krull et al., 2017a; Liu et al., 2020; Nieder-Heitmann et al., 2018; Pedroso et al., 2017; Yang et al., 2020, 2019).

Apart from *A. terreus,* some other *Aspergillus* strains have also been found with the ability to biosynthesize itaconic acid naturally. *A. oryzae* can synthesize itaconic acid and has an excellent stock of hydrolytic enzymes, thus allowing production through simultaneous saccharification and fermentation (Jiménez-Quero et al., 2016). Ramakrishnan et al. (2020) conducted solid-state fermentation on *Citrullus lanatus* rind using an isolated fungal strain, *A. japonicas*. In another study, *A. niveus* MG 183809, isolated from soil, was used for itaconic acid production from inexpensive substrates (sweet potato, wheat flour, and corn starch), and maximum titer of 15.6 g/L was obtained from corn starch (Gnanasekaran et al., 2018). *A. niveus* was also employed for itaconic acid production using ultrasonicated algal biomass hydrolysate and glycerol from the biodiesel industry, thus generating a final titer of 31.5 g/L (Gnanasekaran et al., 2019). Sudarkodi et al. (2012) tested *A. flavus,* isolated from a soil sample, for itaconic acid biosynthesis and obtained 8.8 g/L itaconic acid in the optimized conditions. Table 2 provides a summary of itaconic acid production by natural producers.

Despite many advantageous features, there are several drawbacks with *A. terreus*. *A. terreus* has high fermentation time, low volumetric productivity, and produce many by-products. This leads to high manufacturing costs. The fungus exhibits delimited growth in the optimal media for the itaconic acid production. Additionally, the shear stress slows down the growth. Hence, the conventional conditions remain inappropriate for the fermentation (Blazeck et al., 2015). On the other hand, titer values reported from other native *Aspergillus* sp. are pretty low. Furthermore, the filamentous morphology of these fungi also creates additional handling difficulties due to increased media viscosity and sensitivity towards stressful conditions (Bafana and Pandey, 2018). Also, filamentous fungi have the tendency to form globules under agitation leading to stoppage of oxygen delivery to the cells present in the center of globular mass. Hence, the use of *A. terreus* at an industrial scale for itaconic acid production becomes a very tedious and expensive process with low efficiency.

4.2. *Ustilago* strains

Ustilago is a genus of parasitic smut fungi which infect plants, grasses, and crops. Many members of this genus have economic significance due to their ability to produce value-added chemicals like itaconic acid (Geiser et al., 2014). The itaconic acid production by *U. maydis* was first reported in 1955, and a 15 g/L titer of itaconic acid was obtained as the metabolic product in the submerged culture of fungi (Haskins et al., 1955). Owing to its itaconic acid production efficiency and non-filamentous yeast-like morphology, *U. maydis* is the best candidate for carrying out large-scale fermentation avoiding the problems associated with filamentous fungi (Geiser et al., 2016c; Klement et al., 2012).

Rao et al. (2012) used an isolated strain of *U. maydis* for itaconic acid production through submerged fermentation of glucose and studied the effect of different conditions on yield. They obtained a maximum yield of 29 g/L in 5 days at pH 3.0, 34°C temperature, and glucose concentration of 4% (w/v). Carstensen et al. (2013) presented continuous fermentation for itaconic acid production by *U. maydis* MB215 and product recovery through "reverse-flow diafiltration" in a membrane bioreactor. Studies involving fermentation of biomass substrates by *U. maydis* to synthesize itaconic acid have been carried out in past years. While studying the effect of biomass pretreatment, Klement et al. (2012) observed that itaconic acid production requires nitrogen-limited conditions, and product inhibition takes place at itaconic acid levels

beyond 25 g/L. Maassen et al. (2014) screened different *U. maydis* strains, and *U. maydis* MB215 was the best itaconic acid producer among them. It showed similar results with glucose and xylose substrate under similar conditions. In a medium containing 200 g/L glucose and 75mM ammonium chloride, 44.5 g/L of itaconic acid was produced with a maximum rate of 0.74 g/L/hr.

Geiser et al. (2014) screened 68 Ustilaginaceae strains and reported the itaconic acid production by other *Ustilago* spp. viz. *Ustilago cynodontis* 2217 (3.4 g/L), *Ustilago vetiveriae* 2220 (0.05 g/L) and *Ustilago xerochloae* 2221 (0.05 g/L) along with *U. maydis* strains. In another study, itaconic production by *U. vetiveriae* RK 075 and *U. xerochloae* UMa702 was reported using glycerol as substrate. The *U. vetiveriae* showed itaconic acid production after 21 rounds of re-inoculation. The best single colony (*U. vetiveriae* TZ1) was isolated, which produced 34.7 g/L of itaconic acid from glycerol (Zambanini et al., 2017). A study stated *U. rabenhorstiana* as a robust natural itaconic acid producer that could grow on different monosaccharide substrates and was not influenced by weak acids in the media. The final titer of 50.3 g/L was obtained from glucose media (Krull et al., 2020).

4.3 Other natural producers

Some other fungal species have also been found with the natural ability to produce itaconic acid. Tabuchi et al. (1981) reported that among 140 isolated yeast strains, one strain identified as *Candida* sp. produced itaconic acid, and a 35% yield was obtained in phosphate-limited conditions. *Pseudozyma antarctica* NRRL Y-7808, a non-pathogenic Ustilaginomycetes closely related to *Ustilago* sp., is another natural producer that produced a significant amount of itaconic acid (30 g/L) in nitrogen-limited conditions by glucose fermentation (Levinson et al., 2006). Some amount of itaconic acid productivity was also reported in *Helicobasidium mompa* and *Rhodotorula* sp. (Kuenz and Krull, 2018; Sayama et al., 1994).

5. Strategies for improvement of itaconic acid production

5.1 Random mutagenesis for strain improvement

Since itaconic acid is synthesized through a secondary pathway in microbes, its yield remains obscure in natural conditions. To boost the itaconic acid productivity of a microbe in a biotechnological setup, researchers classically relied on mutagenic treatments through special

physical (UV, X-rays) or chemical (LiCl, NTG, HNO_2, colchicine, etc.) agents. Each mutagen causes random alterations in DNA, which may be in the form of deletion, addition, substitution of bases, or cross-linking and breakage in DNA strands (Heerd et al., 2014). Random mutagenesis provides a simpler route towards better yields over tedious rational engineering strategies, which are more strategic incorporations of genetic manipulations and require detailed information about the genetic makeup and structure of the proteins in the biosynthetic pathway. Several researchers have successfully improved strain performance through this technique. For example, a mutant strain *A. terreus* TN-484, generated through NTG (*N*-methyl-*N*′-nitro-*N*-nitroso-guanidine) treatment of *A. terreus* IFO-6365, produced itaconic acid titer (82 g/L), which was 1.3 times higher than the wild parent strain on using glucose as the carbon source. This mutant generated 60 g/L itaconic acid by fermentation of corn starch hydrolyzed by nitric acid (Yahiro et al., 1997, 1995). A 48.2 g/L titer and a yield of 0.34 g/g were obtained from sago starch using the same strain (Dwiarti et al., 2007). To improve the efficiency of itaconic acid production, Reddy and Singh (2002) conducted ultraviolet, chemical, and mixed mutagenic treatments on *A. terreus* SKR10. The two obtained mutants, N45 and UNCS1, produced itaconic acid to almost twice the parent strain using corn starch and fruit waste substrates, respectively.

The presence of inhibitory compounds in complex lignocellulosic substrates greatly hampers the growth of a microbe; hence prior detoxification becomes a necessity. Some studies have found that the micro-organism showed better tolerance towards the presence of toxic compounds and product inhibition upon mutagenesis. Li et al. (2016) applied plasma exposure on *A. terreus* CICC 2452 to generate a mutant *A. terreus* AT-90. The mutant could efficiently grow on nondetoxified hydrolysate of steam-exploded corn stover and secrete itaconic acid (19.3 g/L), unlike the parent strain, which could only produce a minimal titer of itaconic acid (0.5 g/L). UV-induced mutagenesis of *A. terreus* CICC40402 enhanced metabolic flux towards the itaconic acid. It also suppressed the inhibitory effect of product accumulation. The mutant strain yielded an increased itaconic acid titer (by 33.4%) with a product concentration of 49.65 g/L, using wheat bran hydrolysate as substrate (Wu et al., 2017). Similarly, a mutant obtained by UV-LiCl treatment of *A. terreus* CICC 2433, produced a substantial amount of itaconic acid (19.3 g/L) upon fermentation of undetoxified hydrolysate of bamboo residues (Yang et al., 2020). Rafi et al. (2014) carried out mutagenesis of *U. maydis* MTCC-1474 by UV exposure and treatment with

colchicine. A significant yield was observed in solid-state fermentation of different kinds of agricultural wastes.

5.2 Metabolic engineering in native itaconic acid producers

Genetic engineering strategies can serve as a potential route to boost the performance of natural producers of itaconic acid. Fig. 2 summarizes some genetic manipulation techniques used to enhance itaconic acid productivity in microorganisms. Enhanced primary metabolism could be a critical step towards improved production of secondary metabolites. Hence, upregulated glycolytic flux could enhance itaconic acid productivity. Post-translational modifications in a key glycolytic enzyme, 6-phosphofructo-1-kinase (encoded by *pfkA* gene), cause downregulation of the glycolytic flux. Truncation of the gene could be instrumental in preventing the post-translational modifications and thus maintaining high metabolic flux. The expression of a modified *pfkA* gene (truncated t-*pfk*A10) from *A. niger* in *A. terreus*, upregulated the glycolytic flux upon phosphorylation (activation). This led to a substantial increase in itaconic acid production. Further, the site-specific mutation in the gene eliminated the need for phosphorylation, and a titer of 31 g/L was obtained from the transformant carrying six copies of mt-*pfk*A10 gene (Tevž et al., 2010). When starch is used as the substrate, it needs to be hydrolyzed into glucose which is enzymatically done in two steps, initial liquefaction with α-amylase followed by glucoamylase-mediated saccharification, before fermentation. These additional steps make the process more intense and time-consuming. The heterologous overexpression of glucoamylase gene *glaA* (of *A. niger*) in *A. terreus* CICC 40205 under native *PcitA* promoter generated a transformant that could ferment liquefied corn starch directly without an additional saccharification step. The itaconic acid titer (77.4 g/L), thus obtained, was comparable to the industrial titer (80 g/L) obtained from the fermentation of hydrolyzed starch (Huang et al., 2014a). The biosynthesis of itaconic acid is a non-fermentative process and the production of itaconic acid results in a net production of NADH. Therefore, continuous sparging of oxygen is required to oxidize NADH to regenerate NAD^+. Lin et al. (2004) improved the performance of a mutant strain *A. terreus* M8 by reducing its sensitivity towards disruption in oxygen supply. This was achieved by expressing the hemoglobin gene from *Vitreoscilla* (*vbg*) in the *A. terreus* strain.

Overexpression of the genes in itaconic acid gene cluster could escalate its production in the corresponding strain. Shin et al. (2017) attempted overexpression of each gene in the gene cluster, namely *reg*, *mtt*, *cad* and *mfs*, in a high yielding mutant *A. terreus* AN37 and compared the yield of transformants with that of the parent strain. They observed a 5% and 18.3% increase in titer by the overexpression of *cad* and *mfs* genes, respectively, compared to the parent strain. Surprisingly, no remarkable change was observed in the productivity of transformants with overexpressed *reg* (regulator gene) and *mtt* genes. Another study involving an industrial strain *A. terreus* LYT10 showed that overexpression of genes *cadA* and *mfsA*, led to enhancement in itaconic acid production by 9.4% and 5.1%, respectively (Huang et al., 2014b).

Although the itaconic acid yield of *U. maydis* is relatively lower than industrially used *A. terreus*, its properties like unicellular, non-filamentous morphology, potential to utilize unconventional substrates, tolerance to medium impurities and product accumulation are advantageous for large scale production. Therefore, many researchers have worked out metabolic and morphological engineering strategies to boost its performance (Becker et al., 2021; Wierckx et al., 2020). Geiser et al. (2016b) created a hyper-productive strain by simultaneous overexpression of regulator gene *ria1* and deletion of P450 monooxygenase gene (*cyp3).* This strain generated 4.5 times more itaconic acid than the wild-type strain, and with further media optimization, a yield of 67% (0.48 g/g) of the maximum theoretical yield (0.72 g/g) was achieved. Deletion of the *cyp3* gene ceased the conversion of itaconic acid to (S)-2-hydroxyparaconate. By knocking out the genes of competing pathways which generate other metabolites (ustilagic acid, malic acid, mannosylerythritol lipids, triacyl glycerols) of *U. maydis*, metabolic flux towards itaconic acid could be improved (Becker et al., 2020).

Hosseinpour Tehrani et al. (2019b) expressed transporter proteins from *A. terreus* in *U. maydis* MB215 and analyzed the obtained metabolic products. Expression of *At_mttA* led to itaconic acid production, which was 2.3 times higher than its *U. maydis* counterpart (*Um_mttA),* while expression of *At_mfsA* decreased itaconic acid titer as it showed more affinity towards (S)-2-hydroxyparaconate. Highest reported itaconic acid titer (220 g/L) to date has been obtained from *U. maydis* through integrated metabolic and morphological engineering accompanied by process optimization and in-situ crystallization. Fuz7 is a dual-specificity protein kinase that plays a crucial role in conjugation tube and filament formation in *U. maydis* and thus acts as a

critical target for morphology engineering. Deletion of the *fuz7* gene abolished filamentous growth and stabilized the yeast-like morphology. Additionally, deletion of *cyp3,* overexpression of native regulatory gene *ria1*, and heterologous overexpression of *A. terreus* transporter gene *At_mttA* elevated the itaconic acid concentration. *In situ* crystallization of the product with $CaCO_3$ mitigated the problem of product inhibition (Hosseinpour Tehrani et al., 2019a). Using the same system along with deletion of genes for glycolipid by-products, Becker et al. (2021) designed a strain, *U. maydis* K14, that generated itaconic acid equivalent to theoretical yield in a low-density continuous fermentation system. Schlembach et al. (2020) used a co-culture of cellulase producing *Trichoderma reesei* and the genetically modified *U. maydis* for consolidated bioprocessing of cellulose substrate. Through this process, they obtained an itaconic acid titer of 34 g/L titer and 0.16 g/g yield from a fed-batch cultivation system.

Metabolic engineering has also been attempted for other itaconic acid-producing *Ustilago* sp. The combination of multiple genetic modification strategies in *U. cynodontis* NBRC9727, which included deletion of *fuz7* and *cyp3 genes* and along with overexpression of *ria1* and *At_mttA,* intensified the itaconic acid production by 6.5 times to that of parent strain while maintaining its pH tolerance (Hosseinpour Tehrani et al., 2019c). In *U. vetiveriae* TZ1*,* itaconic production from glycerol was increased to 2.5-fold and 1.5-fold by overexpression of regulator gene *ria1* and mitochondrial transporter gene *mtt1* from *U. maydis*, respectively (Zambanini et al., 2017).

5.3 Metabolically engineered non-native itaconic acid producers

Native itaconic acid producers are primarily dependent upon the carbon source for ultimate itaconic acid yields. Despite its high itaconic acid yield, the selection of glucose as a chief carbon source ends up in high expenditures. Several studies involved the utilization of inexpensive substrates such as lignocellulosic biomass also employed pretreatment steps. This resulted in inhibitory compounds generation, which are toxic to the microbial producers and eventually decreased the itaconic acid yield. Moreover, the sensitivity of native producers (*A. terreus*, *U. maydis*) towards low pH, ions (Mn^{2+}) and product accumulation hampers the itaconic acid production. The above insights direct the researchers to come up with advanced techniques to counter these ambiguities. This paved the ground for exploring the metabolically engineered

non-native producers. Some of the microorganisms genetically engineered for itaconic acid production are mentioned in table 3.

5.3.1 Engineering fungi and yeasts for itaconic acid production

A. niger, a close relative of *A. terreus,* is an excellent industrially used producer of citric acid (>200 g/L), an itaconic acid precursor, but cannot produce itaconic acid due to the absence CAD enzyme. Thus, it is a suitable candidate for metabolic engineering as high amount of accumulated citric acid can be transformed to itaconic acid. Heterologous expression of *cadA* from *A. terreus* confer the itaconic acid-generating ability to *A. niger* (Li et al., 2011). Xie et al. (2020) studied the influence of promoter and linker for co-expression of *cadA* and *acoA* in an industrially used strain *A. niger* YX-1217. They found that the fusion construct of *cadA* and *acoA* linked by a short peptide linker, under the control of the PglaA promoter, increased itaconic acid production to 7.2 g/L, 71.4% more than the parent strain. Li et al. (2013) proposed that enhanced itaconic acid can be achieved by knocking out the genes responsible for generating by-products like oxalic acid and overexpression of fungal hemoglobin gene *hbd1* in low dissolved oxygen conditions.

The citric acid cycle occurs in mitochondria while the key enzymes for itaconic acid production (CadA, Adi1, and Tad1) are located in the cytosol, and therefore, itaconic acid production requires shuttling of cis-aconitate from mitochondria to cytosol. The permeability of cis-aconitate through the mitochondrial membrane is limited in comparison to citric acid. The transport of cis-aconitate from mitochondria to cytosol is a rate-limiting step, and it is a real challenge to make this transport smooth which will eventually result in high-level production of itaconic acid (Klement and Büchs, 2013a). Therefore, proper organelle targeting of the enzymes and channeling of substrates towards the enzyme is a crucial factor to consider. Overexpression of native aconitase gene (*acoA*) along with heterologous expression of *cis*-aconitate decarboxylase gene (*cadA*) in the mitochondria generated 0.75 g/L itaconic acid, which was twice to that obtained through the cytosolic expression of these enzymes (Blumhoff et al., 2013). The heterologous expression of the entire itaconic acid gene cluster (*cadA, mttA,* and *mfsA*) of *A. terreus* in an *A. niger* chassis showed itaconic acid production 25 times more than the strain with only *cadA* gene. The *A. niger* chassis did not produce by-products like oxalic and gluconic due to

mutations in two genes, namely *oahA* (oxaloacetate hydrolase) and *goxC* (glucose oxidase) (van der Straat et al., 2014).

Some metabolic engineering approaches on *A. niger* aim to enhance the production of primary metabolite precursors and thus ultimately improve metabolic flux towards itaconic acid. Overexpression of truncated *pfkA gene* escalated the glycolytic flux in *A. niger* transformant having itaconic acid gene cluster and led to the enhancement of itaconic acid production rate (van der Straat et al., 2013). Hossain et al. (2016) designed a non-mitochondrial itaconic acid synthesis pathway by overexpression of cytosolic citrate synthase gene *citB* in the *A. niger* strain having itaconic acid gene cluster. Generation of citrate in the cytosol allowed single compartment synthesis of itaconic acid, and a final itaconic acid titer of 26.2 g/L was reported. In another study, additional accumulation of acetyl-CoA in the cytosol was achieved by overexpression of gene encoding for ATP-citrate lyase (*acl1* and *acl2*) in the previous strain. The strain thus prepared was able to reach itaconic acid titer up to 42.7 g/L and 0.26 g/g yield when used in a media enriched with alkalizing nitrogen source to reduce stress caused by acid accumulation (Hossain et al., 2019).

Some microbes that thrive on lignocellulosic substrates have the innate ability to metabolize lignocellulose and its components using special lytic enzymes that are missing in most other micro-organisms. *Neurospora crassa* is a filamentous fungus that bears the ability to metabolize cellulose and hemicelluloses by secretion of degrading enzymes. Thus, the application of this fungus for biomass utilization is well researched. *N. crassa* FGSC 9720 engineered with codon-optimized *CAD1* gene was able to carry out consolidated bioprocessing of cellulose avicel and the lignocellulosic substrates like corn stover and switchgrass. It generated 20 mg/L of itaconic acid (Zhao et al., 2018). Though obtained yield was meager, *N. crassa* was established as a model system for itaconic acid production from inexpensive biomass substrates without external pretreatment and hydrolysis, which could lead to better results with further engineering. *Candida lignohabitans* has emerged as a novel candidate of interest in the biotechnological production of valuable products from biomass. It can metabolize components of lignocellulosic biomass and can grow as efficiently on biomass as on pure sugars. Heterogeneous expression of cadA gene, under GAP promoter and terminator control, generated up to 2.5 g/L itaconic acid from non-detoxified hydrolysate of lignocellulosic biomass (Bellasio et al., 2015).

Among all known micro-organisms, *Pichia stipitis* has the best efficiency for the metabolism of xylose, a major component of lignocellulosic biomass. Adopting an approach of co-expression of heterologous CAD and cytoplasmic ACO (aconitase) in *P. stipitis*, Qi et al. (2017) generated 1.52 g/L titer using xylose as a carbon source.

Saccharomyces cerevisiae has become an accomplished microbial factory for the biotechnological production of valuable chemicals. It is also advantageous for industrial applications due to its tolerance to low pH, low temperature, inhibitors, and many other stressful conditions. Blazeck et al. (2014) employed this yeast for itaconic acid production by incorporating *CAD1* gene under the control of enhanced P_{GPD} promoter and further engineering of native metabolism. They identified three genes outside the itaconic acid pathway viz. *ade3*, *bna2*, and *tes1,* which, when deleted from transformed *CAD1* containing strain, led to a seven-fold increase in itaconic acid titer to 168 mg/L. The *ade3* gene encodes for a trifunctional C1-tetrahydrofolate (THF) synthase involved in purine biosynthesis, *bna2* encodes for tryptophan 2,3-dioxygenase involved in NAD^+ (Nicotinamide adenine dinucleotide) synthesis from tryptophan, and *tes1* encodes for acyl-CoA thioesterase. The deletion of these genes rewired the intracellular metabolic flow and focused it in the TCA cycle, resulting in increased itaconic acid synthesis. Recently, a more acid-tolerant yeast *Pichia kudriavzevii*, had also been engineered for itaconic acid production. Integration of multiple genetic modifications that include heterologous expression of CAD from *A. terreus* (*At_cad),* deletion of the gene for isocitrate dehydrogenase (*icd*), and overexpression of native mitochondrial tricarboxylate transporter (*Pk_mttA*), resulted in the production of 1.23 g/L itaconic acid in a fed-batch system without any pH adjustments (Sun et al., 2020).

Yarrowia lipolytica is an oleaginous yeast that can serve as an attractive and promising host for economic itaconic acid production due to its ability to hyper accumulate citric acid, robustness under low pH, high tolerance against toxicity of organic acids and fermentation inhibitors. In *Y. lipolytica,* nitrogen scarcity induces enzyme AMPD (adenosine monophosphate deaminase) that degrades a cofactor (AMP) of isocitrate dehydrogenase in TCA cycle, thus rendering the TCA cycle broken, with a high accumulation of initial cycle metabolites, which can be directed towards itaconic acid by metabolic engineering. To achieve the purpose, Blazeck et al. (2015) carried out heterologous overexpression of CAD enzyme along with expression of a

modified aconitase, lacking mitochondrial expression signal (ACOnoMLS) which results in cytosolic localization of this enzyme, and obtained 4.6 g/L itaconic acid. In another study, *Y. lipolytica* Po1f carrying *cadA* was further modified by overexpression of *A. terreus* genes *acoA, mfsA, and mttA.* It was reported that the introduction of *mttA* greatly enhanced the itaconic acid titer (22g/L) by ensuring transfer to the cytoplasm for CAD to act on it (Zhao et al., 2019).

5.3.2 Engineering bacteria for itaconic acid production

Although bacterial systems don't possess well-compartmentalized structure as fungi, many researchers have successfully engineered them for itaconic acid production. Bacteria have a short generation time, easy genetic manipulation, and simpler organization rendering them suitable hosts for itaconic acid production. Moreover, all the reactions take place in the cytosol. The key to confer itaconic acid-generating capability into an unconventional host is the heterologous expression of the CAD enzyme. *Corneybacterium glutamicum*, when transformed with CAD enzyme, showed less initial itaconic acid yield. Hence a newer strategy was employed involving a fusion of CAD and MBP (maltose-binding protein) along with site-directed mutagenesis of isocitrate dehydrogenase, which resulted in a final titer of 7.8 g/L, a yield of 0.29 g/g, and maximum productivity of 0.27 g/L/h (Otten et al., 2015). In another study having *Synechocystis* sp. PCC6803 (a photosynthetic bacterium) transformed with the CAD gene showed a very small itaconic acid titer of 14.5 mg/L when it was cultivated in a medium aerated with 5% CO_2 as a sole carbon source (Chin et al. 2015). In a study by Lim et al. (2019) *Methylorubrum extorquens* AM1 was engineered to express CAD enzyme, and itaconic production was analyzed using acetate, succinate, and methanol as carbon sources. The best titer (31.6 mg/L) was obtained from methanol which is an inexpensive feedstock. Transcriptomic analysis identified certain genes related to the process, which opens up the possibilities for further yield improvement through metabolic engineering.

Escherichia coli is the most researched bacterial model in biotechnology owing to its short life cycle, simple organization, well-mapped genetic framework, and easy gene manipulation. Okamoto et al. (2014) carried out the heterologous expression of *cadA* from *A. terreus* in *E. coli.* They reported a low initial itaconic acid titer of 0.07 g/L, which increased to 4.34 g/L by the overexpression of the aconitase (*acnB*) gene and inactivation of isocitrate

dehydrogenase (*icd*). Similarly, Vuoristo et al. (2015) obtained a meager titer of 10 mg/L from *E. coli* transformed with *cadA*, in initial LB media and 37°C, which hiked up to 240 mg/L by media and temperature optimization. To further increase itaconic acid production, the intracellular concentration of itaconic acid precursors, citrate and aconitate, was enhanced by overexpression of genes for citrate synthase (*gltA*) and aconitase (*acoA*) from *Corneybacterium glutamicum.* Termination of the routes towards by-products acetate and lactate by deleting genes for phosphate acetyltransferase (*pta*) and lactate dehydrogenase (*ldhA*) elevated the supply of precursors for itaconic acid, and a final titer of 690 mg/L was reported. It was found that the CAD enzyme had low activity due to the formation of inclusion bodies in *E. coli*; hence it produced a feeble amount of itaconic acid (Vuoristo et al., 2015). Jeon et al. (2016) screened a library of cadA variants with the first ten codons as synonymous to generate CAD enzyme with higher solubility and functionality. When expressed in the *E. coli*, one of the variants generated enzyme with 95% solubility and gave rise to 7.2 g/L titer of itaconic acid from glycerol under nitrogen-limited conditions in a two-stage system.

Harder et al. (2016) engineered a strain *E. coli* ita23 using a model-based approach that gave exceptionally high titer values. A plasmid (pCadCS) with *cadA* and *gltA* (citrate synthase) was introduced into *E. coli* along with abolition of undesired metabolic reactions by deleting genes for pyruvate kinases (*pykA* and *pykF*), isocitrate lyase (*aceA*), phosphate acetyltransferase (*pta*), succinyl-CoA synthetase (*sucCD*), and downregulation of isocitrate dehydrogenase. Up to 32 g/L titer and maximum productivity of 0.45 g/L/h was achieved in a fed-batch system using the developed strain. In their subsequent study, exceptionally high productivity was achieved through dynamic control of metabolic flux in a two-stage process by temperature variation. With further modification in the previous strain, they developed a strain, i.e., *E. coli* ita36A, in which instead of downregulating isocitrate dehydrogenase, it was expressed under Lambda promoter controlled by temperature-sensitive repressor *CI857*. At 37°C temperature of the first stage, bacterial growth and metabolism were fast, thus allowing a rapid accumulation of sufficient biomass. But below 30°C, in the second stage, the repressor is activated, causing downregulation of the TCA cycle and thus diversion of metabolic flux towards itaconic acid. The strain functioning in this situation showed maximum productivity of 0.86 g/L/h and generated titer of

47 g/L, which are the highest values reported for any genetically engineered non-native producer (Harder et al., 2018).

Whole-cell bioconversion is a suitable strategy for the fast production of biochemicals with minimum cost and processing requirements. Use of recombinant *E. coli*, with overexpressed *cadA* (3 gene copies from *A. terreus*) and *acn* (single copy from *C. glutamicum*), as whole-cell biocatalyst generated 319.8 mM (41.6 g/L) itaconic acid from 500 mM citrate. In this system, 64% conversion was accomplished in 19 h with remarkable productivity of 2.19 g/L/h without generating by-products (Kim et al., 2017). Yang et al. (2017) achieved 8.7 g/L titer of itaconic acid from sodium citrate using a whole-cell biocatalytic system of *E. coli* carrying self-assembled CAD and ACO enzymes. Luo et al. (2020) developed a similar whole-cell bioconversion system using *Shewanella livingstonensis* Ac10, a psychrophilic bacterium, as a host. By the expression of *cadA* from *A. terreus* and *acnB* from *E. coli*, itaconic acid was produced from citric acid at the rate of 0.22 g/L/h, which got amplified to 1.41 g/L/h upon 15 min heat treatment (47°C) due to an increase in membrane permeability.

Lignocellulosic feedstocks are mainly composed of lignin, cellulose, and hemicelluloses. While cellulose and hemicelluloses, being carbohydrates, are readily metabolized by most microbes, lignin and its aromatic components generally remain unmetabolized in a biomass fermentor. *Pseudomonas putida* is a robust micro-organism that can digest aromatic compounds present in lignin. Pseudomonas putida KT 2440 was recently engineered to produce itaconic acid utilizing alkali-treated lignin from corn stover and p-coumarate, a lignin component bioconverted to acetyl-CoA in *P. putida,* as carbon sources in a two-stage dynamically regulated process. Cis and trans itaconic acid pathways were heterologously induced by the expression of *cadA* from *A. terreus* and *tad1/adi1* from *U. maydis*, respectively. The trans pathway, being thermodynamically more favored, showed better results as compared to the cis pathway. The carbon flux was further modulated by deleting genes participating in the competing PHA pathway under nitrogen-limited conditions. Finally, titers of 1.3 g/L and 1.4 g/L were obtained from p-coumarate and alkali-treated lignin, respectively (Elmore et al., 2021a).

6. Hurdles for achieving a high level and low-cost itaconic acid production

Considering its significant utility in the polymer industry, the large-scale production of itaconic acid becomes indispensable. Such mass production of itaconic acid has been endeavored in the past using native and engineered microorganisms but faced economic drawbacks or marginal yields. There are several technical constraints that limit the economic viability and efficiency of the production process.

Challenges with native producers: Currently, itaconic acid is produced from biological routes. *Aspergillus terreus* is the most dominant host for bio-production of itaconic acid, reaching titers of >100 g/L. *A. terreus* is a pathogenic and slow-growing fungus with the formation of various byproducts. The filamentous fungus suffers from several other disadvantages such as low yields (<0.50 g/g), long fermentation time (>150 h), high sensitivity of filaments to hydro-mechanical stress, laborious handling of spores, elevated viscosity, interruption of oxygen supply and poor growth in media optimal for itaconic acid production. All these factors limit the process efficiency and increase production costs which hold back the commercial application of itaconic acid (Steiger et al. 2013; Klement and Büchs, 2013; Bafana and Pandey, 2018). The non-filamentous native producer *U. maydis* could be an alternative itaconic acid producer suitable for mass production. The challenge suffered in itaconic acid production using *U.maydis* is its naturally lower itaconic acid-producing ability and high pH sensitivity (Klement and Büchs, 2013a; Zhao et al., 2018). These drawbacks have been addressed recently by genetic engineering approaches. Also, natural producers like *A. terreus* and *U. maydis* lack the enzymes needed to metabolize biomass substrates; thus, additional hydrolysis steps are required for using complex feedstocks (lignocellulose, starch, etc.), which contributes to the additional cost.

Low performance of metabolically engineered non-native strains: Few other organisms have been investigated for itaconic acid production in the last decade. Apart from *A. terreus*, some other hosts (*E. coli*, *Corynebacterium glutamicum, Yarrowia lipolytica*) have been engineered for itaconic acid production (Otten et al. 2015; Chang et al. 2017); however, the production parameters are too far from commercialization. This can be attributed to the fact that engineered metabolic pathways, made up of enzymes that are not homologous to the producing host, are prone to flux imbalances because they lack the regulating mechanisms found in natural

metabolism. Also, heterologously expressed proteins and enzymes in recombinant systems suffer from problems of insolubility due to high-level production and lack of proper protein folding machinery. For instance, the heterologous expression of CAD enzyme in *E. coli* was reported to have low activity due to the formation of inclusion bodies, which resulted in a feeble amount of itaconic acid production (Vuoristo et al., 2015). Hence, the industrial applications of such systems require substantial improvement, which leads to an enormous scope of research in this field.

Low tolerance against fermentation inhibitors: The sourcing of inexpensive non-edible and waste biomass as a feedstock to replace high-cost carbon sources is a key strategy to curb the production cost. However, *A. terreus* has a high degree of sensitivity to inhibitory compounds in a culture medium. So, it is crucial that sugar extraction from feedstock generate minute or no fermentation inhibitors, such as organic acids (formic acid, acetic acid, etc.), furan derivatives (furfural and hydroxymethylfurfural) and phenolic compounds, which are commonly generated during the processing of lignocellulosic feedstocks, which is unlikely. These inhibitory compounds, present in the hydrolysate of lignocellulosic biomass, slow down the growth and metabolism of the fungi and thus reduce itaconic acid yields (Saha et al., 2019). A study showed that enzymatic hydrolysate of wheat straw pretreated with dilute acid contained acetic acid, furfural, and ions like Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, S^{2-}, Al^{3+}, Fe^{3+}, Mn^{2+}, Cu^{2+}, etc. which inhibited the production process, and itaconic acid was formed only upon 1000 fold dilution of hydrolysate (Saha and Kennedy, 2018). There have been reports that wild-type *A. terreus* strains have shown minimal growth on biomass substrates without prior detoxification (Li et al., 2016). According to Tippkötter et al. (2014), *A. terreus* NRRL 1960 could not grow on untreated organosolv beech wood hydrolysate. Hence, to eliminate the fermentation inhibitors, they applied a range of detoxification approaches, including successive treatments with NaOH, zeolite, and ion exchangers. Such approaches become a necessity for the effective valorization of biomass into valuable products.

7. Current status and future perspectives

Itaconic acid production is classically based upon the fermentation of filamentous fungi *A. terreus*. Another natural producer *U. maydis* shows lower productivity of itaconic acid, but its non-filamentous morphology is ideal for large-scale production. Few other *Aspergillus* and *Ustilago* sp. have also been reported to produce itaconic acid naturally. Researchers have carried out several metabolic engineering approaches to address the challenges associated with natural producers and enhance their productivity. In addition, several alternative and unconventional producers are being created through genetic engineering strategies. Overexpression of genes belonging to the itaconic acid gene cluster of natural producers and genes which direct the metabolic flux towards itaconic acid are the basic steps in metabolic engineering to enhance productivity. Deletion of genes of side reactions and by-products could further improve the yield.

With increased market demands for sustainable products, the manufacture of value-added compounds using microbial biocatalysts appears to have a bright future. Genetic engineering strategies developed in recent years have created a plethora of possibilities to modulate the biochemical pathways of a micro-organism and impart the desired characteristic into it. A critically designed fermentation route integrated with well-coordinated bioprocess and metabolic engineering strategies using a robust micro-organism is required to generate high yields of itaconic acid. The high cost of production is the primary constraint to large-scale production and consumption of itaconic acid-derived products. Further rational studies on each contributing parameter are needed to improve the efficacy of each step of itaconic acid production: pretreatment, fermentation, and downstream processing, in terms of yields as well as economic viability. By heterologous incorporation of genes of a suitable lytic enzyme into the host genome, the requirement of additional hydrolysis steps involving intense pretreatment of the substrate could be avoided. Several other less-explored inexpensive lignocellulosic substrates, including forest and industrial wastes, could also be investigated to reduce production costs. With production-cost low enough to compete with conventional fossil-based products, itaconic acid could establish a strong position in the commercial market to produce everyday-use polymeric products.

8. Conclusion

Itaconic acid is an interesting bio-based building-block chemical that has the potential to act as a green substitute for many petroleum-based chemicals in the polymer industry. The itaconic acid production processes need cost-effectiveness, utilization of inexpensive substrates, and scaling up of itaconic acid production to the industrial level. As the itaconic acid synthesis pathways and metabolism are well defined in today's research scenario, a meaningful improvement could be achieved that imparts the transfer of the itaconic acid synthesis to a varied organism of interest. With effective strategies such as metabolic engineering, the time is near when natural producers of itaconic acid would be sufficiently replaced by engineered organisms that could prove efficient microbial cell factories. Nevertheless, a more precise and small-scale screening platform would remain instrumental in developing a process successfully. Moreover, the advent of better genetic improvement strategies and tools will pave the grounds for effective manipulation in itaconic acid production. Hence, achieving the current demands as well as increasing the itaconic acid production as per its utilities in the polymer industry could be the newer scope of such research.

Credit author statement

Deeksha Gopaliya has designed as well as prepared the manuscript. Sunil Kumar Khare has conceived the idea of the review manuscript. Sunil Kumar Khare and Vinod Kumar have critically reviewed the manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Legends to tables

Table 1. Genes related to microbial itaconic acid biosynthesis

Table 2. Summary of itaconic acid production by native producers

Table 3. Itaconic acid production by genetically engineered micro-organisms

Legends to figures:

Fig. 1. Metablic pathways for biosynthesis of itaconic acid in *Aspergillus terreus* (red) and *Ustilago maydis* (green) (Created with BioRender.com)

Fig. 2. Engineering approaches to enhance itaconic acid production by microbial cell factories.

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Table 1:

Gene	Enzyme/ Protein
cadA, *cad1*	*cis*-Aconitate decarboxylase
tad1	*trans*-Aconitate decarboxylase
adi1	Aconitase-Δ-isomerase
mttA, *mtt1*	Mitochondrial tricarboxylate transport protein
mfsA	Major facilitator superfamily protein
itp1	Itaconate transporter protein
reg, ria1	Regulatory gene of itaconic acid gene cluster
cyp3, cypC	P450 monooxygenase
rdo1 rdoC	Ring-cleaving dioxygenase
acoA, acnB	Aconitase
gltA, *citA, citB*	Citrate synthase
Icd	Isocitrate dehydrogenase
pfkA	6-Phosphofructo-1-kinase

Table 2.

Strain	Carbon source	Fermentation system	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
A. terreus NRRL 1960	Corn starch	Shake flask	18.4	0.34	0.13	(Petruccioli et al., 1999)
	Soft wheat flour		17.8	0.31	0.12	
	Cassava flour		9.8	0.19	0.07	
A. terreus DSM 23081	Glucose	15 L bioreactor (pH 3.1)	86.2	0.62	0.51	(Kuenz et al., 2012)
A. terreus DSM 23081	Alkali treated wheat chaff hydrolysate	Shake flask	27.7	0.41	0.19	(Krull et al., 2017a)
A. terreus DSM 23081	Glucose	1.5 L bioreactor (fed-batch)	160	0.46	0.99	(Krull et al., 2017b)
		15 L bioreactor (fed-batch)	150	0.56	0.64	
A. terreus BD	Synthetic food waste	2.5 L bioreactor (45°C, pH 2.3)	41.1	0.27	0.19	(Narisetty et al., 2021)
	Glucose		44.7	0.3	0.21	
A. terreus NRRL 1972	Glucose	Shake flask	37.2	-	0.22	(Saha and Kennedy, 2020)
		2 L bioreactor	43.1	-	039	
A. terreus NRRL 1971	Mannose	Shake flask	36.4	0.46	0.17	(Saha and Kennedy, 2017)
A. terreus NRRL 1961	Glucose	Shake flask	49.8	0.62	0.3	(Saha et al., 2017)
	Xylose		38.9	0.49	0.23	
	Arabinose		34.8	0.44	0.2	
A. terreus M69	Activated charcoal-treated corn stover hydrolysate	Shake flask	33.6	0.56	0.28	(Liu et al., 2020)
A. niveus MG183809	Corn starch	Shake flask	15.6	-	0.09	(Gnanasekaran et al., 2018)
	Wheat flour		9.2	-	0.05	
	Sweet potato		7.4	-	0.04	
A. niveus MG183809	*Gracilaria edulis* algal biomass and glycerol	Shake flask	31.5	-	0.18	(Gnanasekaran et al., 2019)
A. flavus	Sucrose	Shake flask	8.8	-	-	(Sudarkodi et al., 2012)
U. maydis MB215	Glucose	Shake flask (pH 3, 34°C)	29	-	0.24	(Rao et al., 2012)
U. maydis MB215	Glucose	2.5 L bioreactor (pH 6)	44.5	0.24	0.74	(Maassen et al., 2014)
U. Rabenhorstiana	Glucose	Shake flask	33.3	0.26	0.13	(Krull et al., 2020)
		1L bioreactor (fed-batch)	50.3	0.31	0.73	
U. vetiveriae TZ1	Glycerol	Shake flask	34.7	0.18	0.09	(Zambanini et al., 2017)
P. antarctica NRRL Y-7808	Glucose	Shake flask	30	0.29	0.23	(Levinson et al., 2006)

Table 3.

Parent Strain	Engineering strategy/ Genetic manipulation/ Mutagenic treatment	Carbon source	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
A. terreus IFO-6365	*A. terreus* TN-484 mutant generated by NTG treatment	Glucose	82.3	0.54	0.57	(Yahiro et al., 1995)
		Hydrolyzed corn starch	62	0.55	0.43	(Yahiro et al., 1997)
		Hydrolyzed sago starch	48.2	0.34	0.4	(Dwiarti et al., 2007)
A. terreus SKR10	N45: Mutagenesis by NTG treatments	Hydrolyzed corn starch	46-50	0.42	0.3	(Reddy and Singh, 2002)
	UNCS1: Mutagenesis by NTG, colchicine and sodium azide	Fruit waste extract	31-32	0.36	0.19	
A terreus CICC 2452	*A. terreus* AT-90 : Plasma induced mutagenesis	Cellulase hydrolysate of corn stover	19.3	0.36	0.16	(Li et al., 2016)
A. terreus CICC 40402	UV induced mutagenesis	Wheat bran hydrolysate	49.6	-	0.41	(Wu et al., 2017)
A. terreus CICC 2433	*A. terreus* AtYSZ-38: UV-LiCl induced mutagenesis	Bamboo residues	19.35	0.36	0.16	(Yang et al., 2020)
A. terreus NRRL 1960	NTG mutagenesis and heterologous expression of *vbg* (haemoglobin gene from *Vitreoscilla*)	Glucose	46.8	-	0.49	(Lin et al., 2004)
A. terreus A 156	Expression of truncated and mutated *pfkA* (mt-*pfk*A10) gene from *A. niger*	Glucose	31	-	0.31	(Tevž et al., 2010)
A. terreus CICC 40205	Overexpression of *glaA* (glucoamylase gene from *A. niger*) under native *PcitA* promoter and a strong signal peptide	Liquified corn starch	77.6	-	1.07	(Huang et al., 2014a)
U. maydis MB215	Deletion of *cyp3* (P450 monooxygenase gene) and overexpression of regulator gene *ria1* under P_{etef} promoter	Glucose	54.8	0.48	0.33	(Geiser et al., 2016b)
U. maydis MB215	Deletion of byproduct genes (*cyp3*, MEL, UA, *dgat*); overexpression of *ria1*	Glucose	53.5	0.47	0.27	(Becker et al., 2020)
U. maydis MB215	Overexpression of native *rai1* and *mttA* (from *A. tereus*); deletion of *cyp3* and *fuz7*	Glucose	220	0.33	0.46	(Hosseinpour Tehrani et al., 2019a)
U. maydis MB215	*U. maydis* K14: Overexpression of native *rai1* and *mttA* (from *A. tereus*); deletion of *fuz7* and by-product genes (*cyp3*, *dgat*, MEL and UA)	Glucose	74.9	0.54	0.53	(Becker et al., 2021)
U. cynodontis NBRC9727	Overexpression of native *rai1* and *mttA* (from *A. tereus*); deletion of *cyp3* and *fuz7*	Glucose	22.3	0.42	0.07	(Hosseinpour Tehrani et al., 2019c)
A. niger AB 1.13	Heterologous expression of *cadA*	Glucose	0.60	-	0.006	(Li et al., 2011)
A. niger ATCC 1015	Mitochondrial expression of *acoA* (*A. niger*) and *cadA* (*A.terreus*)	Glucose	1.2	-	0.005	(Blumhoff et al., 2013)

A. niger YX-1217	Co-expression of *acoA* and *cadA* linked by a peptide linker under P*glaA* promoter	Cornmeal	7.2	-	0.7	(Xie et al., 2020)
A. niger NW186	Mutation in *oahA* and *goxC;* heterologous overexpression of *cadA*, *mttA* and *mfsA*	Sorbitol and xylose	7.1	-	0.09	(van der Straat et al., 2014)
A. niger AB 1.13	Overexpression of *citB* (cytosolic citrate synthase), *cadA*, *mttA* and *mfsA*	Glucose	26.2	0.37	0.35 (max.)	(Hossain et al., 2016)
A. niger AB 1.13	Overexpression of *acl1*2 (ATP-citrate lyase) , *citB* (Cytosolic citrate synthase), *cadA*, *mttA* and *mfsA*	Glucose	42.7	0.26	0.18 (avg.)	(Hossain et al., 2019)
Neurospora crassa FGSC 9720	Heterologous eexpression of *CAD1*	Lignocelluose biomass	20.4 mg/L	-	-	(Zhao et al., 2018)
Saccharomyces cerevisiae	Deletion of *ade3*, *bna2*, and *tes1* Heterologous eexpression of *CAD1*	Glucose	0.168	-	-	(Blazeck et al., 2014)
Yarrowia lipolytica PO1f	Cytosolic coexpression of CAD and ACOnoMLS (native aconitase without mitochondrial expression signal)	Glucose	4.6	0.058	0.045 (max.) 0.027 (avg.)	(Blazeck et al., 2015)
Yarrowia lipolytica PO1f	Heterologous expression of cadA, acoA, mttA and mfsA from *A. terreus*	Glucose	22	0.056	0.111 (max.) 0.05 (avg.)	(Zhao et al., 2019)
Pichia stipitis FPL-UC7	Heterologous expression of *CAD* and overexpression of native truncated *ACO (*without mitochondrial signal)	Xylose	1.52	-	0.013	(Qi et al., 2017)
Pichia kudriavzevii YB4010	Heterologous expression of *At_cad*, overexpression of native *Pk_mttA* and deletion of *icd* (isocitrate dehydrogenase)	Glucose	1.23	29 mg/g	51mg/L/h	(Sun et al., 2020)
Candida lignohabitans CBS 10342	Heterologous expression of *cadA* under the control of GAP promoter and terminator	Undetoxified Lignocellulose hydrolysate	2.5	-	0.04	(Bellasio et al., 2015)
E. coli BW25113	Heterologous expression of *cad* ; overexpression of native *acnB* (aconitase); inactivation of *icd* (isocitrate dehydrogenase)	LB medium with 3% glucose	4.34	-	0.04	(Okamoto et al., 2014)
E. coli BW25113	Heterologous expression of *cadA* from *A. terreus; gltA* (citrate synthase) and *acnA (*aconitase) from *C.glutamicum;* Deletion of *pta* (phosphate acetyltransferase) and *ldhA* (lactate dehydrogenase)	M9 minimal media with Glucose	0.69	0.09 mol/mol	0.012	(Vuoristo et al., 2015)
E. coli XL1-Blue	Expression of synonymous codon variant *scv_cadA* with highest solubility	Glycerol	7.2	-	0.1	(Jeon et al., 2016)
E. coli MG1655	ita23: plasmid (pCadCS) with *cadA* and *gltA*; downregulation of *icd;* deletion of *aceA*, *sucCD*, *pta, pykA* and *pykF*,	Glucose	32	0.68 mol/mol	0.45 (max.) 0.38 (avg.)	(Harder et al., 2016)
E. coli MG1655	ita36A: pCadCS expression; *icd* expression under Lambda promoter and its temperature sensitive repressor for temperature controlled two-stage process; deletion of *aceA, pta, pykF pykA*	Glucose	47	0.62 mol/mol	0.86 (max.) 0.39 (avg.)	(Harder et al., 2018)
E. coli BL21(DE3)	Whole cell bioconversion system with overexpressed *acn* (*C. glutamicum*) and 3 copies of *cadA* (*A. terreus*)	Citric acid	41.6		5.43 (max.) 2.19 (avg.)	(Kim et al., 2017)

Shewanella livingstonensis Ac10	Whole cell bioconversion system with overexpressed *cadA* (from *A. terreus*) and *acnB* (from *E. coli*); membrane permeability increased by heat treatment (47°C) for 15 min	Citric acid	-	-	1.41	(Luo et al., 2020)
Corneybacterium glutamicum	Expression of CAD and its fusion with maltose binding protein; mutation in isocitrate dehydrogenase	Glucose	7.8	0.29	0.27 (max.)	(Otten et al., 2015)
Synechocystis sp. PCC6803	Heterologous expression of CAD	CO_2	14.5 mg/L	-	919µg/L/day	(Chin et al., 2015)
Methylorubrum extorquens AM1	Heterologous expression of CAD	Methanol	31.6 mg/L	-	-	(Lim et al., 2019)
Pseudomonas putida	Heterologous expression of *tad1* and *adi1* from *U. maydis*; deletion of PHA synthetases *phaC1* and *phaC2*	Alkali treated-depolymerized lignin	1.4	-	0.79g/g	(Elmore et al., 2021b)
		p-coumarate	1.3		1.2mol/mol	

Figures

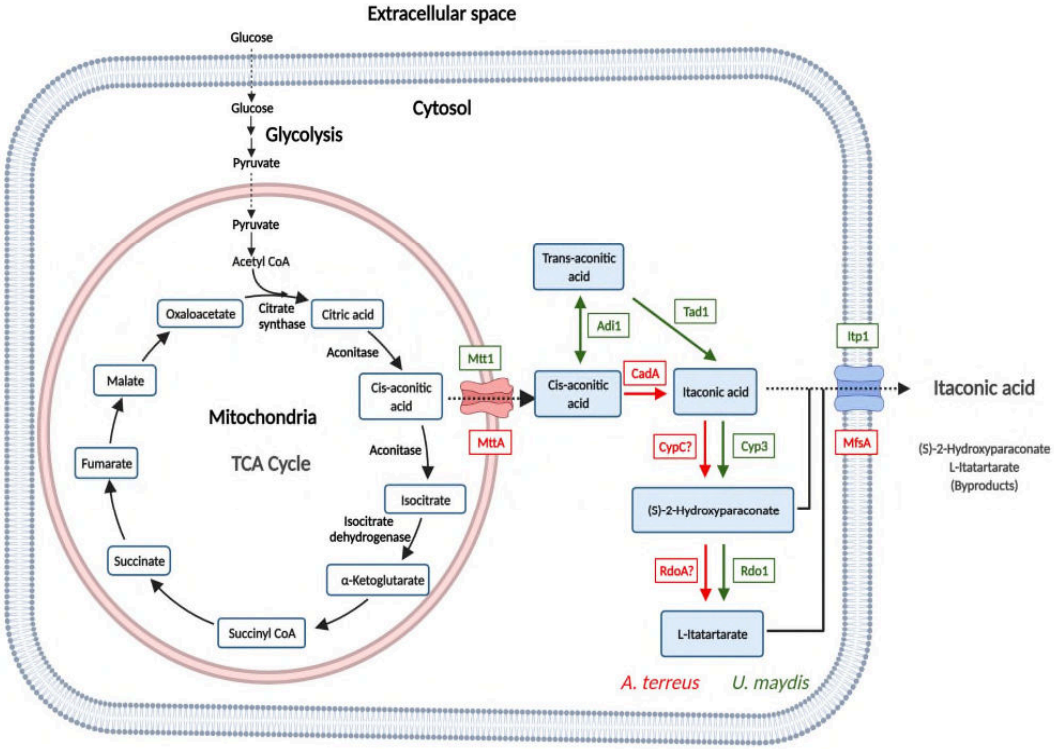

Fig. 1a

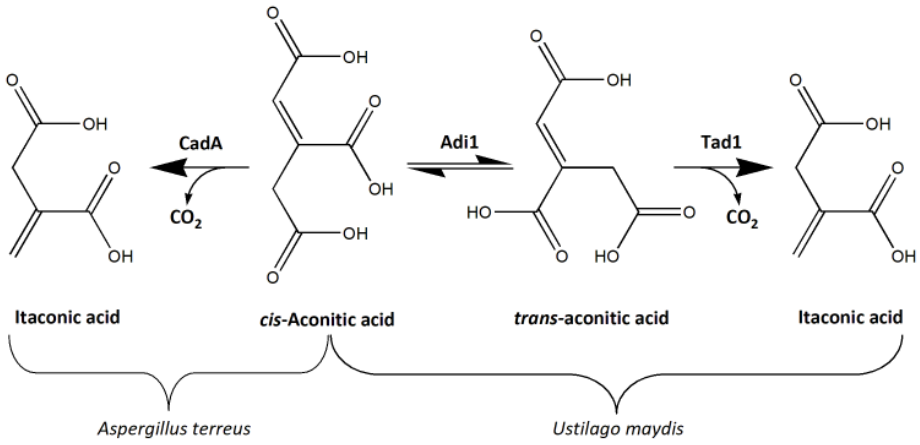

Fig. 1b

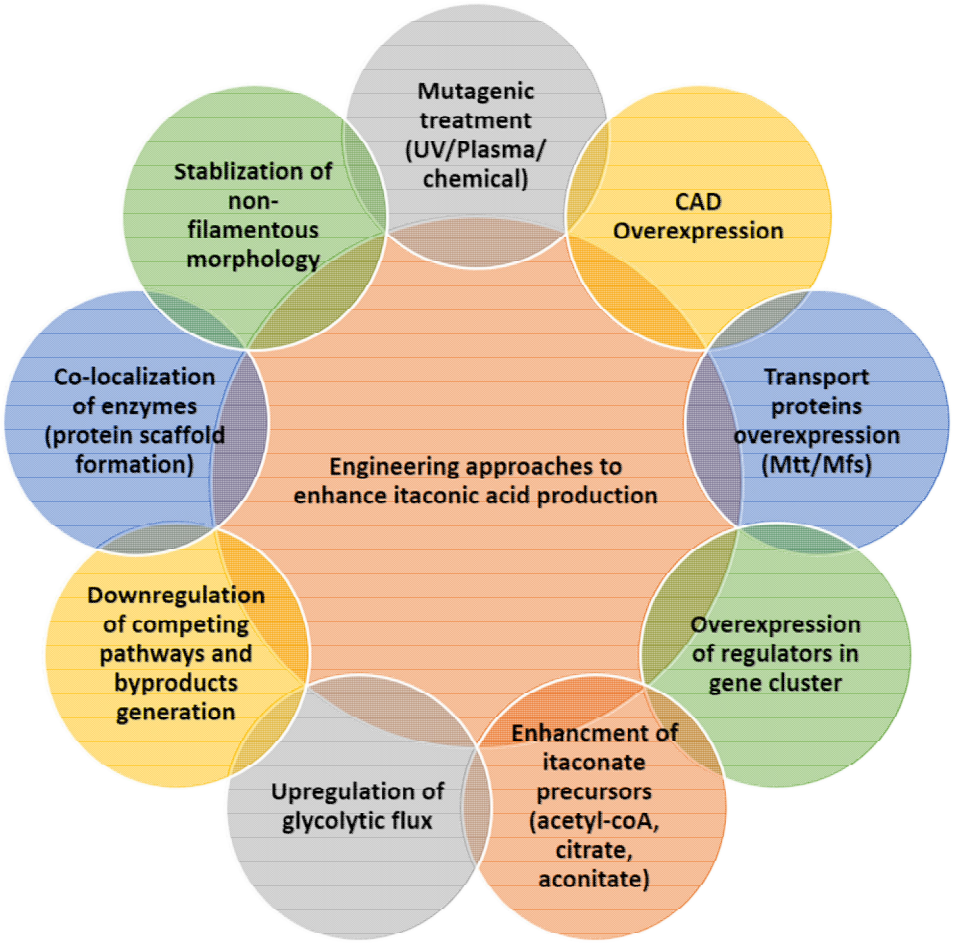
Mutagenic treatment (UV/Plasma/ chemical)
CAD Overexpression
Transport proteins overexpression (Mtt/Mfs)
Overexpression of regulators in gene cluster
Enhancment of itaconate precursors (acetyl-coA, citrate, aconitate)
Upregulation of glycolytic flux
Downregulation of competing pathways and byproducts generation
Co-localization of enzymes (protein scaffold formation)
Stablization of non-filamentous morphology
Engineering approaches to enhance itaconic acid production

Fig. 2

2021-08-18

Recent advances in itaconic acid production from microbial cell factories

Gopaliya, Deeksha

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