AIMS Bioengineering, 4(4): 418-430.
DOI: 10.3934/bioeng.2017.4.418
Received: 23 September 2017
Accepted: 19 October 2017
Published: 23 October 2017

http://www.aimspress.com/journal/Bioengineering

Review

Bio-succinic acid production: *Escherichia coli* strains design from genome-scale perspectives

Bashir Sajo Mienda [1,*] and Faezah Mohd Salleh [2]

[1] Department of Microbiology & Biotechnology, Federal University Dutse, PMB 7156, Dutse, Jigawa State, Nigeria
[2] Faculty of Biosciences & Medical Engineering, Universiti Teknologi Malaysia, 81310 Skudai Johor Bahru, Malaysia

*** Correspondence:** Email: b.mienda@fud.edu.ng; bsmienda@gmail.com; Tel: +234-803-052-2701.

Abstract: *Escherichia coli* (*E. coli*) has been established to be a native producer of succinic acid (a platform chemical with different applications) via mixed acid fermentation reactions. Genome-scale metabolic models (GEMs) of *E. coli* have been published with capabilities of predicting strain design strategies for the production of bio-based succinic acid. Proof-of-principle strains are fundamentally constructed as a starting point for systems strategies for industrial strains development. Here, we review for the first time, the use of *E. coli* GEMs for construction of proof-of-principles strains for increasing succinic acid production. Specific case studies, where *E. coli* proof-of-principle strains were constructed for increasing bio-based succinic acid production from glucose and glycerol carbon sources have been highlighted. In addition, a propose systems strategies for industrial strain development that could be applicable for future microbial succinic acid production guided by GEMs have been presented.

Keywords: bio-succinic acid production; *Escherichia coli* strain design; genome-scale metabolic models; proof-of-principle strains

1. Introduction

Succinic acid is a platform chemical with a variety of applications in various field and it served as starting chemical for the production of other commodity and specialty chemicals Figure 1 [1]. *E. coli* has been established to be a native producer of succinic acid under anaerobic conditions in a

mixed acid fermentation reactions using glucose carbon source [2,3]. Metabolic engineering strategies involving various gene manipulations to overproduce succinic acid in *E. coli* using experimental trial and error approach [4,5] have been reported elsewhere [2,4,6,7,8].

E. coli genome-scale metabolic models (GEMs) have been developed and published [9,10] with ability to predict metabolic engineering capabilities for increasing production of desired compound of interest consistent with that of experimental approaches [11,12,13]. *E. coli* GEMs have been applied for predicting metabolic engineering interventions for increasing succinate production faster than the conventional approach of experimental trial and error [14,15,16]. We recently constructed proof-of-principles strains of *E. coli* using GEMs for increasing succinic acid production from glucose, and glycerol carbon sources [14,15,16].

Systems metabolic engineering integrating genome-scale metabolic modelling and omics analysis could help in developing superior microbial strains that can produce industrially relevant titer of a desired compound. Although constraint based modelling can serve as starting point to identify novel gene targets (knockouts, over and/or under expression) to create proof-of-principle strains and it can be used iteratively at various stages of the ten strategies previously reported by lee and colleagues [17]. The implementation of the strategies (see Table 3) proposed by lee and colleagues [17], enabled bio-based succinic acid production from *Mannheimia succiniciproducens* [18] and *Basfia succiniciproducens* [19].

Here we discuss *E. coli* genome-scale metabolic model's perspective in guiding much faster metabolic engineering strategies for constructions of proof-of-principle strains that could serve as a starting point for systems strategies in industrial strain development. Specific case studies of proof-of-principle *E. coli* strains constructed have been summarized in Table 2 and further proposed that ten strategies (see Table 3) applied elsewhere to produce L-arginine [20], L-lysine [21], and nylon from *Corynebacterium glutamicum* respectively [22]. This approach could also be applicable for bio-based succinic acid production using *E. coli* predictive potentials (since its GEMs have been published) and/or other succinic acid producing microorganism that could be discovered in the future.

2. Succinic Acid

Succinic acid is a bulk chemical with an estimated world production rates ranging between 30,000 to 50,000 tons annually [23]. The compound annual growth rate of its market is expected to reach 18.7% from 2011 to 2016 [23]. The conventional production of succinic is achieved via petrochemical process from butane or benzene through the conversion of maleic anhydride to succinic anhydride followed by subsequent hydrolysis. Other alternative routes for succinic acid production include the carbonylation of ethylene glycol and the oxidation of 1, 4-butanediol [24]. These petrochemical-based productions are expensive and pose serious environmental concerns.

2.1. Application and market for succinic acid

Succinic acid has a wide range of industrial applications. Succinic acid and its derivatives have applications as food ingredients, starting material for the manufacture of active pharmaceutical ingredients and/or pharmaceutical additives. It also has applications as surfactants/detergent extender, ion chelator, as flavoring agent [1] and as well as de-icing agent in aviation sector [25]. Succinic acid

has been reported to be in the list of the US department of Energy's 12 top bio-based chemicals that are produced using fermentation of microorganisms from renewable feedstock [5,23]. In addition, it was identified to be a building block chemical with a variety of applications [23]. Four major existing markets for succinic acid has been previously identified [1]. These markets include: (i) surfactant/detergent extender, which is considered as the largest, (ii) used in electroplating for prevention of corrosion and painting of metals (ion chelator), (iii) used as food additives, flavouring agents, and (iv) used in the production of pharmaceuticals, antibiotics, amino acids and vitamins (health-related applications).

Succinic acid derived from fermentation could have the potentials to become a precursor for the synthesis of commodity chemical that could serve as routes for providing a number of essential intermediates with applications in other industries. Succinic acid, has the potential to replace a number of commodities based on petrochemical intermediates and benzene, which could prevent pollution from manufacturers and the consumption of benzene-derived chemicals [1]. The potential routes map leading to succinic acid-based intermediates and specialty chemical is illustrated in Figure 1. These routes indicates where succinic acid can be a building block chemical for syntheses of commodity and specialty chemicals. Succinic acid is a linear saturated dicarboxylic acid, as such it can be used as a starting chemical to synthesize 1,4-butanediol, tetrahydrofuran, γ-butyrolactone and other chemicals, that could have a range of global application in various fields.

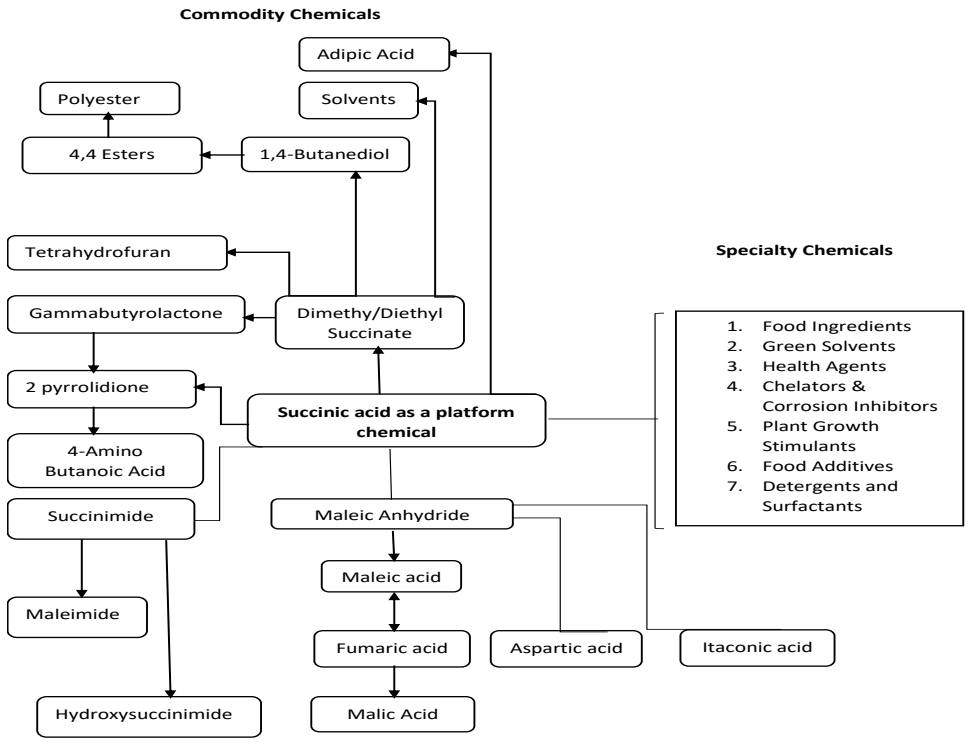

Figure 1. Succinic acid as a platform chemical for syntheses of other specialty and commodity chemicals. Partially adopted from Zeikus and colleagues [1].

3. *Escherichia coli* Genome-Scale Metabolic Models

Interaction of cell components with one another within the cell serve as bases for cellular functions from biochemical reactions. Cheap DNA sequencing and increase genome data availability, lead to a large number of biochemical data from cell component interactions, that can be organized and assembled in a form of reconstruction known as genome-scale models [26]. The computational model nowadays called reconstruction or GEM is generated from the wealth of biochemical information or data obtained by experimentation. The model has the capability to predict biological phenomena that are linked to genotypic and phenotypic functions, which usually uses a user define environmental and genetic parameters [26]. Both the parameters are well accounted for in an ideal GEM, paving a conspicuous way for increasingly accurate genotype-phenotype relationship prediction in a designated environmental conditions [26]. Genome-scale metabolic model has recently become foundational to understanding cells metabolic capabilities at systems-level and its noticeable uses in systems metabolic engineering [27]. A genome scale model (GEM) is usually constructed based on extensive collections and curation of the known biological information that contained gene annotation and functions, metabolites, metabolic reactions, enzymes and their overall reactions within an organism [27].

The *E. coli* GEM represent the best-validated GEM so far. This is largely due to the wealth of its experimental data availability and the simplicity of its network structure [27]. The first *E. coli* model was reported in 2000 and later updated as more is known on its biochemical reaction network (reactome) [26]. There are two most popular *E. coli* GEMs available in the literature and both can be downloaded from either the Biomodels database [28] or BiGG [29]. The Feist model designated as *i*AF1260, was published in 2007 which contained up to 1260 open reading frame [9]. This model contains 1,668 metabolites, 2,382 metabolic reactions (see Table 1) [9]. Using the new biochemical information (data) and conditional essentiality analysis, the model was further updated to the most recent version called the Orth model, designated as *i*JO1366 (see Table 1). This model was reported to accounts for 1,366 genes, 1,805 unique metabolites and 2,583 metabolic reactions [10] (see Table 1). In addition, an expanded GEM of *E. coli* has been published in 2013 called genome-scale metabolism and gene expression, designated as *i*OL1650-ME model [30]. This model was equally reported to have 1,683 genes, 12,009 reactions, and 6,563 unique metabolites [30]. These most recent models mentioned above were reconstructed by taking into account, elements and charged balance reactions, thermodynamic consistency and gene-reaction protein associations [9,10] These features are considered critical in improving the ability of models' accurate predictions of cellular phenotype and gene essentiality [9].

Table 1. *E. coli* genome-scale metabolic models with different size and scope.

Models	Reactions	Genes	Metabolites/Components	References
***i*AF1260**	2,382	1,260	1,668	[9]
***i*JO1366**	2,583	1,366	1,805	[10]
***i*OL1650-ME**	12,009	1,683	6,563	[30]

3.1. Escherichia coli proof-of-principle strains constructed using GEMs for succinate production

Strain design strategies for bio-based industrial production of succinic acid requires

system-wide approaches combining systems and synthetic biology tools to create superiors strains taking into consideration the fermentative strain performance at industrial scale. GEMs have been developed and widely used for strain design and biological discovery [9,10,11,31,32]. GEMs of *E. coli* have varying scope and their capabilities to predict metabolic engineering strategies considerably varies from one GEM to the next (see Table 1).

E. coli GEMs have been used for construction of proof-of-principle strains that enhances succinic acid production from two most important carbon sources, glucose, an glycerol [14,15,16,33,34,35]. The proof-of-principle strains constructed from our previous studies leverages the predictive metabolic engineering strategies using *E. coli* GEMs (in silico-driven hypotheses building), which reduces, time, cost, and labor intensive processes involved in identification of novel gene deletion targets, when compared with the conventional experimental trial and error approach (which is time consuming, expensive and labor intensive). A reasonable increase production of succinic acid have been achieved, though not industrially relevant titer but rather at proof-of-principle stage. This stage is considered as one of the fundamental stages in strain design and development strategies for industrially relevant performance. The strain constructed using GEM include BMS1 (Δ*atpE*), BMS2 (Δ*gnd*), BMS4 (Δ*fdoH*), with both predicted and experimentally confirmed increase in succinate production from glucose and glycerol carbon sources (see Table 2). Additionally, in silico

Table 2. *E. coli* proof-of-principle strain constructed for increasing succinic acid production from glucose and glycerol carbon sources.

Strains	Target genes	Carbon source used	Predicted using GEMs?	Experimentally confirmed?	Fermentation time (days)	Succinate production (g/l)	References
Wild-type	-	glucose	Yes	Yes	2	0.16	[36]
BMS1	ΔatpE	glucose	Yes	Yes	2	0.44	[36]
BMS2	Δgnd	glucose	Yes	Yes	2	0.29	[14]
BMS4	ΔfdoH	glucose	Yes	Yes	2	0.45	[16]
Wild-type	-	glycerol	Yes	Yes	7	0.02	[36]
BMS1	ΔatpE	glycerol	Yes	Yes	7	1.39	[36]
BMS2	Δgnd	glycerol	Yes	Yes	7	0.67	[14]
BMS4	ΔfdoH	glycerol	Yes	Yes	7	2.06	[16]
ptsG/b1101	ΔptsG	glycerol	Yes	Not yet	-	-	[35]
pntA/b1603	ΔpntA	glucose	Yes	Not yet	-	-	[33]
glpC/b2243	ΔglpC	glycerol	Yes	Not yet	-	-	[34]
Wild-type	-	glucose	Yes	Yes	1	0.29	[37]
W311OGFA	ΔptsG, ΔpykFA	glucose	Yes	Yes	1	0.96	[37]
W311OGFA	ΔptsG, ΔpykFA	glucose	Yes	Yes	3	2.05	[37]
W311OGFAP	ΔptsG, ΔpykFA, Δpfl	glucose	Yes	Yes	3	0.99	[37]
W311OGFAP	ΔptsG, ΔpykFA, Δpfl, ΔldhA	glucose	Yes	Yes	3	0.05	[37]

strains were constructed namely: *ptsG*/b1101, *pntA*/b1603, and *glpC*/b2243 that shows predicted increase in succinate production with not yet experimentally verified outcomes [33,34,35].

Other researchers have reported the use of *E. coli* GEMs for gene knockout simulation predicting increase in succinic acid production from glucose [37]. Their predicted and experimentally confirmed findings indicated increase succinic acid production in *E. coli* from glucose (see Table 2). The strains constructed contained combinatorial gene knockouts to increase succinic acid production from glucose. These strains include: W311OGFA (Δ*ptsG*, Δp*ykFA*), W311OGFA (Δ*ptsG*, Δ*pykFA*), W311OGFAP (Δ*ptsG*, Δ*pykFA*, Δ*pfl*) W311OGFAP (Δ*ptsG*, Δ*pykFA*, *Δpfl,* Δ*ldhA*) [37]. These strains were found to have increase in succinic acid production higher than their wild-type counterparts (see Table 2). None of the proof-of-principle strains constructed have reached industrially relevant titer, as it is considered as an initial stage of strain design strategies that could be further improved by applying recent ten strategies described by lee and colleagues [17]. For step by step brief description of the strategies that can be applicable to obtain industrially relevant performing strain with high yield titer and productivity, please kindly see Table 3.

Table 3. Ten systems strategies for constructing superior industrial strains.

Strategies	Brief descriptions	Expected inferences
1. Project design	Project design should be conducted for the target product and other plausible scenarios should be explored such as, cost-effective carbon source, aerobic and/or anaerobic fermentation, and downstream strategies and equipment to be used. Other key performance indices to be considered are: product titer, yield, and productivity in the context of bioprocess development and whether it could be economically competitive. In addition, systems and synthetic biology tools are becoming more available to make microorganisms of interest tractable to genetic manipulations within shortest possible time. With the recent development in synthetic biology gene-editing technology called CRISPR (clustered, regulatory interspaced, short palindrome repeats)-Cas9 (CRISPR-associated protein)-based systems offered considerable advantage for engineering microorganisms that were previously reported to be not amenable to genetic manipulations [17].	Technical, economical and regulatory factors are considered during project design and they should be strictly adhered to, as microbial engineering are involved and are considered as genetically modified organism (GMO) [17]. The microbial chassis strain constructed and their target products are classified as GMOs and they fall under different GMOs international regulations and different jurisdictions [17]. The regulations should be adhered to for safety.
2. Selection of chassis host	Chassis host should be carefully selected based on the availability of tools for its easy improvement or genetic manipulation for the increase production of target compound. *E. coli* and *S. cerevisiae* have been used more often than not, but some researchers are of the opinion that new host should be explored for biobased production of certain compounds, but this depends on the availability of resources and researchers' wisdom to explored the new host for the targeted project objectives.	Microbial chassis host selection is expected to take into consideration the target products, substrate utilization, strain tolerance to certain metabolic and physiological profiles such as pH in case of acids (e.g. lactic acid and succinic acid). The consideration for downstream processing, and purification cost should be carefully considered.

	Additional factors are considered in careful strain selection, such as carbon substrate utilization range, ease of fermentation on a cheap medium, ease of scale up, and requirements for aerobic and/or anaerobic conditions [17]. For example, succinic acid and lactic acid production requires a strain that is tolerant to low pH, because the purification of dissociated acid end product could be expensive when fermentation are performed under neutral pH [17].	
3. Metabolic pathway reconstruction	The candidate metabolic pathways leading to the production of the target compound may or may not be present in the select chassis host. If it is present, then the host is a native producer of the target compound e.g. succinic acid in *E. coli.* If the pathway is absent in the chassis host e.g. 1, 4-butanediol production in *E. coli* , the researchers need to establish the pathway by carefully identifying the candidate enzymes and/or genes via mining genomes and metagenomes.	Natural producer cells are expected to undergo re-engineering to block byproduct formation, decrease and/or increase precursor formation for the target compound and/or otherwise. Sometimes researcher need to introduce foreign genes that could help in increasing compound titers in native producer chassis host. On the other hand, heterologous pathways can be designed from other organisms for non-native producers of the target compound of interest. In this case, it is expected that optimization strategies should be employed to increase the performance of the foreign pathway introduced into the chassis host for optimal performance.
4. Tolerance of product Toxicity	Some products at certain requisite level of say 50–80% tends to inhibit cell's growth. Increasing tolerance level of the target strain design is of utmost importance. This can be achieved by developing product-tolerance strains by serial subculturing of cells with increasing concentration of product with or without mutagen treatment, followed by identification of cells that grow faster [17]. This could gradually increase the strain design tolerance to its target product.	It is expected that the strategies could increase product tolerance in the chassis host. This will be interesting so that it will not be toxic to the production host, giving it ability to produce industrially relevant titer and productivity. A chassis host that has been designed using this strategy is expected to be tolerant to the target compound which will offer considerable advantage in terms of industrially relevant performance.
5. Removal of negative regulatory circuits	Biological networks have negative feedback loops that influences genes expression and signal transduction profiles. Negative regulatory circuits that affects metabolic engineering cause transcriptional attenuation control and feedback inhibition during amino acids biosynthesis [17]. Transcriptional attenuation control could be addressed by replacing promoters of the target	The production of certain compounds from microbial chassis could be affected by negative regulatory circuits. There are, as yet, other regulatory circuits that could be uncovered in the future. If they serve as bottlenecks for overproducing a target compound in a chassis host, their removal

	metabolic enzymes with constitutive ones and/or removing genes encoding regulators that represses amino acids biosynthesis [17].	and/or attenuation become necessary to increase titer and productivity. Therefore, a chassis host devoid of negative regulatory circuit could ultimately increase the production of the target compound that can reached industrial relevant titer.
6. Changing flux directions to optimize cofactor and/or precursor availability	Increasing availability of cofactors increases the production of target compounds. Cofactor such as NADH is involved in many biochemical reactions and sometimes serves as precursor for the production of certain compound, eg Succinic acid production in *E. coli* require 2 mole of NADH to make 1 mole of Succinic acid from glucose. Therefore, deleting competing reactions (metabolic gene knockouts) that consumes NADH will ultimately increase precursor availability to be utilized in target compound production such as succinic acid in *E. coli*.	Changed flux directions by metabolic gene knockout of competing pathways (preserving additional cofactor and/or precursors such as NADH, NADPH) and minimizing by-products formation. Chassis host that is engineered to optimized cofactor and/or precursor availability would ultimately increase target compound production that might reach industrially relevant titer and productivity. Succinic acid production can be increased by increasing the availability of NADH in microbial chassis host.
7. Optimizing metabolic fluxes toward product formation and its diagnosis	Fluxes should be optimized using fed-batch fermentation at laboratory conditions, as the fed-batch fermentation are often required for standard industrial fermentative production of the target compound. This should be clearly taken into consideration during strain design and development. This involve iterative design and diagnosis at the laboratory scale before moving to industrial scale production. Fluxes to fermentative end product can be optimized by removing identified bottlenecks, diverting flux from branch pathways or even blocking secretion of byproducts, which can reduce the operation costs for product separation and purification in downstream processes 5 [17,38,39].	Production performance of the target strain need to evaluated and diagnosed using key indicators such as productivity, yield and titer. The metabolic intermediates should be carefully evaluated and diagnosed by optimizing metabolic fluxes towards the end product of interest. This strategy is expected to have effect on the substrate consumption by the chassis and its ability to produce the required end product to reach industrially relevant titer. Depending on its outcome, the subsequent objective that could address the next round of metabolic engineering can be proposed.
8. Optimization of microbial culture conditions and its diagnosis	In order to obtain an optimized microbial culture conditions for high performance, productivity, yield and titer of the target compound, one need to examine the availability of substrates and/or feed stocks with its surrounding economics. The ability of the host chassis to efficiently utilize the chosen carbon source is also an important consideration. Although chemically defined media is preferred compared to complex media, because of its desirability in both laboratory and industrial scale	Selection of suitable feedstock or carbon source that is cheaper, and ultimately abundant for microbial utilization is of utmost importance. The strain should have efficient substrate utilization rates in relation to the target compound production. The strain should be re-examined on different carbon sources, such as glycerol, glucose, xylose, and

	fermentation, and thus allow precise metabolic analyses of the chassis host and high efficiency in experimental reproducibility when compared to the complex media that could be expensive. e.g. optimized culture conditions using glucose and/or glycerol substrate could be used to produce succinic acid from microbial chassis host.	sucrose depending on their availability, economic profiles, and the target compound being produced. Careful diagnosis of these carbon sources and other conditions are expected to produce high performance microbial chassis for the production of the compound of interest.
9. System-wide gene manipulation for increasing production	This strategy seek to identify system-wide evaluation of gene manipulation targets that could ultimately increase production capability of the target strain. This strategy is often considered as the final round of engineering approach for final industrial strain. The strategy can be achieved by applying systems and synthetic biology methods such as cultivation-based profiling and systems wide analyses, high-throughput genome-scale engineering, in silico metabolic simulations and/or omics-based approaches.	Though require development of new tools and strategies for engineering microbial host cells other than *E. coli* and *S. cerevisiae*, it is considered as a stage that can evaluate, diagnose, and reexamined the final laboratory fed batch fermentation of the target strain in relation to the different approaches employed before industrial scale-up fermentation. Certain outcomes obtained at this stage may suggest iterative revisiting of an earlier decision and vice versa.
10. Scale-up fermentation of the designed strain and its diagnosis	Strain designed at bench scale and/or laboratory scale with desired characteristics could have a different performance under real pilot plant or demo plant fermentation for scale up [17]. Such discrepancies of strain displaying different characteristics at laboratory and pilot scales are often very difficult to be predicted at the inception. Therefore, this stage requires actual demo or pilot scale fermentation to be conducted for the desired chassis of interest. The outcomes of such fermentation profile of the constructed strain should be evaluated and diagnosed for possible discrepancies that might be attributed to genetic instability of the chassis host and/or substrate consumption or utilization at different fermentation stages [17].	The strain at this stage is expected to have stability and industrially relevant performance (e.g. titer, yield, and productivity) at the laboratory stage and pilot or demo plant fermentation stages. Failure in scale-up fermentation stage may occur when certain stages in the systems strategies were not adequately and carefully conducted and diagnosed [17]. e.g. failure to do actual pilot or demo plant characterization after the flask or fed-batch culture fermentation conducted at the laboratory scale.

4. Systems Strategies That Could Be Used to Increase Industrial Bio-Succinic Acid Production in the Future

To achieve microbial bio-based succinic acid production at industrial scale, an integrated approach combining systems-wide metabolic engineering and optimization of cellular metabolism is of utmost importance. This approach is expected to combine strain development (upstream), fermentation (midstream), and separation and purification (downstream). This approach entails ten different strategies that should be carefully adhered to in an iterative fashion to achieve biosynthetic goal of developing microbial chassis strains that can produce industrially relevant titer, yield, and productivity of succinic acid using cost effective manner. The ten strategies demonstrated

elsewhere [17] and summarized in Table 3 could be used in the future for bio-based succinic acid production using cheaper carbon substrate with microbial chassis host. The ten systems strategies for developing industrially relevant strain are briefly summarized with their corresponding expected outcomes in Table 3. For detailed strategies and inner working of the strategies, we refer the reader to the most recently published perspective reported elsewhere [17]. A noteworthy contribution of the ten strategies summarized in Table 3 is evident, as it has been used for bio-based production of L-arginine [20], L-lysine [21], and nylon [22] all from metabolically engineered *Corynebacterium glutamicum.* In a different study, the ten strategies for industrial strain development was used to directly overproduce 1,4-butanediol in metabolically engineered *E. coli* strain [40].

5. Conclusions and Perspectives

In this mini review, we describe brief applications of succinic acid as a platform chemical and the need to produce it via bio-based route, as a green technology. We further discuss the constructions of proof-of-principle strains, with specific published case studies from *E. coli* GEMs for increasing succinic acid production from glucose, and glycerol carbon sources. We additionally proposed that systems-wide strategies combining, metabolic engineering, systems biology and synthetic biology tools could be deployed to address the current challenge of achieving industrially relevant titers for bio-based succinic acid production from microbial chassis host.

The need for identification of new microorganisms capable of producing succinic acid is of utmost importance in this post genomic era. Genome-scale metabolic models of these organisms need to be developed and make them tractable and/or amenable for genetic manipulation to allow the deployment of toady's systems and synthetic biology tools including CRISPR-Cas9 gene editing technology to create robust microbial chassis for the production of succinic acid and/or any target compound of interest.

A noteworthy contribution of quality GEMs in systems strategies for strain development is highly recognized [32,37], although currently we have different GEMs of *E. coli* with varying scopes and gaps. For example, there are two types of *E. coli* GEMs namely, metabolism model (M-Model) [9,10] and metabolism and gene expression model (ME-model) [30]. The former (M-model), predict only reaction fluxes in the metabolic network while the later (ME-model), has the capability of predicting the cell's entire proteome [12]. Therefore, careful development and selection of GEMs in predicting metabolic engineering strategies could reduce the time, cost and labor intensive processes involved in systems strategies for strain development. The use of accurate GEMs is of great importance, and the deployment of recent advanced systems metabolic engineering tools in systems strategies for creation of superior industrial strains is also of utmost importance.

Acknowledgements

We are grateful to Dr. Pablo Carbonell, and Prof. Jean Marie Francois, for their unshakeable support, and advice.

Conflict of Interest

All authors declare that they have no conflict of interest in this paper.

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