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Metabolic pathway engineering: Perspectives and applications

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ABSTRACT

Background: Metabolic engineering aims at contriving microbes as biocatalysts for enhanced and cost-effective production of countless secondary metabolites. These secondary metabolites can be treated as the resources of industrial chemicals, pharmaceuticals and fuels. Plants are also crucial targets for metabolic engineers to produce necessary secondary metabolites. Metabolic engineering of both microorganism and plants also contributes towards drug discovery. In order to implement advanced metabolic engineering techniques efficiently, metabolic engineers should have detailed knowledge about cell physiology and metabolism. *Principle behind methodologies:* Genome-scale mathematical models of integrated metabolic, signal transduction, gene regulatory and protein-protein interaction networks along with experimental validation can provide such knowledge in this context. Incorporation of omics data into these models is crucial in the case of drug discovery. Inverse metabolic engineering and metabolic control analysis (MCA) can help in developing such models. Artificial intelligence methodology can also be applied for efficient and accurate metabolic engineering. *Conclusion:* In this review, we discuss, at the beginning, the perspectives of metabolic engineering and its application on microorganism and plant leading to drug discovery. At the end, we elaborate why inverse metabolic engineering and MCA are closely related to modern metabolic engineering. In addition, some crucial steps ensuring efficient and optimal metabolic engineering strategies have been discussed. Moreover, we explore the use of genomics data for the activation of silent metabolic clusters and how it can be integrated with metabolic engineering. Finally, we exhibit a few applications of artificial intelligence to metabolic engineering.

1. Introduction

During last century, industrial biotechnology has evolved to deal with production of fuels and foodstuffs. Emergence of metabolic engineering has been acting a significant boost in this domain [1]. Currently, metabolic engineering has improved a lot to procure desired cell phenotype [2,3] by redirecting appropriate metabolic fluxes with the help of metabolic flux analysis. In the post-genomic era, a tottering volume of metabolite, protein and gene data, introduces more accurate techniques to explore cellular metabolism. Consequently, it exponentially reduces the costs of oligonucleotide synthesis. Thus systems metabolic engineering [4–7] have evolved with various novel approaches, such as, rational gene deletion, expressing heterologous genes and tools to control gene expression/cell regulation [8,9]. Application of systems metabolic engineering to microbial production strain (*viz*., *Escherichia coli* and *Saccharomyces cerevisiae*) enables metabolic engineers to overproduce miscellaneous secondary metabolites for both industrial and pharmaceutical benefits [10,11]. These secondary metabolites are salient sources of industrial biofuels, chemicals and biopolymers [12].

Like microorganisms, plants can also be treated as important sources of secondary metabolites for efficient production of foods, pharmaceuticals and industrial compounds. For example, more than thirty thousand terpenoids can be synthesized from plants [13]. Here most of the terpenoid classes are secondary metabolites which can provide anticancer drugs and different defensive compounds [14]. Metabolic engineering can also improve agricultural production by enhancing nitrogen supply and nutrient in plant. In this context, improvement of photosynthetic efficiency and efficient control of plant diseases by advanced engineering technology play significant roles.

Metabolic engineering of both plant and microorganism helps to get different secondary metabolites with pharmacological values. It leads to efficient and cost effective drug discovery process [15,16]. In the first part of this review, we have discussed metabolic engineering perspectives from application point of view. Besides,

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we have elaborated its applications on microorganism, plant and drug discovery. Besides, we have elaborated different objectives, strategies and technologies behind such metabolic engineering on microorganism and plant.

Metabolic engineering often needs detailed inspection of the cellular physiology and metabolism using high-throughput genomic experimental procedures. Besides, it should involve clear concept on cellular processes which can be described with mathematical models. However, lack of detailed pathway (for example, protein secretion pathway) models is a major limitation in metabolic engineering. Although different kinetic models [17,18] of the parts of such pathways have been developed, modeling genome-scale integrated metabolic, signaling, gene regulatory and protein-protein interaction networks is still in nascent stage. Such genome-scale models will enable metabolic engineers to analyze the integrated complex pathways leading to rational design [19] of optimal alternative routes [20] for enhanced protein secretion [21]. Thus metabolic engineers need both *in silico* model for prediction, screening and directed evolution, and experimental analysis for suggesting novel hypotheses and confirming *in silico* predictions [22]. Subsequently, incorporation of omics data into the *in silico* models [23] leads to more accurate prediction of appropriate drug targets for certain complex diseases. In this context, it may be mentioned that silent metabolic clusters can be activated with the help of genomics data [24], leading to the integration of metabolic engineering and genomics data. In addition, artificial intelligence can play an important role in advanced metabolic engineering.

In order to explore cellular dynamics, inverse metabolic engineering and metabolic control analysis (MCA) play significant roles. Inverse metabolic engineering can help to identify particular gene(s) for improved production or desired phenotype using high-throughput screening techniques. On the other hand, MCA can determine accurate flux/pattern involved in a metabolic pathway. In the second part of this review, we have elaborated inverse metabolic engineering and MCA in the context of metabolic engineering. In addition, we have discussed some important steps to be carried out for developing both optimal and efficient metabolic engineering strategies. Moreover, we have explored some aspects of integrating genomics data and metabolic engineering. Besides, we have discussed how artificial intelligence can be applied to metabolic engineering. Finally, we have concluded with a discussion pointing towards the advanced technology and future direction of modern metabolic engineering.

2. Perspectives of metabolic engineering

Till today, metabolic engineering has immense contributions to agriculture, medicine and basic science. Metabolic engineering has emerged as advanced future technology providing efficient strategies to utilize renewable resources (*viz*., microorganism and plant) to generate alternative fuels [25]. Genome-scale models of integrated metabolic, signal transduction, protein-protein interaction and gene regulatory networks have significant roles in developing metabolic engineering strategies. Subsequently, advanced tools for gene editing, deleting and cloning have pushed metabolic engineering a few steps ahead. However, such tools need detailed understanding about cellular properties at molecular level, pathway regulations as well as dynamics, enzyme kinetics, flux distributions and gene-protein relationships among others, particularly in response to various perturbations under consideration. Figures 1 and 2 depict a close relationship among metabolic engineering, MCA and inverse metabolic engineering. Here MCA and inverse metabolic engineering provide necessary knowledge about the properties of cell and concerned biochemical pathways so that efficient metabolic engineering strategies can be formulated. Computational analysis based on multiple omics data (*e.g.*, genomics data), with the help of artificial intelligence, can also facilitate such efficient and optimal metabolic engineering strategies. We will discuss MCA and inverse metabolic engineering in details in next sections. Besides, we exhibit some glimpse of the role of genomics data and artificial intelligence in modern metabolic engineering in the following sections.

Efficient metabolic engineering strategies have been applied on microorganism as well as plant to produce alternative fuels, food, industrial compounds and pharmaceuticals. Fig. 1 demonstrates different strategies of metabolic engineering applied on microorganism and plant. It includes synthetic pathway construction and production enhancement strategy for microorganism. In case of plant, enhancement of nitrogen supply, nutrient, biofuel and photosynthesis as well as disease control can be achieved through advanced metabolic engineering strategies. More details about such strategies, advanced technologies, tools, *in vivo*/*in vitro* methods and *in silico* algorithms can be found in following sections. Such strategies, technologies, tools, experimental and computational methods, and application of metabolic engineering on microorganism as well as plant lead to successful drug design for various complex diseases, such as type 2 diabetes and cancer among others. Later we shall discuss in details how metabolic engineering influences drug discovery.

3. Metabolic engineering of microorganism

In pursuit of exploring various chemicals and materials as essential and renewable resources for mankind, metabolic engineering of microorganism plays an important role nowadays [26]. Recently, metabolic engineering of microbial cells can produce desired amount of natural and non-natural chemicals as well as necessary materials [27,28]. Here, metabolic engineers mainly face two kind of problems. One of them is to construct appropriate synthetic pathways to produce such important chemicals/compounds that are not generated from native pathways. Another one deals with enhancing the production through the identified synthetic metabolic pathways. Now, we are going to discuss about different techniques applied to solve the aforesaid problems.

3.1. Synthetic metabolic pathway construction

Formation of synthetic metabolic pathways for efficient production of targetted biochemicals is a popular strategy in metabolic engineering. We can further classify the strategies behind synthetic metabolic pathway formation into three subdivisions, such as *in vitro* and *in vivo* pathway generation, *in silico* optimal pathway prediction, and enzyme engineering.

3.1.1. In vitro *and* in vivo *synthetic pathway generation*

Here, in search of optimal pathways, appropriate enzymes from different organisms and metagenomes are heterologously/combinatorially used to form new synthetic metabolic pathways. For example, a previous investigation [29] has produced fatty acid ethyl ester from hemicelulosic biomass in an engineered *E. coli* strain in combination with genes from other plants and bacteria. β-carotene, applied in pharmaceuticals, neutraceuticals, cosmetics and food, can be generated from a synthetic pathway in *E. coli* due to modulation of single ATP synthesis gene, pentose phosphate and TCA modules [30]. Engineered *E. coli* can produce perillyl alcohol (POH), an anti-cancer agent [31]. In this study, a cytochrome P450 is coupled with a heterologous mevalonate and limonene synthase pathway. Introduction of heterologous *ispS* genes encoding isoprene synthase from *Populus nigra* and *Pueraria montana* into *E. coli* and cyanobacterium *Synechocystis* sp. PCC 6803 can produce a jet fuel, isoprene [32,33]. Researchers show

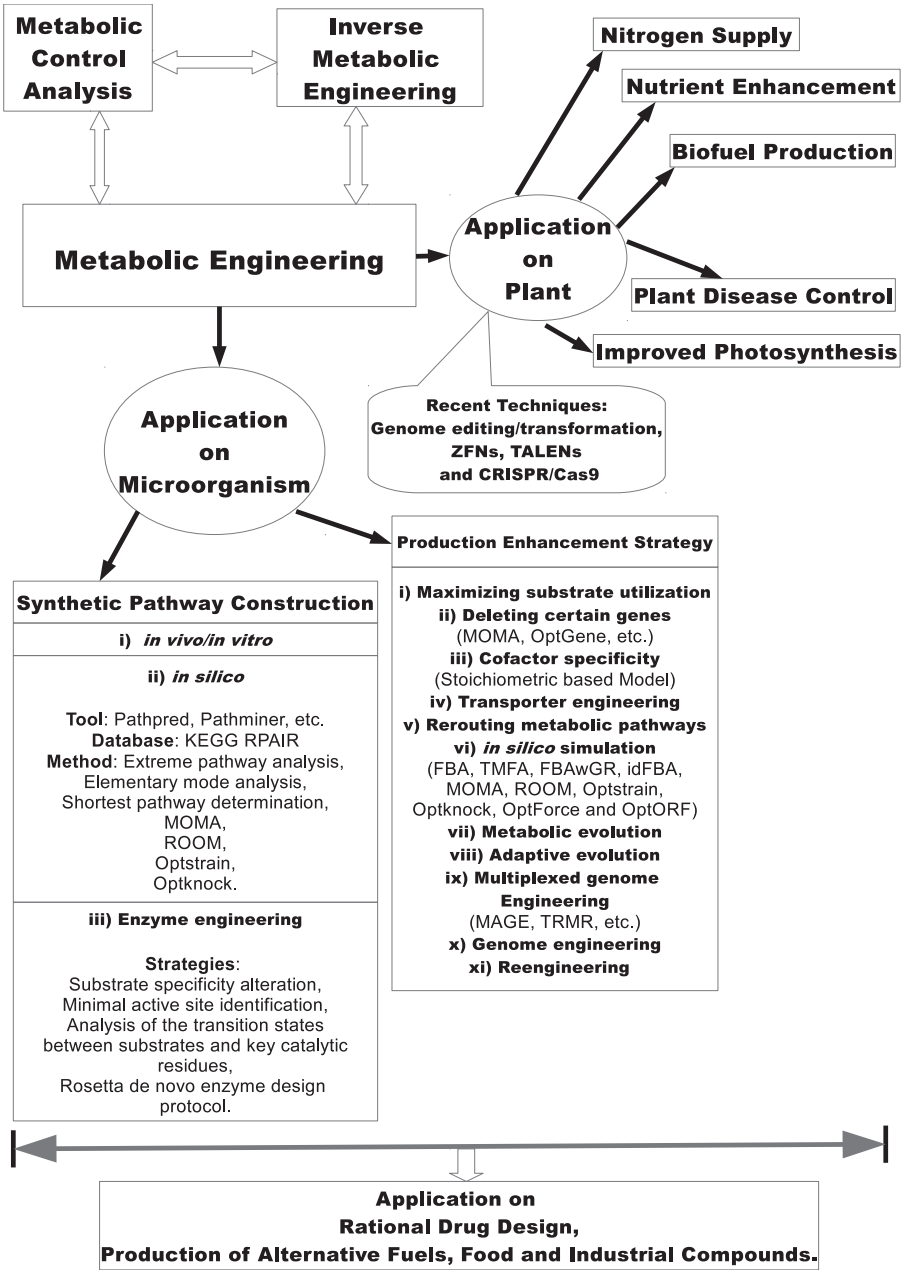

Fig. 1. Perspectives of metabolic engineering from application point of view with the help of metabolic control analysis and inverse metabolic engineering.

how the incorporation of 2-ketoacid decarboxylase from *Lactococcus lactis* and alcohol dehydrogenase from *Saccharomyces cerevisiae* converts 2-ketoacids into various natural alcohols (for example, isobutanol) in *E. coli* [34]. In this context, a previous investigation [35] shows similar kind of strategy in engineered biosynthesis of butanol. First autotrophic 1-butanol has been produced using an engineered metabolic pathway in a cyanobacterium, *Synechococcus elongatus* PCC 7942 [36].

Styrene is useful as a monomer for many important polymer synthesis. Microbial styrene production has been reported first in a recent work [37]. In this study, a synthetic pathway is formed, by introducing phenylalanine ammonia lyase2 from *Arabidopsis thaliana* and ferulate decarboxylase from *Saccharomyces cerevisiae* into *E. coli* host, to produce 260 mg/L styrene from phenylalanine. Combination of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase, chorismate mutase, phenylalanine ammonia lyase, 4-coumarate:CoA ligase, chalcone synthase, chalcone isomerase, malonate synthetase and malonate carrier protein can produce the flavonoid precursor (2S)-pinocembrin from glucose directly [38]. Flavonoid has a valuable role in human health and nutrition. A previous investigation [39] has produced α-farnesene, useful in pharmaceuticals and industry, using a codon optimized enzyme α-farnesene synthase and mevalonate (MVA) pathway on an engineered *E. coli* host. Long-chain polyunsaturated fatty acids (for example, eicosapentaenoic acid (EPA)) help in preventing and treating cardio-vascular diseases, obesity and diabetes. Here, engineering a bacteria, *Pseudomonas putida* [40] and a yeast *Yarrowia lipolytica* [41] can produce the important long-chain polyunsaturated fatty acids nowadays. Even recently, metabolic engineers have shown how bioelectricity can be generated from one molecule of glucose through de novo *in vitro* synthetic enzymatic pathway [42].

Metabolic engineers show a great interest in *Saccharomyces cerevisiae* to produce fatty acid derived biofuels and chemicals from

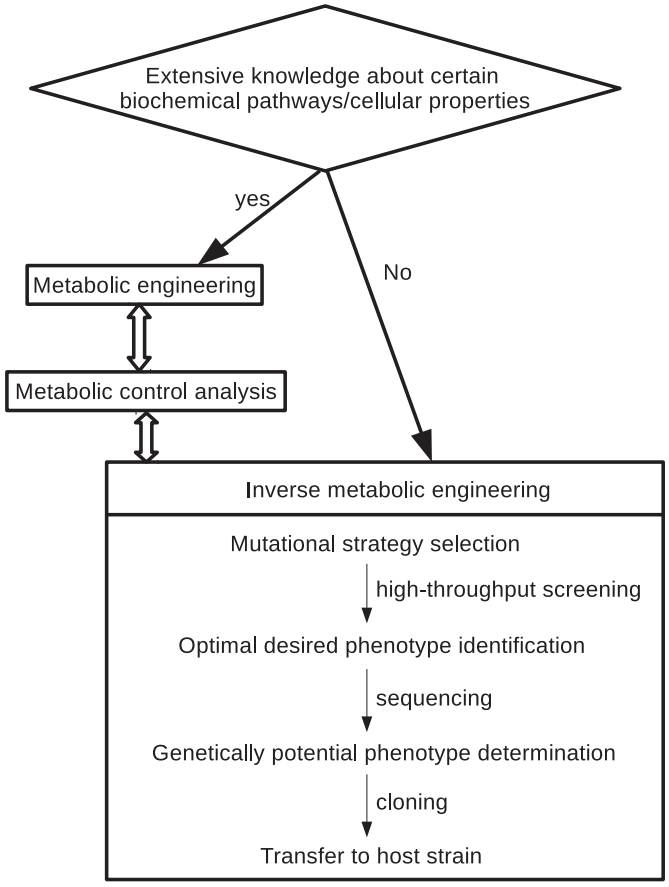

Fig. 2. Different steps of inverse metabolic engineering.

sugars using overexpressed acetyl-CoA carboxylase, fatty acid synthase1 and fatty acid synthase2 in the same organism [43]. A previous investigation [44] shows how *Saccharomyces cerevisiae* can be a promising host to be engineered to generate succinic acid on a large scale. Ceremide-NS, responsible for the defence mechanism and moisture control of human skin, is produced from *Saccharomyces cerevisiae* by replacing the genes for sphingolipid hydroxylases of yeast by the genes translating human sphingolipid desaturase [45]. Similar kind of work [46] has been performed to improve growth on glycerol of *Saccharomyces cerevisiae*. Recently, some researchers aim at developing industrial yeast (*Saccharomyces cerevisiae*) strains to be engineered to produce commercially available cellulosic biofuels (for example, ethanol) in experimental lab [47].

3.1.2. In silico *optimal pathway prediction*

Many tools can predict the optimal routes to produce a specific target molecule by inspecting all possible routes in a certain metabolic pathway. The relative distance between the substrate and product, network analysis, substrate binding sites, reaction mechanism, structural analysis and thermodynamics specifications are taken into account in this regard [48]. Pathpred [49] is an example of such tool to predict probable pathways of multi-step reactions using chemical structure transformation patterns from KEGG RPAIR database. PathMiner [50] is another heuristic approach to identify plausible routes to produce certain molecules efficiently. Here, the chemical structure based methods have also been used.

Unlike chemical structure based methods, experimental information and/or knowledge about the reactions and pathways from different databases contribute mostly to the knowledge based methodologies. Here, the most efficient pathway is chosen using extreme pathway analysis [51,52], elementary mode analysis [53] and the shortest pathway determination based on the knowledge. Construction of efficient routes to produce 3-hydroxypropanoate from pyruvate in *E. coli* host, using Biochemical Network Integrated Computational Explorer (BNICE) framework [54], can be considered as an example of knowledge based method. Often some of the *in silico* models/methodologies, [55] such as Minimization of Metabolic Adjustment (MOMA) [56], Regulatory On/Off Minimization (ROOM) [57], OptStrain [58] and Optknock [59], are combined with the knowledge based methods to identify optimal routes as well as candidate genes to be knocked out. For example, a previous investigation [60] predict gene targets to generate medium chain length PHAs on glucose in *Pseudomonas putida* host using elementary mode analysis. Another *in silico* model [61] predicts the optimal pathway for synthesis of L-lysine in *Corynebacterium glutamicum* host.

3.1.3. Enzyme engineering

Enzyme engineering is a process of creating new enzymes with desired functions to activate/deactivate certain reactions synthesizing target molecules in a pathway. Specificities of the substrates for the most probable enzymes have been altered through mutagenesis and guided evolution in this regard. For example, propionate CoA transferase from *Clostridium propionicum* and PHA synthase from *Pseudomonas sp.* MBEL 6–19 have been selected among all screened plausible enzymes to produce a non-natural biodegradable polymer, polylactic acid (PLA) in one-step fermentation from *E. coli* host [62]. However, substrate specificity alteration strategy has some restrictions [63]. Thus, minimal active site identification, analysis of the transition states between substrates and key catalytic residues and some other de novo design strategy have also been considered for enzyme engineering [64,65]. Recently, new enzymes have been designed to catalyze some well known non-natural reactions such as Kemp elimination [66], Diels-Alder reaction [67] and retro-aldol reaction [68]. Further development in enzyme engineering includes a recent investigation following Rosetta de novo enzyme design protocol [69].

3.2. Production enhancement strategy

After generation of synthetic pathways, metabolic engineers have followed several strategies to enhance the production of certain target molecules/chemicals. Multiple rational and intuitive approaches are combined to achieve multiple objectives in this regard.

In order to enhance the production of a certain target molecule, the process of maximizing substrate utilization is very common in practice. A previous investigation has enhanced succinate production from glycerol in *E. coli* host by retaining all routes of glycerol consumption [70]. The native glucose utilizing phosphotransferase system of *E. coli* has been replaced with non-phosphotransferase system of *Corynebacterium glutamicum* for increasing L-lysine production significantly [71]. This kind of strategy helps scientists in utilizing glucose and galactose as well as to produce an industrially important chemical, enantiopure (2R,3R)-butanediol in *Saccharomyces cerevisiae* simultaneously [72]. A recent work shows how methanol, an essential biological product for chemicals and fuels, can be utilized in *E. coli* host with the help of a NAD-dependent methanol dehydrogenase (Mdh) from *Bacillus stearothermophilus* and ribulose monophosphate (RuMP) pathway enzymes from *Bacillus methanolicus* [73]. Moreover, engineers have shown how *Saccharomyces cerevisiae* can successfully produce bioproducts from feedstocks which are generated from biomass [74]. However, substrate utilization approach has some limitations such as difficulty in handling heterologous expression of genes [75] and different rates of using multiple carbon sources [76].

Another simple production enhancement approach is to delete certain genes for minimizing byproduct formation, and to amplify specific genes for the enrichment of precursor. This kind of strategy has successfully been applied to enhance sesquiterpene biosynthesis in *Saccharomyces cerevisiae* [77]. Here, OptGene [78] and MOMA have played an important role to assess gene deletion effect on the flux distributions in the metabolic model of *Saccharomyces cerevisiae*. Similar approach has been used to engineer *E. coli* for enhancing cadaverine production [79]. Here, metabolic engineers should care about the possible accumulation of toxic intermediates due to imbalanced expression of certain genes.

Availability of necessary cofactors is an important issue to drive the production of target molecules/chemicals at desired level. Site-directed or random mutagenesis driven target enzyme evolution [80,81], and screening of different heterologous enzymes [82] play a crucial role to alter cofactor specificity. A previous investigation [83] shows how enhanced cofactor specificity of the heterologously expressed lactate dehydrogenase leads to photosynthetic production of lactic acid by *Synechocystis sp.* PCC6803. Researchers have used a stoichiometric-based model to enhance availability of the cofactor, NADPH in *E. coli* for natural product biosynthesis [84]. However, NADPH dependent enzymes catalyzing the required reaction may not be naturally available [85].

Transporter helps in excreting the target chemical from cytoplasm to extracellular space. As a result, intracellular concentration of the target molecule decreases to avoid feedback inhibition. Ultimately, it maximizes the production of the concerned molecule [79,86]. Similar effects can be found if import of excreted required molecule has been restricted using transporter [79,86]. A recent study [87] has used this kind of transporter engineering technique to demonstrate how heterologously expressed pumps in *E. coli* can enhance production of the target biofuel.

There are more efficient pathways than conventional ones to produce certain target products efficiently. Several instances exist to reroute metabolic pathways purposefully. For example, a previous study [88] shows that alcohols and carboxylic acids of different chain length and functionalities can be synthesized by reversal of β-oxidation cycle. Another study [89] has reported how carbon flux of *E. coli* can be redirected to the pathways generating malonyl-CoA. Similar strategy [90] has been carried out to produce ethanol from *Geobacillus thermoglucosidasius* strain.

In order to find out appropriate gene targets, cell growth rate and maximum production rate of target chemicals, *in silico* models and simulation strategies are quite successful. Flux balance analysis (FBA) [91,92] is very popular to determine the reaction fluxes using an objective function and mass balance equations. There are different variation of this methodology, such as thermodynamic based metabolic flux analysis (TMFA) [93], flux balance analysis with grouping reaction constraints (FBAwGR) [94] and integrated dynamic FBA (idFBA) [95]. Moreover, MOMA [56], ROOM [57], OptStrain [58], Optknock [59], OptForce [96] and OptORF [97] can successfully predict gene manipulation targets to achieve efficient production rate of target molecules. Even computational optimization, based on mRNA sequences including their secondary structures, DNA microarray, metabolic profiling and RNA devices including ribozymes and aptazymes, can predict how minutely gene expression levels can be controlled for specific purpose [98,99].

Finally, there exist some other techniques such as metabolic evolution, adaptive evolution, multiplexed genome engineering [100], genome resequencing and reengineering, which are popular nowadays to enhance production of target chemicals. Recently, metabolic evolution has been applied to produce succinic acid in *E. coli* [101]. In case of adaptive evolution, cells are exposed in perturbed external condition to obtain desired phenotype. Observing gene adaptation during this process, gene resequencing and reengineering have been performed to identify appropriate genes for the desired phenotype. This adaptive evolution process is applied to improve isobutanol tolerance in *E. coli* [102]. Recently, multiplexed automated genome engineering (MAGE) helps in tuning multiple gene target more efficiently [103]. Trackable multiplex recombineering (TRMR) is more advanced version of multiplexed genome engineering technique. It helps in engineering and monitoring thousands of microbial genes simultaneously [104]. For example, genetic modifications to *Yarrowia lipolytica* can improve its lipid metabolism pathways (*i.e.*, synthesis of β-oxidation and TAG) to produce higher amount of conjugated linoleic acids (CLA), which is a beneficial dietary supplement for human health [105].

4. Metabolic engineering of plant

Apart from microorganism, plant metabolic engineering advances rapidly over last few decades. Golden rice [106–110] is a significant example of the progress in plant metabolic engineering. Here, metabolic engineers face great challenges including self sufficient plant creation in case of nitrogen requirement, enhancement of the nutrient in crop plants, biofuel production from plants, photosynthetic efficiency improvement [111] and plant disease control [112]. In this context, metabolic engineering strategies should integrate different factors, such as highly compartmentalized plant metabolism and cellular/subcellular localization of metabolites and enzymes [113,114].

4.1. Nitrogen supply

Nitrogen fertilizers damage the soil, environment and nearby water resources. Thus, it will be a great advantage if a plant itself can manage its own required nitrogen without the dependency on fertilizers. Metabolic engineering helps in this regard. An enzyme, *viz.*, nitrogenase [115], found in some bacteria, converts atmospheric nitrogen (N_2) into a biologically available form ammonia (NH_3). Plants are able to produce the required nitrogen if somehow nitrogenase is expressed in plant host. Here, the necessary and sufficient condition for nitrogenase production is to express eighteen genes from *Klebsiella oxytoca* simultaneously. Some previous engineering methods [116,117] successfully transfer the biosynthetic gene cluster from one microbe to other for nitrogenase to make it active in the host. However, metabolic engineers are able to make express only eight genes for nitrogenase in plat host till now [118]. Subsequently, determining the tissue location and cell type, where the gene cluster for nitrogenase needs to be expressed, raises a big question to plant metabolic engineers.

In order to overcome the difficulties in making nitrogenase active in plant host, engineering the interaction between the nitrogen generating microorganism and plant host through rhizosphere becomes an important technique to supply required nitrogen for the plant [119]. Here, both plant and microbial metabolic engineering play their crucial roles.

4.2. Nutrient enhancement in plant

Metabolic engineering contributes a promising role in enhancing the nutrient contents in food crops. Here, the metabolic pathways, producing phytoalexins, flavonoids and other molecules helpful in chemopreventive mechanism of vegetables and fruits, become the main targets for metabolic engineering. An innovative plant breeding technology, leading to the Beneforte strain of broccoli with enhanced content of the glucosinolate glucoraphanin, has been reported recently [120]. Brococli with high glucoraphanin reduces the risk of cancer in human. This process does not require the knowledge about genes involved in the biochemical pathways. Flavonoid anthocyanin has been enhanced 3-fold by expressing two transgenes from snapdragon in tomato [121]. Here, tomato

with high anthocyanin extends the life span of the cancer susceptible mice. The metabolic and genetic engineering approaches are also helpful in increasing the iodine content in certain plant species [122]. For example, a previous investigation [123] shows how iodine content in a plant species *Arabidopsis thaliana* can be enhanced by expressing sodium-symporter (NIS) of human thyroid gland and knocking out HOL-1 gene.

Rice with higher vitamin-A can be produced by adding beta-carotene pathway to it. This strain is known as golden rice [106]. Apart from rice, the nutrient content in maize, wheat and cruciferous vegetables can also be enhanced [124]. The effects of transferring 13-gene glucoraphanin pathway into rice, maize, and wheat are reported in a recent work [125]. Another recent finding shows that docosapentaenoic acid (DHA) can be produced from a plant crop *Camelina sativa* by introducing a transgenic D6-desaturase pathway from yeast [126]. In this context, it should be mentioned that DHA is an essential omega-3 fatty acid which helps in developing brain of a premature baby during first four months of life span [127]. Besides, DHA plays important roles to treat type2 diabetes, hypertension and thrombosis among others [127]. Thus, exploring the genes and their activities in plant biochemical pathways are very much essential in this regard. In this scenario, new genome editing tools can perform multiple changes simultaneously in plant host. Recently, zinc finger nucleases (ZFNs) [128], transcription activator-like effector nucleases (TALENs) [129] and clustered regularly interspaced short palindromic repeats-CRISPR-associated gene 9 (CRISPR-Cas9) [130–133] play promising role in systematic engineering of plant genomes. Although advanced tools in metabolic engineering can enhance the nutrient contents in plant crops, they result in alteration of the taste and palatability of the plant. Thus, there is another challenge to improve or maintain the taste of the plant crops. For example, researchers have included the steviol glycosides and mixed esters in strawberry plants to give an unique flavor [134].

4.3. Biofuel production from plant

Plant takes carbon dioxide (CO_2) from nature and converts it into sugar. Besides, most of the carbon is preserved in dehydrated crystalline cellulose wrapped in a phenylpropanoids, called lignin [135]. The sugar can be used as an ideal substrate for biofuel production. Though polymers of glucose in the forms of starch and glycogen can easily be degraded into sugar monomers, it becomes a challenging task to break beta-1,4-linked chains of cellulose down into sugar monomers. Even it is very hard to separate cellulose from lignin [136]. Researchers have tried different techniques [137,138] to degrade lignin into aromatic monomers (biofuels) by chemical application or enzymes found in microorganisms. However, these works do not meet success in real world applications.

Recently, it is reported that knocking out a lignin biosynthetic gene, called caffeoyl shikimate esterase, from *Arabidopsis thaliana* can increase the efficiency to break oligosaccharides down into monosaccharides [139]. However, this technique affects the growth and development of the plant by decreasing 25% and 40% cellulose content and size of the plant respectively than wild type [140]. In this context, another protein engineering investigation [141] has reduced lignin content in *Arabidopsis thaliana* by expressing a 4-O-methyltransferase without affecting the growth phenotype significantly.

It will be better if somehow plant can itself degrade its own lignin and generate pure cellulose. No doubt, it is a daunting task. However, existence of some enzymes (for example, lignin peroxidases from white rot fungi), that can degrade lignin [142], shows the possibility of future work in this field. Here, identification of appropriate enzymes as well as the proper modification to lignin backbone should be carried out carefully to avoid structurally altered plant phenotype susceptible to pathogens and pests.

4.4. Photosynthetic efficiency improvement

Rapid increase in world population and urbanization gives rise to the importance of improving the photosynthetic efficiency of plant. There are some methods [143,144] to improve the photosynthetic efficiency with the active concentration of CO_2 and the reduction of the oxygenase activity of Rubisco [145]. In this context, two systems, namely C4 and Crassulacean acid metabolism (CAM) photosynthesis, are available nowadays. However, the evolution of C3 plant to C4 plant requires new metabolic engineering techniques [146]. In other words, it requires to develop more efficient and sophisticated genome editing or transformation methods.

4.5. Plant disease control

Metabolic engineering of biosynthetic pathways contributes to generate plant chemical defence compounds with antimicrobial properties, useful in crop protection [112]. Commercially grown transgenic crops, such as maize, cotton and soybean, are more tolerant to herbicides, such as glyphosate, and resistant to insects. They can successfully be produced by transferring a single gene (for example, endotoxin encoding genes from *Bacillus thuringiensis*). However, multiple gene transfer technique is still under microscopic investigation. The most extensive metabolic engineering strategy for plant disease control is to introduce a primarily resveratrol synthase, stilbene synthase [147]. To apply multiple gene based transfer of a pathway into another species of plant to produce a defence compound, it requires extensive knowledge about the plant biochemical pathways, enzyme dynamics and MCA to identify the genes of interest [148].

Plant genome sequences (available in http://www.phytozome.net/) and trascriptome data including co-expression analysis help in identifying the candidate genes [149,150]. Sometimes, these genes are co-localized in the genome to form a biosynthetic gene cluster [151,152]. Here, different kinds of enzymes, involved in the biosynthesis of a specific defence compound, can be encoded from the non-homologous genes contained in the gene cluster. In this context, ten candidate genes are identified by a biosynthetic gene cluster for the anti-tumour alkaloid noscapine found in opium poppy, *i.e., Papaver somniferum* [153]. Here, six among these identified genes are experimentally found to be involved in the biosynthesis of noscapine. Thus biosynthetic genes of a pathway can be identified by a gene cluster. Another promising approach is to imitate regulatory mechanisms of natural plant. Expressing the transcription factors and use of artificial gene clusters [113] may serve in this occasion. However, it may lead to undesirable side-effects reflected in certain phenotype of the plant [154]. Besides, gene silencing may arise due to expression of multiple genes of a pathway simultaneously. There exist several technologies [155], such as polyprotein technology and synthetic promoters, trying to solve the issues as mentioned.

We have already mentioned that some advanced technologies, such as ZFNs, TALENs and CRISPR-Cas9, play an important role to modify genome of an organism precisely. Here, the chimeric proteins, ZFNs and TALENs use DNA binding modules and target site cleavage by a fused nuclease domain [156] to detect specific DNA target sequence. On the other hand, CRISPR-Cas9 uses a guide RNA molecule to do the same. These technologies allow one to knock out target genes and modify as well as repair a particular nucleotide sequence.

Thus it concludes that metabolic engineering of the chemical defence compounds in plant depends upon the knowledge about the pathway and the interactions among different targeted genes.

Different technological and experimental developments in pathway analysis, DNA sequencing, gene expression analysis and finally genome editing have been carried out nowadays. In this context, trait stacking [157] plays a crucial role in increasing the scope and efficacy of transgenic immunity from pests, pathogens and various abiotic stresses [158].

5. Metabolic engineering in drug discovery

Development of a successful drug involves many years and high cost [159]. Thus pharmaceutical companies must introduce as many as possible candidate molecules in drug discovery pipeline for identification of successful one to become an effective drug [159]. However, very little amount of natural candidate molecules for drug discovery have been found in their native hosts. In this context, metabolic engineering can be useful strategy for cost effective production of the candidate molecules in short period of time. Here metabolic engineering can efficiently utilize cellular resources in biosynthetic pathways to produce such molecules. Moreover, in order to enhance pharmaceutical productivity, it needs intelligent strategies to analyze biological systems at molecular level. Recently, expression of multiple genes can be controlled simultaneously with the help of metabolic engineering to analyze molecular dynamics of the biological system in response to perturbation.

Metabolic engineering has shown how significant cellular resources can be diverted into biosynthetic pathways of heterologous hosts (*Saccharomyces cerevisiae* and *E. coli*) to produce different anticancer, antibacterial and antifungal agents, such as polyketides, isoprenoids and carbohydrates [159]. It results in less expensive drugs in short span of time. Activation of silent genes through ribosome engineering to myxobacteria, *Streptomyces* and fungi has successfully synthesized significant secondary metabolites required for drug development [160]. Identification of biosynthetic gene clusters involved in such secondary metabolite production is an important step in this regard. Here improved whole genome sequencing as well as genome mining helps in cloning the gene clusters so that their enhanced expressions can be achieved in heterologous hosts. For example, the effective metabolic engineering of actinomycetes can be done based on the aforementioned principle for antibiotic development [161].

Thus metabolic engineering in combination with synthetic biology and systems biology can enable efficient engineering of microorganism as well as plant to produce cost and time effective pharmaceutical agents from heterologous hosts [162]. However, exploring the relation among metabolism, transcription, translation and enzyme activities is the most important factor in this context. It can be done by analyzing an *in silico* model of human metabolic pathway [163], appropriately integrated with gene regulatory, signal transduction and protein-protein interaction networks. For example, Recon 2.2 [164], a genome-scale human metabolic network reconstruction, is capable of predicting the variation of metabolism from single cell to tissues in response to perturbation. However, development of an effective integrated metabolic, signal transduction, gene regulatory and protein-protein interaction network model is still in a nascent stage. In addition, integration of metabolomics and genomics may efficiently predict optimal biosynthetic pathways to produce necessary amount of secondary metabolites for drug discovery [15]. In the following section, we are going to explore the role of genomics data in activating silent metabolic clusters. Consequently, it leads to integration of genomics data and metabolic engineering. Inverse metabolic engineering and metabolic control analysis (MCA) play an important role in this context. In addition, in order to design optimal and efficient metabolic engineering strategies, it needs to incorporate the notion of artificial intelligence nowadays. We are going to discuss them in following sections.

6. Inverse metabolic engineering

Extensive knowledge about a certain biochemical pathway, responsible for the production of a particular metabolite or for a particular cell phenotype, is very important to implement metabolic engineering techniques successfully. Based on that knowledge, target gene for modification can be identified appropriately. However, there are many cellular properties relevant to research and industries, which are not properly understood and/or most of the cases are unknown still now. Therefore, efficient identification of a target gene is not feasible. In this scenario, a new strategy, called inverse metabolic engineering [3] has been adopted nowadays. Here, a recognizable phenotype (for example, color and cell growth) is connected with the desired cell properties that metabolic engineers want to achieve. In addition, genetically diverse cell library has been generated by carrying out random genetic modification (for example, gene deletion and chromosomal point mutation) in the host. The genotype with desired phenotype is selected from the library. Genetic analysis of the selected genotype reveals the underlying mechanism responsible for the desired cell properties. More diverse the cell library is, more successful identification of the required genotype is possible [165].

There are four basic steps to implement inverse metabolic engineering [166] as depicted in Fig. 2.

- First one is to select appropriate mutational strategy so that large genetically diverse cell library can be formed. Different types of mutational strategies include overexpression of plasmid library [167], transposon mutagenesis [168], site directed mutagenesis [169] and others.
- Second one is to carry out high throughput screening of the library to identify mutants showing desired phenotypes. In this context, a previous investigation [170] demonstrates how high throughput screening can be used to isolate mutants of *E. coli* and *Synechocystis* sp. strain PCC6803 with enhanced poly-3-hydroxybutyrate (PHB) accumulation.
- In the third step, genetically potential phenotype is determined among the said desired phenotypes through sequencing and quantitative polymerase chain reaction.
- Finally, the genotype of the ideal phenotype is cloned into the host strain.

Selection of appropriate mutational strategy is very crucial in inverse metabolic engineering. Adaptation and specific growth conditions can help in this case [171]. Some previous investigations [172,173] use mutagens to select mutational strategies. However, modern technologies, such as global transcription machinery engineering (gTME) [174,175], genome shuffling [176–178], trackable multiplex recombineering (TRMR) [104,179] and ribosome engineering [180,181], play important roles in this regard. Phenotype screening is a major step in inverse metabolic engineering. Recently, single cell technology has been used for this purpose [166,182,183].

7. Metabolic engineering in relation with metabolic control analysis

Recombinant DNA technique helps in modifying the intermediary metabolism purposefully in metabolic engineering. Here, metabolic engineering tries to introduce new pathways in microorganisms to achieve desired amount of target products/metabolites/heterologous peptides, *viz.*, polyketides, human insulin, erythropoitin, tPA, antibiotics, industrial enzymes among others [184]. In this context, identification of optimal genetic

changes, appropriate target for modification, flux distribution as well as control of fluxes and regulation of enzymes/genes play important roles in metabolic engineering [185]. Consequently, it needs analysis of underlying mechanisms of biological pathways at micro level. For this purpose, there are different methodologies, including physiological studies in detail, *viz*., FBA, MCA, thermodynamic analysis and kinetic modeling. In this section, we are going to discuss about the significant role of MCA in metabolic engineering.

Metabolic engineering needs to quantify metabolic fluxes in order to maximize the production of target molecules from certain substrates. FBA is an useful technique to calculate metabolic flux distribution over the whole metabolic pathways by applying mass balances on the stoichiometric model of the pathways [91,92,186]. Although FBA is efficient to determine the steady state flux values, it is incapable of capturing the transient responses. Subsequently, it does not find how the fluxes are controlled and how the concentrations of different metabolites change with respect to time and perturbations. It also fails to capture key enzyme regulations. In this scenario, MCA takes the responsibility to explore the dynamic behaviour of the pathways, including enzyme regulation, flux quantification as well as control, changes of metabolites with respect to time and perturbations and allosteric effects of enzymes [184,185,187]. Thus, MCA helps metabolic engineers in understanding the behavior of underlying complex biological systems/pathways so that the pathways can be manipulated in an efficient way.

In case of drug discovery, MCA helps metabolic engineers in understanding about suitable candidate enzymes as targets for a disease therapy (for example, cancer), correlation between the contribution of individual genes and phenotypic characteristics in metabolic diseases (*e.g.*, diabetes), the threshold effect due to manifestation of metabolic diseases and the activities of different "silent" genes [188]. It builds a bridge between biochemistry and functional genomics. It shows how the expressions of different genes control the cellular, biochemical and physiological activities. Thus, it has a crucial impact on deciding suitable target in drug discovery. Subsequently, MCA is capable of predicting target enzyme sites also. Metabolic engineers can asses where to intercede within a given metabolic network using the control coefficient distribution among different enzymatic steps in the network, as determined by MCA. After this assessment, engineers can apply enzyme inhibitors directly or manipulate the enzymatic steps genetically to achieve the desired amount of metabolites or flux values so that the network parameters (for example, cell proliferation rate of cancer cells) can transform themselves to its normal values [189,190].

MCA has helped an enzyme kinetics based model of pyruvate distribution in *Lactococcus lactis* to point out key flux control in the production of acetoin, diacetyl and other compounds. Depending on this analysis, researchers have knocked out lactate dehydrogenase and NADH oxidase to enhance flux through the path of acetolactate synthase up to 75% compared to the wild type [191]. In an another work [192], MCA has demonstrated that interaction of *E. coli* native promoter pGAP and *Bacillus subtilis* transcription factor FapR generates two malonyl-CoA sensors which are characterized by opposite transcriptional activities. Based on this investigation, the expressions of genes in *E. coli* responsible for supplying and consuming malonyl-CoA have been controlled optimally to maintain balance between cell growth and metabolite production. As a result, fatty acid production rate improves significantly compared to that of the wild type. In this context, availability of ideal promoters is an important factor to control the required heterologous gene expression [193].

Recently, our previous work shows how a state space based central carbon metabolic pathway model, in combination with proportional-integral (PI) controller(s), can capture the altered dynamics behind the "Warburg effect" in human cancer cells. Here, metabolic engineering leads to identify the altered metabolic control in cancer cells. This work predicts that regulation of pyruvate kinase (M2 isoform), in coordination with enzymes catalyzing pentose phosphate pathway, leads to manage both energy and macromolecule synthesis in cancer cells [194]. In another approach [195], we have introduced a fuzzy logic controller (FLC) based model to identify some probable solutions to destabilize mutated metabolic control in human cancer cells. FLC based model is efficient in handling unknown and frequently changing parameters of biological networks. This work has also used metabolic engineering to analyze metabolic control in cancer cells so that specific drug targets can be identified. Thus, it concludes that metabolic engineering is closely connected with MCA.

8. Prerequisites to develop metabolic engineering strategies

In this section, we are going to discuss about some significant prerequisites required to develop efficient and optimal metabolic engineering strategies. Fig. 3 depicts different essential steps that need to be followed to develop such metabolic engineering strategies.

Computational biologists should determine thermodynamically consistent flux profiles of concerned biochemical pathways. In this context, FBA [91,92,186] is a useful tool to determine flux distribution over the pathways. However, conventional FBA only depends on stoichiometric knowledge of the pathways. In order to determine thermodynamically consistent flux profiles, additional constraints, particularly those based on thermodynamics of the reactions involved in the pathways and transport processes, should be incorporated into FBA. A previous investigation [196] demonstrates how incorporation of such additional constraints ensures thermodynamically consistent flux profiles generated by FBA so that directions of fluxes and changes of Gibb's free energies are consistent to each other. In modern era, more additional constraints based on genomics, proteomics, metabolomics and fluxomics need to be considered for accurate and reliable flux profiles of the concerned biochemical pathways. Here in the following section, we are going to discuss an example depicting integration of genomics data and metabolic engineering for activation of silent metabolic clusters. The concentrations of metabolites also affect the gross thermodynamic feasibility of the biochemical pathways under consideration. Thus sampling of metabolite concentrations along with Gibb's free energies of reactions is a crucial step to characterize and analyze the thermodynamic space of the concerned metabolic networks [197]. In order to accomplish this task, the network-embedded thermodynamic analysis (NET analysis) [198,199] and thermodynamics-based metabolic flux analysis (TMFA) [93] have been widely used. In this context, it should be mentioned that incorporation of such thermodynamic constraints into FBA can remarkably narrow down the solution space. Subsequently, it renders a solid platform to integrate metabolomics and fluxomics data [200].

Similar to metabolite concentrations, sampling of enzyme states and its degree of saturation is quite important to develop and parameterize stoichiometrically, thermodynamically, concentration and flux consistent integrated pathway models. Analysis of reaction kinetic mechanisms, availability of experimental values of kinetic parameters and efficient algorithms to estimate unknown kinetic parameters play significant roles in this context. A recent investigation has developed a general reaction assembly and sampling palatform (GRASP) [201] for sampling and parameterizing kinetics of required enzymes with the help of nominal biochemical mechanistic and reference data.

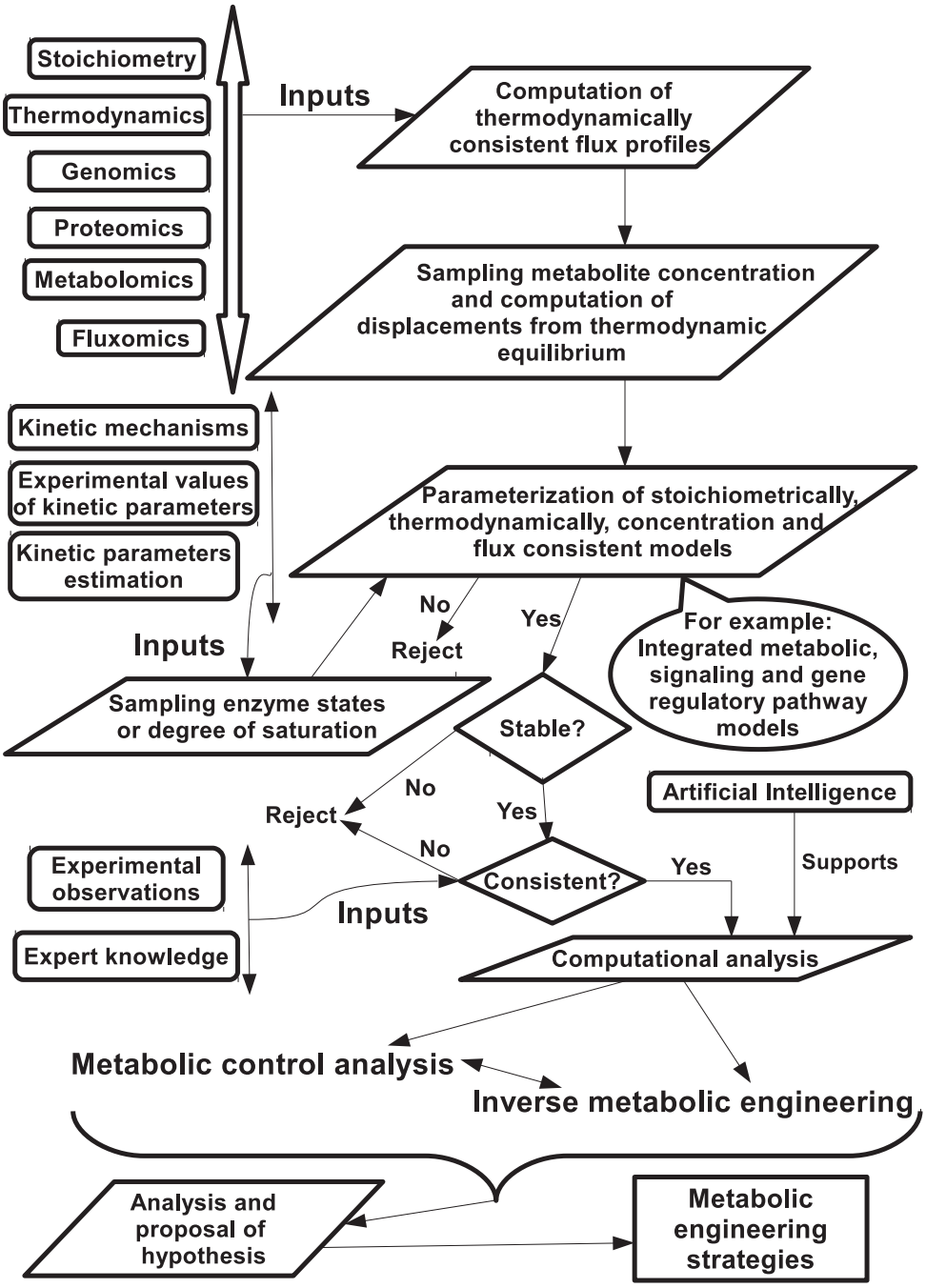

Fig. 3. Prerequisite steps to develop both efficient and optimal metabolic engineering strategies.

The aforesaid integrated pathway models should be explored for its stability and robustness under parametric uncertainty [202]. Satisfactory stable and robust pathway models should be investigated if their molecular behaviour is consistent with experimental observations and expert knowledge. Finally, the consistent integrated pathway model qualifies for computational analysis with the support of artificial intelligence (discussed in the following section) leading to metabolic control analysis (MCA) and inverse metabolic engineering. Based on the outcome of all the aforesaid steps, appropriate hypotheses have been formulated to help in developing efficient and optimal metabolic engineering strategies.

9. Integration of omics data and metabolic engineering: An example with genomics data

Recently, efficient and optimal systems metabolic engineering can be implemented using the combination of machine learning and omics data sets with high throughput capacity. Consequently, metabolic strain design using data driven modeling enables metabolic engineers in maximizing metabolite (particularly, secondary metabolites) production, exploring appropriate phenotypes and constructing efficient synthetic metabolic pathways [203]. Such synthetic metabolic pathways can be utilized to enhance the production of required secondary metabolites with

multiple chemical structures. However, these biosynthetic pathways are frequently assembled as biosynthetic gene clusters [204]. Thus it involves extremely complex biosynthetic logic. In this context, genome mining techniques can be used to explore core biosynthetic enzymes. Genome mining strategies use sequence similarity based approaches applied to the enzymes/genes involved in these biosynthetic pathways. Application of these strategies depends on phenotype of the specific compounds/enzymes, chemical structure of the secondary metabolites to be targeted, and potentiality and uniqueness of the species to be isolated from native environment [205]. Here, it may be mentioned that manual mining for different biosynthetic gene clusters (BGC) is no longer possible. Consequently, various bioinformatics tools, such as antiSMASH [204], PRISM [206] and BAGEL3 [207] among others, have been developed to accomplish automated mining of various BGC [205]. In addition, some standard databases, such as MIBiG [208], have been developed to store the information connecting biosynthetic genes with metabolites having specific chemical structures. These databases can be used for automated genome mining using the aforementioned bioinformatics tools.

The main problem is that the aforementioned BGC is found to be 'silent' mostly in laboratory. Consequently, sufficient amount of required secondary metabolites cannot be produced helping in industrial purposes. In order to address this problem, scientists [209] have reported a CRISPR-Cas9 strategy to activate 'silent' BGC in *Streptomyces viridochromogenes*. This strategy helps them in producing pentangular type II polyketide efficiently from *Streptomyces viridochromogenes*. Likewise, there are various methods to activate silent 'BGC' nowadays. These methods can be three types mainly [24]. One of the techniques has tried to alter the entire metabolome of target strain. In addition, it has aimed at producing pleiotropic effect activating a particular metabolic pathway randomly in the strain under consideration. However, this technique does not take the advantages of next generation sequencing and aforesaid genome mining. On the other hand, BGC specific technique can concentrate only on desired pathways, but its high throughput implementation is difficult without the intervention of artificial intelligence or machine learning techniques. Whereas, targeted genome wide technique has been developed to take advantages of both pleiotropic activation methods and BGC specific techniques. Thus there are different methodologies which can be chosen by metabolic engineers for activating biosynthetic pathways. However, there is no single superior method for the same to date. In addition, intervention of artificial intelligence or machine learning techniques using high throughput genomics data is quite necessary to develop such superior technique(s) to activate silent BGC for producing commercial amount of necessary secondary metabolites from appropriate biosynthetic metabolic pathways, leading to future drug discovery. In the following section, we are going to discuss specifically the role of artificial intelligence in modern metabolic engineering.

10. Role of artificial intelligence in metabolic engineering

In order to predict appropriate and significant target genes for perturbing pathway dynamics, the role of machine learning techniques on high throughput multiomics data cannot be repudiated nowadays. These techniques have outperformed the conventional kinetic modelling in terms of qualitative and accurate quantitative prediction, leading to mitigate the efforts of metabolic engineers [210,211]. As discussed earlier, efficient and optimal system metabolic engineering nowadays is driven by high throughput bio big data which includes transcriptomics, proteomics and metabolomics data among others [212]. Consequently, modern machine learning algorithms including deep learning approaches can facilitate metabolic engineers to analyze the bio big data helping in predicting optimal way to activate silent BGC as mentioned earlier. For example, scientists have invented a deep learning based technique DeepRibo based on convolutional neural networks (CNN) and recurrent neural network (RNN) to annotate genes efficiently in prokaryotes [213] without any help of gene homology analysis. DeepEc [214] is an another CNN based method with in-built homology analysis for predicting enzyme commission numbers taking input of protein sequences.

In the field of metabolic pathway reconstruction, a machine learning based method 3N-MCTS [215] has been developed. It is a retrosynthesis method based on integration of three distinct artificial neural networks implementing Monte Carlo algorithm. The goal of this technique is to explore efficient synthetic routes to produce a target molecule from a host species. Even machine learning can help in optimizing metabolic flux of the explored synthetic routes. Scientists have already shown how partial least squares regression [216] and artificial neural networks (ANN) [217] can be used to optimize up regulation of promoter and ribosome binding sites. In addition, CNN combined with random forest and linear regression [218,219] can be used to help in optimizing plasmid copy number and selecting promoter region among others. Recently, an advanced deep learning based method DeepCRISPR [220] is capable of predicting on-target to be knocked out and off-target sites of single-guide RNAs efficiently. Optimization of fermentation of a manufacturing host depends on several parameters including agitator type, pH, temperature, reactor type and aeration rate among others. Machine learning techniques, such as ANN, fuzzy logic, RNN and SVM, may be helpful in optimizing the multivariate system of fermentation [221,222].

It is clear from the aforesaid discussion that machine learning and artificial intelligence have a vast role in modern systems metabolic engineering. Even nowadays automated processes using robots [223,224] in systems metabolic engineering are much more needed. However, in order to utilize machine learning techniques and artificial intelligence efficiently, careful planning is required to generate high quality datasets with standard data format (preferably in machine readable format), data type and content [225]. In addition, the predictions from machine learning techniques should be appropriately validated through appropriate experimental mechanistic models to get into the insights of biological processes deeply.

11. Discussion and future direction

Metabolic engineering has a significant role in efficient production of required chemicals and/or materials for drug discovery as well as renewable resources from heterologous microorganism hosts. Besides, plant metabolic engineering enables pharmaceutical scientists in developing preventive medicines/strategies to combat chronic human diseases by utilizing higher amount of phytonutrients in fruit and vegetables [226]. For these purposes, metabolic engineers have focused on up or down regulation of required genes to redistribute steady state fluxes of specific metabolic pathways. Recently, dynamic regulation strategies [227] can rebalance metabolic fluxes depending on the alteration of cell phenotypes. Moreover, CRISPR-Cas9 technology has revolutionized genome editing strategy in this regard [228–230]. ZFNs [128] and TALENs [129] have also the capacity to make specific alteration to genomic DNA. In addition, site-specific recombinases [231–233] can provide tools to manipulate DNA by modifying certain DNA segments.

Successful implementation of aforesaid approaches needs thorough understanding of the dynamics of synthetic metabolic pathways, particularly in eukaryotic cells. Besides, exploring enzyme functionalities and its relation with other omics information is crucial here. In addition, exploration of interactions among different

metabolic, gene regulatory, signal transduction and protein-protein interaction networks drive engineers to employ more sophisticated advanced technology for improved metabolic engineering in near future [234]. In this context, analysis of thermodynamic feasibility of metabolic pathways along with their fluxes and pathway design through kinetic modeling, FBA and MCA contribute a solid platform to implement efficient metabolic engineering technologies. The analytical tools (*viz*. constraint-based reconstruction and analysis (COBRA) [235]) provide metabolic engineers to identify target enzymes/genes for modification leading to cost effective production of high-volume commodity chemicals as well as therapeutics. Thus combinatorial methods including inverse metabolic engineering approaches enable one to select improved mutants as well as to identify accurate genes for advanced metabolic engineering [236]. However, metabolic engineers need more effective computational models, with the support of artificial intelligence, integrating metabolic, signaling, gene regulatory and protein-protein interaction networks, mainly in eukaryotic cells, to gather detailed system knowledge including proper regulatory guidelines.

Author contributions statement

AD and RKD conceptualized the content of the article. AD wrote the first draft of the article, and made the figures. AD and RKD revised the article. RKD and NC read the article and gave fruitful suggestions to edit the manuscript.

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The authors declare that they do not have any financial or non-financial conflict of interests.

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Supplementary material

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