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Microbial chemical factories: recent advances in pathway engineering for synthesis of value added chemicals

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The dwindling nature of petroleum and other fossil reserves has provided impetus towards microbial synthesis of fuels and value added chemicals from biomass-derived sugars as a renewable resource. Microbes have naturally evolved enzymes and pathways that can convert biomass into hundreds of unique chemical structures, a property that can be effectively exploited for their engineering into Microbial Chemical Factories (MCFs). *De novo* pathway engineering facilitates expansion of the repertoire of microbially synthesized compounds beyond natural products. In this review, we visit some recent successes in such novel pathway engineering and optimization, with particular emphasis on the selection and engineering of pathway enzymes and balancing of their accessory cofactors.

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Introduction

We rely heavily on the dwindling resource of crude petroleum to supply not only fuels but also an array of valuable chemicals. Globally, roughly 20% of crude petroleum consumed is used for the synthesis of products other than transportation and industrial fuels, a sizeable fraction of which are petrochemicals [1]. Petrochemicals are used as raw materials in the manufacturing of a variety of products such as polymers, textiles, paints, solvents, pharmaceuticals, detergents, waxes and lubricants that find applications in our day-to-day lives. Reducing our dependence on petroleum by utilizing biomass as a renewable resource requires an integrated approach towards engineering of microorganisms into 'microbial chemical factories' (MCFs) that can be used in a 'biorefinery' for the conversion of biomass into both fuels and value added biochemicals. Biosynthesis of high value chemical compounds from biomass using natural or engineered pathways in microorganisms also serves as a promising alternative to chemical synthesis processes that employ expensive, hazardous and non-renewable raw materials and reagents as well as harsh processing conditions.

The evolution of the field of metabolic engineering has led to the development of principles and tools that enable construction and optimization of MCFs by tapping into naturally occurring pathways in specific host organisms, heterologous expression of non-native pathways in well-characterized hosts, or engineering *de novo* biosynthetic pathways for synthesis of various natural and non-natural products. *De novo* pathway engineering refers to the design and construction of novel pathways (hitherto unknown in nature in any single organism) by assembling multiple existing partial pathways from different organisms or using promiscuous or engineered enzymes as biocatalysts to catalyze a series of biotransformations with non-natural substrates [2•]. This capability is of particular importance for truly expanding the repertoire of value added products that can be synthesized microbially.

Figure 1 outlines the steps involved in the design of novel pathways for MCFs and various tools and approaches used for their optimization. Once a pathway has been designed and selected for experimental exploration [3•], suitable pathway enzymes need to be selected to catalyze the reaction steps. Pathway enzymes are the tireless machines of the MCFs that sequentially process raw materials into desired value added products and govern the pathway rates, selectivity, yield and overall productivity. Whenever possible, enzymes known to specifically catalyze reactions with the respective pathway intermediates are selected. If no enzyme has been documented to catalyze the required biochemical reaction with the specific pathway intermediate, other enzymes exhibiting the required biocatalytic activity known to act with structurally and chemically similar substrates may be selected to take advantage of natural enzyme promiscuity exhibited by many enzymes. The selected enzymes are then expressed in a suitable organism grown in a culture medium supplemented with the required starting materials for the microbial synthesis of the desired value added product.

Once a novel or natural pathway has been established and the synthesis of the product of interest has been demonstrated, enhancing pathway yields is critical to attain

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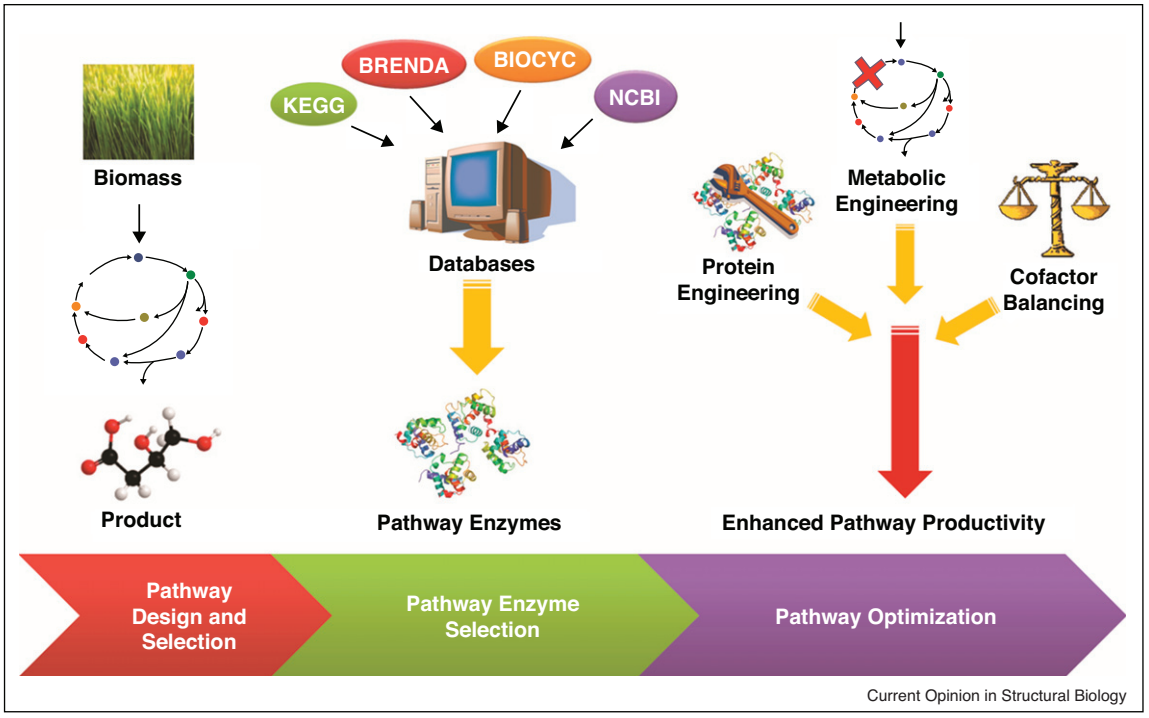
Figure 1



Design and engineering of pathways for microbial chemical factories (MCFs). The first step in engineering novel or natural pathways for MCFs is to identify potential natural cell metabolites or biomass derived feedstocks that can serve as starting materials and the series of biochemical reactions required to convert these into the desired product. Martin *et al*. [3•] have reviewed some of the computational tools available for identifying and selecting from the multiple possible pathways connecting different starting materials to a product of interest. Once a pathway is selected, appropriate natural enzymes expected to catalyze pathway reactions need to be selected using enzyme information from various databases. *In silico* approaches such as protein BLAST searches and molecular docking may help in such enzyme selection. Further pathway optimization to enhance product titers relies on an integrated approach composed of (1) *metabolic engineering* to enhance precursor metabolite availability using gene knockouts and enzyme expression level manipulation, (2) *protein engineering* to enhance pathway enzyme specificity and activity and (3) *cofactor balancing* via effective cofactor recycling.

industrially viable product titers. Protein engineering can be used for improving the selectivity and activity of the pathway enzymes and can effectively complement conventional metabolic engineering approaches such as increasing the precursor supply by varying pathway enzyme expression levels or knocking out competing pathways to enhance productivity. Further, pathway enzymes often require cofactors for catalysis. Imbalances and limitations arising out of an inability to effectively recycle cofactors can hamper pathway productivity, warranting specific efforts for cofactor regeneration.

A number of research groups have employed combinations of these strategies towards developing novel pathways and enhancing productivities of already established pathways for the microbial synthesis of a number of value added biochemicals including the polymer and pharmaceutical building blocks putrescine [4], cadaverine [5], succinic acid [6,7], 3-hydroxybutyric acid [8] and 3-hydroxyvaleric acid [9], native silk protein [10], the high value flavoring agent natural vanillin [11,12], pharmaceutical drug precursors such as taxadiene [13], amorpha-4,11-diene [14] and D-glucaric acid [15] and plant secondary metabolites such as flavanoids, stilbenoids [16] and isoprenoids [14,17]. In this review, we visit some recent studies focusing on the design, engineering and optimization of MCFs for synthesis of value-added products, with particular emphasis on exploiting enzyme promiscuity for *de novo* pathway construction and engineering of pathway enzymes and cofactor balances to enhance pathway productivity.

Exploiting enzyme promiscuity for design and engineering of novel pathways

All living cells metabolize a limited set of common metabolites into a wide range of molecules essential for the sustenance of various life processes. The key to the generation of such molecular diversity is the array of enzymes coded by the genomes, capable of catalyzing a wide variety of biochemical transformations, often with more than one substrate. Enzyme promiscuity [18,19•] refers to the ability to catalyze the same reactions with a variety of structurally and chemically similar substrates

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(substrate promiscuity) or multiple different chemical reactions (catalytic promiscuity) sharing certain mechanistic features and is critical for expanding natural cell metabolism for the biosynthesis of valuable new chemicals. While the use of catalytic promiscuity for development of novel biocatalysts has been extensively reviewed [20–22], exploiting natural substrate promiscuity for catalysis in novel pathways has received less attention. Indeed, many enzymes naturally exhibit a broad substrate range by accommodating alternative molecules in their active site, a property that can be effectively employed for catalysis with non-native substrates in novel pathways.

The Liao group at UCLA [23••,24••,25] has effectively employed such promiscuity for the production of various non-natural alcohols and amino acids in *Escherichia coli* from various 2-ketoacid intermediates in the native branched chain amino acid synthesis pathways. The inherent ability of the enzymes LeuA, LeuC and LeuD of the Leu operon to initiate chain elongation with their natural substrate, 2-ketoisovalerate during L-leucine synthesis was extended to the structurally similar natural metabolite 2-keto-3-methylvalerate (precursor to L-isoleucine) followed by decarboxylation by the broad substrate range enzyme 2-ketoisovalerate decarboxylase (KIVD) from *Lactococcus lactis* and reduction by alcohol dehydrogenase VI (Adh6) from *Saccharomyces cerevisiae* Adh6 for synthesis of the novel branched chain alcohol (*S*)-3-methyl-1-pentanol. In a separate study, the authors explored the ability of the *E. coli* transaminase IlvE and valine dehydrogenases from various *Streptomyces* species to catalyze amination of 2-ketobutyrate for the synthesis of the non-natural amino acid L-homoalanine [24••]. Our group has effectively used enzyme promiscuity exhibited by enzymes of the butanol and polyhydroxyalkanoate synthesis pathways from different organisms for the microbial synthesis of enantiopure forms of the biodegradable polymer precursor 3-hydroxyvaleric acid as a value-added product [9].

In each of these studies, enzymes chosen to be tested for promiscuity were selected based on knowledge of catalytic activity on structurally similar native substrates or functional groups. Screening of multiple possible enzymes exhibiting the required biocatalytic activity from different organisms can be an effective strategy for sampling different naturally evolved enzyme activities and specificities to identify an enzyme exhibiting activity towards a required pathway intermediate. For example, in the synthesis of non-natural alcohols, the Liao group screened 7 different ketoacid decarboxylases from four different organisms before identifying KIVD as a broad substrate range ketoacid decarboxylase [25]. Computational approaches may be effectively employed to comb through the extensive genomic, metabolic and enzyme databases such as KEGG [26], BRENDA [27], BioCYC [28] and NCBI (http://www.ncbi.nlm.nih.gov/guide/) that classify well-characterized natural enzymes on the basis of the specific and generalized reactions they catalyze, in order to identify such enzyme candidates for screening [29,30]. BLAST searches (blastp) using a protein sequence of known activity as a query may be used to identify homologous enzymes from various organisms capable of exhibiting similar biocatalytic functions with different substrate specificities, an approach effectively employed for identifying a PHA synthase enzyme capable of acting on lactate for the synthesis of polylactic acid (PLA) [31]. Knowledge of the catalytic active site or specific motifs important for catalytic activity and specificity can facilitate narrowing these searches towards isolation of enzyme variants exhibiting differences in order to sample alternative activities and specificities. Kim *et al.* [32•] used this strategy to identify new fructosyl peptide oxidase enzymes that find applications in diagnostics for diabetes. Molecular docking is yet another valuable *in silico* tool to predict promiscuity *a priori* by studying interactions of a given enzyme active site with a range of different substrates of interest and estimating activities and specificities when sufficient information about enzyme crystal structures and active sites is available [18,33].

Indeed, identification of candidate enzymes for novel pathways relies on continued investigation of enzyme crystal structures and identification of catalytic active sites as well as development and validation of reliable bioinformatic approaches for utilizing this information for *a priori* prediction of enzyme activity and specificity. Simultaneously, the information garnered from the experimental exploration of enzymes in novel pathways can in turn offer useful insights into enzyme catalytic activity and serve to validate and refine the developed computational tools. Further, such substrate promiscuity also serves as an excellent starting point for engineering novel biocatalysts with higher activity and specificity towards the non-native substrates.

Enhancing pathway productivity through protein engineering

Improving pathway productivity requires selective diversion of the precursor metabolites and pathway intermediates away from natural metabolism or competing side reactions and specifically towards the pathway of interest, without hampering cell growth and viability. Conventionally, this is achieved by enhancing precursor metabolite pools by knocking out host genes encoding enzymes catalyzing competing reactions and controlled overexpression of pathway enzymes to enhance flux along the pathway and reduce metabolic burden [34•,35•]. Overexpression of pathway enzymes and enhancement of precursor metabolite pools cannot, however, overcome inherent limitations associated with activities and specificities of pathway enzymes. Protein engineering allows one to enhance these properties by altering key amino

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acid residues and can effectively complement metabolic engineering efforts for selective and efficient synthesis of the desired products. Leonard *et al*. demonstrated the implementation of such an integrated metabolic and protein engineering approach for effectively enhancing titers of levopimaradiene (a valuable diterpenoid precursor to pharmaceutical ginkgolides) over 2600-fold in *E. coli* by engineering the key rate limiting pathway enzymes levopimaradiene synthase (LPS) and geranylgeranyl diphosphate synthase (GPPS) for increased activity and specificity, in addition to amplifying fluxes towards the precursor metabolites isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMPP) [36••]. For evolution of GPPS exhibiting higher activity towards geranylgeranyl diphosphate (GPP) synthesis from IPP and DMPP, random mutagenesis using error prone PCR was used to create a library of GPPS mutants. Lycopene biosynthesis from GPP was used as a colorimetric reporter for high throughput screening of this library for isolating an improved GPPS variant. Yang *et al*. used directed evolution of the enzyme propionyl-CoA transferase (Pct) from *Clostridium propionicum* to effectively activate lactate to lactyl-CoA to serve as a substrate for microbial synthesis of the valuable polymer, polylactic acid (PLA) and other lactic acid co-polymers using an engineered PHA synthase [31,37•].

Completely combinatorial strategies towards altering protein activity and specificity, such as random mutagenesis or directed evolution do not require detailed enzyme crystal structure or active site information, but are time consuming, laborious and often require several rounds of mutagenesis and selection. They may also be impractical in the absence of high throughput screens or selection strategies. While technologies such as MAGE [38], TRMR [39] and SCALES [40] have thus far focused on modifying regulatory regions to affect protein expression, they offer excellent practical solutions towards accelerated directed evolution across multiple target loci simultaneously within a short period of time. However, they still rely on high throughput screens to isolate improved mutant candidates. On the other hand, site directed mutagenesis of select residues in the active site can limit the size of the mutant library and obviates the need for a high throughput screen, but requires detailed information about the active site and substrate–enzyme interaction to be derived from the enzyme crystal structure. In the absence of such information for an enzyme of interest, constructing homology based crystal structure models by threading residues of the enzyme of interest onto known crystal structures of similar enzymes can allow identification of a putative active site. In the previously mentioned study on levopimaradiene synthesis [36••], the authors used this approach to identify a putative active site for the LPS enzyme and replaced individual residues in this active site with alternative residues from paralogous LPS-type enzymes from other organisms, one at a time to sample alterations in activity and specificity using the resulting distribution of levopimaradiene and by-products as a screen. Mutations in residues that resulted in the most significant productivity enhancements were subjected to saturation mutagenesis and were combined to engineer an LPS enzyme with 10-fold enhancement in levopimaradiene productivity.

Protein engineering can also be employed for building on and improving naturally promiscuous activities employed in novel pathways. Zhang *et al*. adopted this approach to enhance the specificities of the enzymes LeuA and 2-ketoisovalerate decarboxylase (KIVD) towards the non-natural substrates 2-keto-3-methylhexanoate and 2-keto-3-methylvalerate respectively by enlarging the substrate binding pockets by replacing bulky residues with smaller ones to facilitate interaction, resulting in 20-fold enhanced synthesis of the non-natural alcohol (*S*)-3-methyl-1-pentanol [23••]. Yang *et al*. used site directed mutagenesis of key residues identified to confer specificity in 5 different broad substrate range PHA synthase enzymes to evolve higher specificity towards incorporation of lactate [31]. These numerous examples underscore the immense potential of engineered enzymes to enhance yields by reducing by-product formation and accelerating rate limiting steps, and inherent challenges in engineering enzymes stemming from the limited available information about enzyme crystal structures and catalytic mechanisms and lack of convenient high throughput screens.

Enhancing productivity through cofactor balancing

Pathways for synthesis of value-added products often employ oxidation–reduction reactions catalyzed by enzymes using the nicotine amide dinucleotide cofactors NAD(H) and NADP(H) that are as critical for catalysis as the enzymes themselves. In the course of the biocatalytic transformations, these cofactors are oxidized or reduced and need to be recycled back to their active forms via natural cell metabolism or a subsequent pathway step for continued catalysis. In the absence of such recycling, the pathway can come to a grinding halt. These cofactors also play a critical role in redox reactions in natural cell metabolism where NAD^+ is often the cofactor of choice for oxidation of substrates in catabolic reactions with NADPH serving as the reductant in biosynthetic reactions. Thus, pathway enzymes often have to compete with natural cellular enzymes for these essential cofactors. Decreased active cofactor availability due to competition with natural metabolism as well as rapid depletion of cofactor pools due to an imbalance between pathway demands and natural cofactor recycling can pose serious limitations on pathway productivity [41]. For example, NADH and NADPH are oxidized to NAD^+ and $NADP^+$, respectively, during the synthesis of reduced fermentation products such as ethanol, lactate, butanol, succinate,

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3-hydroxybutyrate and polyhydroxybutyrate and their availability can influence both the productivity and product profile of pathways [9,41,42].

Effective recycling of cofactors is hence essential and may be achieved by specific metabolic engineering efforts targeting natural host pathways that can bring about such recycling or via enzyme mediated cofactor regeneration using a suitable sacrificial substrate or transhydrogenases. For example, Chelmer *et al.* employed a stoichiometric model to identify gene knockouts for improving the NADPH availability for the biosynthesis of the natural flavanoid polyphenols (+)-catechins and leucoanthocyanidins from dihydroquercetin, targeting natural *E. coli* metabolic pathways for effective recycling of NADP to achieve a four-fold improvement in the yields [43]. The disruption of the phosphoglucose isomerase gene and simultaneous over-expression of NAD^+ kinase or soluble transhydrogenases can be employed for diversion of glucose flux away from the Embden-Meyerhof-Parnas (EMP) pathway towards the pentose phosphate pathway (PP) to enhance NADP recycling to NADPH as demonstrated in the enhanced synthesis of thymidine [44]. This, however, comes at the cost of loss of valuable carbon in the decarboxylation step in the PP pathway that can decrease pathway yields. Transhydrogenases bring about the reversible transfer of reducing equivalents between NADH and NADP and can be employed to replenish pools of NADPH or NADH (at the cost of the other) to restore the cofactor balance, an approach effectively employed to enhance pathway productivity in polyhydroxybutyrate synthesis [45]. Hwang *et al.* employed enzyme mediated cofactor recycling using glucose dehydrogenase and glucose as a sacrificial molecule to regenerate NADPH consumed in the simultaneous synthesis of the valuable fragrance compound 2-phenylethanol and the non-natural amino acid L-homophenylalanine, enhancing the yields four-fold over the strains without such cofactor regeneration [46]. Altering cofactor specificity via protein engineering is yet another approach to achieve effective cofactor recycling [47,48]. These various examples again highlight the importance of cofactor balancing for enhancing pathway productivity.

Conclusions

Design, engineering and optimization of novel pathways expand the array of value added products that can be synthesized from biomass as alternatives to petroleum derived chemicals. Successful development of MCFs is essential to cope with our dwindling fossil resources but poses a number of challenges, including lack of well characterized enzymes, poor activity of selected pathway enzymes, low product titers, poor yield and selectivity, metabolic burden and unfavorable cofactor balance. Advances in the fields of metabolic engineering, biochemistry, protein engineering and synthetic and molecular biology have produced experimental and *in silico* tools to address these challenges. In the absence of a large number of well-characterized enzymes, exploiting natural enzyme promiscuity is critical for design of novel pathways. Protein engineering can further improve on such promiscuous activity and enhance specificity and activity in natural and novel pathways to improve productivity. Cofactor balancing is essential and can further result in several fold improvement in titers. Indeed, for synthesis of value added chemicals using MCFs to be economically feasible and to compete favorably with chemical synthesis, these approaches need to effectively complement conventional metabolic engineering efforts in the design and engineering of pathways.

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