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J Ind Microbiol Biotechnol. Author manuscript; available in PMC 2012 August 1.

Published in final edited form as:
J Ind Microbiol Biotechnol. 2011 August ; 38(8): 873–890. doi:10.1007/s10295-011-0970-3.

Engineering microbial factories for synthesis of value-added products

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Abstract

Microorganisms have become an increasingly important platform for the production of drugs, chemicals, and biofuels from renewable resources. Advances in protein engineering, metabolic engineering, and synthetic biology enable redesigning microbial cellular networks and fine-tuning physiological capabilities, thus generating industrially viable strains for the production of natural and unnatural value-added compounds. In this review, we describe the recent progress on engineering microbial factories for synthesis of valued-added products including alkaloids, terpenoids, flavonoids, polyketides, non-ribosomal peptides, biofuels, and chemicals. Related topics on lignocellulose degradation, sugar utilization, and microbial tolerance improvement will also be discussed.

Keywords

Synthetic biology; Metabolic engineering; Microbial synthesis; Value-added products; Natural products; Fuels and chemicals

Introduction

Microorganisms have been increasingly used to produce value-added compounds with numerous applications in the food, agriculture, chemical, and pharmaceutical industries. Examples of these value-added compounds include many antibacterial and anticancer drugs, amino acids, organic acids, vitamins, industrial chemicals, and biofuels. Compared to synthetic chemistry methodologies, microbial bio-synthesis has several advantages. First, it avoids the use of heavy metals, organic solvents, and strong acids and bases, thus allowing the synthetic process to take place through an environmentally benign route. Second, enzymes usually have a relatively high substrate specificity, which helps reduce the formation of byproducts. Third, some compounds with complex structures already have natural synthetic pathways while establishing chemical synthetic routes for these complex compounds is very difficult. Finally, metabolic engineering offers ways to further improve

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the yield and productivity of a target compound while combinatorial biosynthesis allows the creation of novel derivatives.

It is straightforward to think about directly extracting natural products from their native producers. However, most of these native producers are not cultivable in the laboratory and many microorganisms grow very slowly and produce minute amounts of the target compounds. It has been estimated that only 1% of bacteria and 5% of fungi have been cultivated in the laboratory [16, 26, 27, 66]. Even when it is possible to cultivate the native producers, their growth conditions have to be extensively optimized. In addition, due to the lack of genetic tools to manipulate these hosts, it is very difficult to improve the product yield and productivity. Therefore, well-characterized microorganisms that can be used as universal platform organisms are highly desired. *Escherichia coli* and *Saccharomyces cerevisiae* are two of the most widely used platform organisms due to their well-characterized physiology and genetics, fast cell-growth rates, and the availability of abundant genetic tools. Other platform microorganisms include *Bacillus subtilis, Pseudomonas putida*, and *Streptomyces* species.

Recent advances in protein engineering, metabolic engineering, and synthetic biology have revolutionized our ability to discover and construct new biosynthetic pathways and engineer platform organisms or so-called microbial factories to produce a wide variety of value-added products such as alkaloids, terpenoids, flavonoids, polyketides, non-ribosomal peptides, biofuels, and chemicals in a cost-effective manner. This review will highlight a few representative examples from the past 5 years. Related topics on lignocellulose degradation, sugar utilization, and microbial tolerance improvement will also be discussed.

Natural products

Alkaloids

Alkaloids are nitrogen-containing compounds of low molecular weight produced by a large variety of organisms, including bacteria, fungi, plants, and animals. Most alkaloids are derived through decarboxylation of amino acids such as tryptophan, tyrosine, ornithine, histidine, and lysine, and possess important pharmacological activities [84]. For example, the antimicrobial agent berberine has cholesterol-lowering activity [62], sanguinarine has shown potential as an anticancer therapeutic [60], bisbenzyliso-quinoline alkaloid tetrandrine has been used to treat autoimmune disorders and hypertension [64, 65], and a number of indolocarbazole alkaloids have entered clinical trials for diabetic retinopathy, cancer treatment or Parkinson's disease [18]. They have a very high diversity and molecular complexity in structure and can be classified into a number of groups, such as morphinane-, protoberberine-, ergot-, pyrrolizidine-, quinolizidine- and furanoquinoline-alkaloids according to the amino acids from which they originate [117]. Even for the plant alkaloids alone, there are over 10,000 structurally characterized members. Due to their high structural diversity and molecular complexity, chemical synthesis of alkaloids has not been very effective. On the other hand, although metabolic engineering strategies have been tried in plants to increase the amount of alkaloid products [3, 36, 116], success was limited and difficult to generalize due to the lack of convenient tools for engineering biosynthetic pathways in plants [115], the complexity of alkaloid biosynthetic pathways and their regulation [150] and the unavoidable transport of synthetic intermediates in and out among multiple intracellular organelles in plants [73].

Reconstitution of alkaloid biosynthesis in an engineered microbe has several advantages including rapid growth and biomass accumulation, abundant availability of genetic tools for pathway expression and optimization, and ease of characterizing and isolating final product and key intermediates due to a relatively cleaner background and the lack of interference

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from other metabolites in plants [48]. To this end, *E. coli* and *S. cerevisiae* were recently explored as production hosts. Sato and coworkers combined microbial and plant enzymes to synthesize benzylisoquinoline alkaloids including magnoflorine and scoulerine from dopamine *via* reticuline by co-culturing *E. coli* and *S. cerevisiae* [84] (Fig. 1a). The key intermediate (*S*)-reticuline was synthesized from dopamine by crude enzymes from recombinant *E. coli*, and was subsequently channeled into *S. cerevisiae*, generating magnoflorine and scoulerine with final yields of 7.2 and 8.3 mg/l, respectively. Smolke and coworkers engineered yeast alone expressing combinations of enzymes from different sources to produce the key intermediate reticuline and downstream metabolites along with two of the major branches from reticuline: the sanguinarine/berberine branch and the morphinan branch (Fig. 1b). In this system, a galactose-inducible enzyme tuning strategy was designed to balance enzyme expression and product yield, conserving cellular resources without compromising pathway flux [48].

In addition to alkaloids produced naturally, combinatorial strategies were applied to further diversify existing alkaloids with the goal of improving their potency as therapeutic molecules. For example, indolocarbazole biosynthetic enzymes possess useful degrees of substrate flexibility, thus they are able to accept different intermediates to yield novel derivatives. More specifically, partial clusters of rebeccamycin and staurosporine biosynthesis were combined and expressed together with additional sugar biosynthetic genes in *Streptomyces albus*. This resulted in generation of a series of novel indolocarbazole derivatives bearing different deoxysugars, some of which showed potent, subnanomolar, yet selective inhibition against kinases, one of the major targets in current drug discovery and development processes [113, 114]. It is believed that the sugar moieties play an important role in the selectivity of protein kinase inhibition.

Terpenoids

Terpenoids (also called isoprenoids), derived from five-carbon isoprene units assembled and modified in thousands of ways, compose the largest class of naturally occurring molecules with important medicinal and industrial properties. They are found in all classes of living organisms and approximately 25,000 structures have already been elucidated [40]. Despite the enormous structural diversity, terpenoids are synthesized from two basic isoprene building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), originated through either the mevalonate pathway or the non-mevalonate pathway depending on the species [22]. They serve a wide variety of functions such as respiration and electron transport (quinones), membrane fluidity and hormone signaling (steroids), and photosynthesis and antioxidant agents (carotenoids). Many terpenoids, especially the ones isolated from plants and marine invertebrates, are bioactive and are used in the pharmaceutical, cosmetic, and food industries [88]. The transformation of IPP and its derivatives to terpenoids of high complexity has been an active area in synthetic biology and metabolic engineering [22]. For example, *S. cerevisiae* has been engineered to produce artemisinic acid, a key precursor for the anti-malarial drug artemisinin [105] (Fig. 2a). The farnesyl pyrophosphate (FPP) biosynthetic pathway was engineered to increase FPP production and its use for sterols was decreased. The amorphadiene synthase (ADS) gene, a cytochrome P450 monooxygenase and its redox partner from *Artemisia annua* were introduced to convert FPP to amorphadiene, which was further converted to artemisinic acid through a three-step oxidation, with a titer of 115 mg/l. This production was further improved to reach 250 mg/l in shake-flask conditions and 1 g/l in bioreactors by modulating the selection markers and the culture composition [104].

One of the most important classes of enzymes involved in plant-derived natural products is the membrane-bound cytochrome P450 superfamily, which is ubiquitously involved in terpenoid biosynthesis and takes part in a wide variety of reactions. For example, eight out

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of the approximately 20 steps in paclitaxel biosynthesis are catalyzed by P450 enzymes [23]. Despite their essential nature, functional expression of plant P450 s in bacteria is extremely challenging and hinders the biosynthesis of many functional molecules by recombinant bacteria. This is mainly due to the absence of cytochrome P450 reductase (CPR) redox partners in bacteria for electron transfer [119] and the absence of an endoplasmic reticulum, which results in the translational incompatibility of the membrane signal modules [141]. In order to enable *E. coli* for production of functionalized terpenoids using plant P450 s, Keasling and coworkers cloned the codon optimized 8-cadinene hydroxylase (CAH) along with a CPR from *Candida tropicalis* in *E. coli* and obtained production of 8-hydroxycadinene at approximately 25 ± 2 mg/l. The N-terminal membrane anchor was subsequently replaced with various N-terminal sequences from three heterologous P450 s, two secretion/ solubilization sequences, or a self-assembling membrane protein, among which the N-terminal sequence of the bovine CAH [11] yielded an additional fiefold improvement in productivity to 105 ± 7 mg/l [21]. Such a strategy of generating a chimeric P450 with a fie-tuned N-terminal domain has also been successfully applied in isoflavone production in *E. coli* [70].

Another successful example of combining protein engineering, metabolic engineering, and synthetic biology strategies to design microbes for production of value-added compounds was demonstrated in the biosynthesis of taxadiene, a taxol precursor (Fig. 2b). Taxol and its structural analogs are potent and commercially successful anticancer drugs [61], originally isolated from the bark of the Pacific yew tree [136]. The traditional direct extraction method [100], the later-developed method of total chemical synthesis [96], and the currently used semisynthetic route [100] all suffered from low productivity and the accompanying constraints of being generalized to other derivatives in the search for more efficacious drugs [53, 106]. As the first step towards the production of Taxol in *S. cerevisiae*, co-expression of *Taxus chinensis* taxadiene synthase (TStc) and geranylgeranyl pyrophosphate synthase (GGPPStc) only resulted in a production of 204 μg/l taxadiene [33]. In yeast, the isoprenoid building blocks are mostly used for steroid biosynthesis, and the mevalonate pathway is subject to complex feedback regulation, with HMG-CoA reductase as the major regulatory target [29]. Expression of a truncated version of yeast HMG-CoA reductase (tHMG1) in combination with *Sulfolobus acidocaldarius* GGPPS, which does not compete with steroid synthesis and the codon-optimized TStc led to a 40-fold increase in taxadiene to 8.7 mg/l [33]. To further improve it, Stephanopoulos and coworkers designed a multivariate modular approach and succeeded in increasing the titer of taxadiene to approximately 1 g/l [1]. In this approach, the pathway was partitioned into two modules at IPP: the upstream native methylerythritol phosphate (MEP) pathway and the downstream heterologous taxadiene pathway. Systematically varying promoters of different strengths and plasmid copy-numbers resulted in identifying conditions that optimally balance the two pathway modules, such that the taxadiene production was maximized with minimal accumulation of any toxic intermediate. Such a modular pathway engineering strategy has the potential for engineering other terpenoid biosynthesis.

Flavonoids

Flavonoids are an important group of plant secondary metabolites, which in general have linear C6-C3-C6 skeletons derived from a phenylpropanoid (C6-C3) starter and three C2 elongation units [31]. Such a 15-carbon phenylpropanoid core is extensively modified by rearrangement, methylations, methoxylations, alkylation, oxidation, *C*- and *O*-glycosylation, and hydroxylation [74, 132], forming a fascinating group of over 9,000 members exhibiting antioxidant, antibacterial, antiviral, and anti-cancer activities [35]. Through the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL) deaminates phenylalanine to cinnamic acid, which is hydroxylated by cinnamic-4-hydroxylases (C4H), activated by 4-

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coumarate/cinnamate coenzymes, and condensed with three malonyl-CoA units to form a chalcone catalyzed by chalcone synthase (CHS). Chalcones are converted by chalcone isomerases (CHI) in a ring closing step to form the heterocyclic C ring [55]. The variability in molecular structure further divides flavonoids into flavones, flavanones, flavonols, isoflavones, anthocyanins, and catechines [74]. Similar to the production of alkaloids and terpenoids, *E. coli* and *S. cerevisiae* are widely used as model systems to produce flavonoids. However, in many cases, the biosynthetic efficacy is greatly limited by precursor and cofactor availability in the host. Therefore, alternative carbon assimilation pathways such as the malonate utilization pathway were introduced and competitive reactions including fatty acid synthesis and UDP-glucose consumption were inhibited and deleted, respectively, in order to improve the availability of malonyl-CoA and UDP-glucose. Such a strain expressing plant 4-coumarate:CoA ligase (4CL), CHS, CHI, anthocyanin synthase (ANS) and 3-*O*-glycosyltransferase (3-GT) produced flavanones up to 700 mg/l and anthocyanins up to 113 mg/l [74] (Fig. 3).

In another study, overexpression of the four acetyl-CoA carboxylase (ACC) subunits under a constitutive promoter resulted in an over fivefold increase in flavanone production. Acetate assimilation pathways were also amplified to convert acetate to malonyl-CoA *via* acetyl-CoA. Auxiliary expression of ACC with a chimeric biotin ligase (BirA) consisting of the N-terminus of *E. coli* BirA and the C terminus of *Photorhabdus luminescens* BirA further increased the production of pinocembrin, naringenin, and eriodictyol to 429, 119, and 52 mg/l, respectively [71].

Due to the relatively broad substrate specificity of the flavonoid biosynthetic genes, providing the pathway with unusual precursors could result in generation of new flavonoids. For example, UDP-glucose dehydrogenase (CalS8) and UDP-glucuronic acid decarboxylase (CalS9) from *Micromonospora echinospora* spp. *calichensis* and 7-*O*-glycosyltransferase (ArGt-4) from *Arabidopsis thaliana* were expressed together with an integrated copy of *E. coli* K12 UDP-glucose pyrophosphorylase (GalU) in background strain *E. coli* BL21 (DE3) with the glucose-phosphate isomerase (Pgi) gene deleted. When the resulting strain was fed with naringenin and naringenin 7-*O*-xyloside, a glycosylated naringenin product, was detected [124]. Due to the importance of flavonoids for human health, further derivatization might offer a chance to create new members with improved or novel properties.

Polyketides and non-ribosomal peptides

Polyketides, mostly derived from bacteria, Wlamentous fungi, and plants, are among the most important metabolites in human medicine, used clinically as antibiotics (erythromycin A, rifamycin S), antifungals (amphotericin B), anticancer drugs (doxorubicin, epothilone), antiparasitics (avermectin), cholesterol-lowering agents (lovastatin), and immunosuppressants (rapamycin) [138]. They are synthesized by a family of multifunctional enzymes known as polyketide synthases (PKSs). The core structures of polyketides are assembled through sequential Claisen-like condensations of extender units derived from carboxylated acyl-CoA precursors mainly including malonyl-CoA, methyl-malonyl-CoA, methoxy-malonyl-CoA, and ethyl-malonyl-CoA [126]. PKSs are classified into types I, II and III based on their biochemical features. The products synthesized by different types of PKSs can undergo different sets of tailoring modifications such as oxygenation, hydroxylation, cyclization, methylation, acylation, and glycosylation to form products with a great level of structural diversity [34, 98].

Research has been directed towards reconstitution of PKS biosynthetic pathways in more technically amenable microbes including *E. coli*, *S. cerevisiae*, *B. subtilis*, *P. putida*, and various *Streptomyces* species [13]. Despite several obvious advantages, *E. coli* and *S. cerevisiae* have their own drawbacks as heterologous hosts, including unavailability of some

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biosynthetic building blocks and lack of post-translational enzymes to modify PKSs [39, 89]. In addition, difficulties in efficient translation and functional folding of key enzymes including megasynthases and the P450 family of enzymes were encountered in *E. coli*. These limitations have to be addressed before synthesis of a wider range of molecules is attempted in these hosts. In fact, some of them were already addressed to a certain degree through metabolic engineering and protein engineering efforts. Nowadays, examples of polyketides synthesized by all three types of PKSs have been reported in *E. coli*, although the titers do vary case by case [39]. On the other hand, *P. putida* has a high GC content in its genome, suggesting it could better support the heterologous expression of genes with a high GC content, especially for the clusters isolated from actinomycetes [13]. Several *Streptomyces* species [10] and *B. subtilis* possess a native ability to provide needed substrates, which is an advantage as a heterologous host. However, the genetic manipulations of these three organisms are not as easy and convenient as the ones developed for *E. coli* and *S. cerevisiae*.

The most successful example of in vivo PKS reconstitution is the biosynthesis of 6-deoxyerythronolide B (6-dEB), the 14-membered macrocyclic core of erythromycin, in *E. coli*. The native and heterologous metabolism were engineered, and the resulting strain BAP1 could supply the required starter unit propionyl-CoA and the extender unit (2*S*)-methylmalonyl-CoA. The three 6-deoxyerythronolide B synthase (DEBS) proteins catalyze six chain extension cycles, converting exogenous propionate into 6-dEB with a specific productivity that compares well with a high-producing mutant of the original host [99]. The titer was further improved by overexpressing the *S*-adenosylmethionine synthetase MetK from *Streptomyces spectabilis* to increase the synthesis of signaling molecules [135] or by deleting the propionyl-CoA: succinate CoA transferase [147]. The DEBS system could serve as a paradigm model to study and engineer modular type I PKSs. Numerous 6-dEB and erythromycin derivatives were synthesized through domain or module insertions, deletions, and replacements [20, 83, 138].

The epothilone PKS currently represents the largest modular type I PKS reconstituted in *E. coli*. The epothilone family is synthesized by a hybrid non-ribosomal peptide synthetase (NRPS)/polyketide synthase in the myxobacterium *Sorangium cellulosum*. The cluster is composed of one NRPS module and eight PKS modules, and the largest gene, the 21.8 kb *epoD* encodes a protein larger than 760 kDa. Through codon optimization, lowered growth temperature, chaperone coexpression, and replacement of the T7 promoter by the arabinose-inducible P_{BAD} promoter, all pathway proteins except EpoD were solubly expressed. The expression of EpoD was finally achieved by dividing the large enzyme into two polypeptides, each consisting of two modules and the compatible linker pairs from related polyketide synthases. The entire cluster was expressed in the strain BAP1 mentioned above, resulting in the production of epothilones C and D. The success of the epothilone example provides an ideal platform for generation of novel epothilone derivatives and shows the importance of using protein engineering strategies to redesign a large gene cluster [90].

Although examples of nearly all major classes of natural products have been synthesized and engineered in *E. coli*, a noticeable exception is type II PKSs from actinomycete, which produce pharmaceutically important aromatic polyketides such as tetracyclines and anthracyclines [50]. The main obstacle is the inability to express the ketosynthase (KS)-chain length factor (CLF) heterodimer in soluble form. To bypass this obstacle using bacterial type II PKSs, Tang and coworkers targeted fungal iterative nonreducing PKSs, dissected and extracted the minimal PKS components of *Gibberella fujikuroi* PKS4, and reassembled it into a synthetic PKS, which synthesized a spectrum of aromatic polyketides in *E. coli* with cyclization regioselectivity not observed among fungal polyketides [148].

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The products of the type III PKSs can be categorized into three groups based on their specific activities [39]. Chalcone synthase cyclizes the intermediates into flavanones, the synthetic precursors of a variety of flavonoids [137]. Stilbene synthase (STS) catalyzes the cyclization of the intermediate polyketide to form a stilbene backbone, which is further modified to stilbenoids [8]. Curcuminoid synthase (CUS) only catalyzes condensation reactions without cyclization, producing curcuminoids [58, 59]. Coexpression of phenylalanine ammonia lyase (PAL) and 4CL with different type III PKSs produced various compounds such as resveratrol, pinosylvin [57], bisdemethoxycurcumin, and dicinnamoylmethane [59]. Precursor-directed biosynthesis [57, 58] and coexpression of post-PKS modification enzymes [85, 144] further led to the generation of a variety of unnatural compounds.

As mentioned earlier, *Streptomyces* are naturally better host microorganisms for the production of polyketides [69]. About half of the bioactive microbial metabolites discovered to date are produced by actinomycetes, with the genus *Streptomyces* being the primary producer [98]. The recent progress on synthesis of bioacitve compounds or their precursors by metabolic engineering of Streptomyces was summarized recently in other reviews [69, 97, 98].

Similar to polyketides, non-ribosomal peptides (NRPs) are synthesized by large modular non-ribosomal peptide synthetases and represent a diverse family of secondary metabolites exhibiting a broad range of biological activities. This family includes antibiotics such as vancomycin and bacitracin, antibiotic precursors like ACV, immunosuppressive agents such as cyclosporine, and siderophores [122]. Baltz and coworkers expanded the modification of the daptomycin amino acid core by replacing single or multiple modules in the DptBC subunit with modules from daptomycin and A54145 NRPSs. A combination of module exchanges, NRPS subunit exchanges, and inactivation of tailoring enzymes enabled *Streptomyces roseosporus* and *Streptomyces fradiae* to generate libraries of novel lipopeptide antibiotics related to daptomycin and A54145 [2, 94, 95]. Nielsen and coworkers initiated the development of a yeast platform for heterologous production of NRPs using ACV as a model NRP. ACV synthetase was expressed in *S. cerevisiae* in a high-copy plasmid together with phosphopantetheinyl transferases (PPTase) from three sources, all leading to production of ACV. The synthesis was improved by lowering the cultivation temperature and integrating the cluster into the genome. This work represents the first success in production of an NRP in yeast [123].

Biofuels

Due to environmental, economic, and energy security considerations, there is an increasing interest in the development of bio-derived fuel alternatives [78]. Dominant biofuel alternatives, such as corn-derived ethanol, however, proved to be only marginally profitable even with the application of governmental subsidies [51]. Thus, the current biofuel production scheme must be modified through the use of cheaper, non-edible lignocellulosic biomass as feedstock or via the production of advanced biofuels (Fig. 4).

Utilization of lignocellulosic feedstocks

Lignocelluloses, the non-edible portion of plant-derived biomass, are considered preferable feedstock for biofuel production due to their low requirement for energy, fertilizer, and pesticide input [77]. Unfortunately, the recalcitrant structure of plant cell wall presents a great challenge for the efficient deconstruction and complete utilization of lignocellulosic feedstocks. Lignocellulosic biomass has a very distinctive structure, with its most abundant component, cellulose, tightly surrounded by a hemicellulose and lignin complex that protects the inner cellulose from hydrolytic enzymes [139].

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Considerable research effort has been dedicated to breaking down the crystalline structure of cellulose to release the fermentable monosaccharide glucose for use in biofuel production. The most recent breakthrough in this area is the development of a microorganism capable of consolidated bioprocessing (CBP). CBP is a process where enzyme production, cellulose hydrolysis, and monosaccharide fermentation for fuel production are combined into a single step. CBP has been proposed to significantly lower biofuel production cost as it eliminates the large-scale production of cellulases [133]. One common strategy to develop a CBP microorganism is to introduce cellulolytic ability into ethanol producing, non-cellulolytic organisms [79].

Gram-negative bacterium *Zymomonas mobilis* is one attractive candidate for CBP microorganism development due to its high ethanol productivity and tolerance. Darzins and coworkers successfully expressed two cellulolytic enzymes, E1 and GH12, from *Acidothermus cellulolyticus*, in *Z. mobilis*. Furthermore, both cellulolytic enzymes can be secreted extracellularly due to the inclusion of native *Z. mobilis* secretion signals. Functional expression and secretion of cellulolytic enzymes in *Z. mobilis* indicate its high potential for serving as a CBP platform microorganism [76].

Another very attractive CBP organism candidate is *S. cerevisiae*, which has served as the major producer of ethanol for thousands of years [134]. van Zyl and coworkers first demonstrated that introducing the endoglucanase of *Trichoderma reesei* and the β-glucosidase of *Saccharomycopsis fibuligera* into *S. cerevisiae* can result in a recombinant strain with the ability to grow on phosphoric acid-swollen cellulose (PASC) as its sole carbon source and produce up to 1.0 g/l of ethanol [28]. Inspired by the structure of cellulosomes, Zhao and coworkers developed a recombinant yeast strain in which endoglucanases, cellobiohydrolases, and β-glucosidases were assembled into a trifunctional minicellulosome through cohesin and dockerin. In the recombinant yeast strain, a miniscaffoldin composed of a cellulose-binding domain and three distinct cohesin modules were expressed using yeast surface display, while three cellulolytic enzymes, each fused with a C-terminal dockerin corresponding to a different cohesin, were co-expressed in the same strain. The cellulolytic enzymes were assembled into minicellulosomes through cohesin–dockerin interaction onto miniscaffoldings anchored onto the yeast's surface. The recombinant yeast exhibited a higher cellulolytic activity due to enzyme–enzyme and enzyme–substrate synergistic effects. As a result, recombinant strains can simultaneously break down and ferment PASC to ethanol with a titer of 1.8 g/l [140]. Around the same time, Chen and coworkers reported their work involving another CBP process that utilized a minicellulosome. In their study, instead of creating a single recombinant strain, a synthetic yeast consortium was used for the expression and assembly of its minicellulosome. The synthetic consortium was composed of four different recombinant yeast strains: a strain displaying a trifunctional scaffoldin and three strains each expressing a dockerin-tagged cellulolytic enzyme. After optimization of the ratio of different populations in the synthetic consortium, a 1.87 g/l ethanol titer was achieved, which is 93% of the theoretical yield [131].

A similar effort was carried out to tackle the problem of hemicellulose utilization. Hemicellulose is the second-most abundant component of lignocellulosic biomass and can make up to 20–30% of the total feedstock. Unlike cellulose, which is composed of glucose, hemicellulose is primarily composed of five-carbon sugars (pentoses) such as D-xylose and L-arabinose [111]. Unfortunately, the industrial microorganism currently used for large-scale production of ethanol, *S. cerevisiae*, cannot utilize pentoses contained within the hydrolysates of the hemicellulose component of biomass feedstocks [49]. The incomplete utilization of sugar substrates present in lignocellulosic biomass hydrolysates is one of the major causes of elevated bioethanol production cost, making this environmentally friendly

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energy alternative typically far less economically competitive compared to fossil fuels [38, 46]. To be utilized by ethanol producing *S. cerevisiae*, pentose sugars need to be first transported into cells and then converted into D-xylulose-5-phosphate, which can then be further metabolized through the pentose phosphate pathway. In order to enable the effcient conversion of D-xylose and L-arabinose into D-xylulose-5-phosphate, heterologous pathways need to be introduced into *S. cerevisiae* and further optimized [46, 63]. For example, to improve the fermentative ability of the fungal D-xylose utilization pathway, much effort was devoted to identify heterologous enzymes with better catalytic efficiency and cofactor specificity [109], balance the cofactor usage of the pathway [75], and optimize ethanol production using more robust industrial ethanol-producing yeast strains [80].

Another factor hampering effcient production of lignocellulosic ethanol in *S. cerevisiae* is the "glucose repression" that occurs during mixed sugar fermentation. Because *S. cerevisiae* preferably utilizes glucose over other carbon sources, utilization of pentose sugars is severely repressed before glucose is depleted [107]. A novel approach was recently developed to overcome glucose repression by introducing a cellobiose transporter and a β-glucosidase into recombinant xylose-utilizing *S. cerevisiae* strains [45, 75] (Fig. 5). In these strains, cellobiose, an incomplete hydrolysis product of cellulose, is fermented instead of glucose in the presence of xylose. Since cellobiose enters *S. cerevisiae* cells through a dedicated cellobiose transporter, the competition of glucose and xylose at the sugar uptake step is eliminated. Cellobiose is then hydrolyzed into glucose intracellularly and continuously consumed for the production of ethanol. The simultaneous hydrolysis and utilization of cellobiose avoids intracellular accumulation of glucose, thus alleviating glucose repression. In the engineered cellobiose and xylose co-utilization strains, cellobiose and xylose are consumed simultaneously and synergistically with an ethanol productivity of 0.65 g/l h and overall ethanol yield of 0.39 g/g glucose.

Production of advanced biofuels

The development of biologically derived ethanol has achieved significant success in the past few decades [4, 9]. However, ethanol exhibits some intrinsic limitations, such as low energy content and corrosiveness, which hampers its large-scale application as a fuel alternative. In contrast, advanced biofuels, such as higher alcohols, fatty acid derived fuels, and hydrocarbons, are considered to be better fuel alternatives as their physiochemical properties are more compatible with the current gasoline-based infrastructure [145].

Isopropanol and *n*-butanol are both better fuel alternatives compared to ethanol due to their higher energy content, higher octane number, and lower water solubility. Fortunately, unlike other long-chain alcohols, both isopropanol and *n*-butanol can be produced by *Clostridium* species in nature. However, since *Clostridium* species are Gram-positive anaerobes with a relatively slow growth rate and spore-forming life cycles, it is hard to control the yield of desired long-chain alcohols in industrial fermentation [145]. To address this issue, long-chain alcohols were produced in non-native hosts such as *E. coli* and *S. cerevisiae* [6, 7, 127, 145]. For heterologous production of isopropanol, various combinations of genes from different *Clostridium* and *E. coli* species have been introduced into *E. coli* for production through the coenzyme-A-dependent fermentative pathway. The resulting optimized recombinant strain can achieve an isopropanol production titer of 5 g/l with a yield of 43.5% mol/mol glucose [47]. Similarly, the *n*-butanol production pathway from a *Clostridium* species has also been introduced into *E. coli* and extensive metabolic engineering efforts have been dedicated to increasing the titer. However, the highest titer for *n*-butanol production only achieved 552 mg/l, most likely due to limitations imposed by low heterologous enzyme activity, insuffcient carbon precursors and inadequate reducing power [6]. At the same time, Keasling and coworkers introduced a similar pathway into *S. cerevisiae* and achieved an *n*-butanol production titer of 2.5 mg/l through the optimization of

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isozymes used in the pathway [127]. One reason for the low efficiency in the heterologous production of long-chain alcohols may be the cytotoxicity caused by the accumulation of intermediate metabolites as well as redox imbalance due to the introduction of heterologous pathways [6]. To address this issue, Liao and coworkers investigated the production of long-chain alcohols through existing non-fermentative keto acid pathways. Using this strategy, 2-keto acids, which are intermediates in amino acid biosynthesis pathways, are converted into aldehydes by broad range 2-keto-acid decarboxylases (KDC) and then reduced to alcohols by alcohol dehydrogenases (ADH). Compared to fermentative pathways, only two heterologous steps need to be introduced for alcohol production through 2-keto-acid pathways. Through the choice of different KDCs, 2-keto acids from various amino acid synthesis pathways can be used to produce long-chain alcohols including isobutanol, 1-butanol, 2-methyl-1-butanol, and 2-phenylethanol [5, 7, 25].

In addition to alcohols, fatty acid-derived fuel alternatives such as fatty acid esters and fatty alcohols are also potential fuel alternatives. In a recent study by Keasling and coworkers [128], recombinant *E. coli* strains were engineered to overproduce free fatty acid through cytosolic expression of a native *E. coli* thioesterase and the deletion of fatty acid degradation genes. Furthermore, the chain length and saturation of the fatty acid chain can be controlled by simply altering the thioesterase used in the pathway. The recombinant strain was further modified to directly produce fatty acid ethyl esters (FAEEs) via the introduction of ethanol production genes from *Z. mobilis* and overexpression of endogenous wax-ester synthase. Finally, hemicellulases from various species were expressed in recombinant fatty acid derivative producers and secreted into the medium to realize consolidated bioprocessing of hemicellulose biomass directly into biodiesels.

Aliphatic hydrocarbons, such as alkenes and alkanes, are highly attractive targets for advanced biofuel production as they are currently the major constituents of jet fuel, gasoline, and diesel. Though alkenes are naturally produced by many species, the genetic and biochemical mechanism for alkene synthesis remains unclear. Keasling and coworkers achieved long-chain alkene production through the expression of a three-gene cluster from *M. luteus* in a fatty acid-overproducing *E. coli* strain. After a series of biochemical characterizations of the strain, a metabolic pathway for alkene biosynthesis was proposed involving acyl-CoA thioester and decarboxylative Claisen condensation catalyzed by OleA [12]. In another report, del Cardayre and coworkers elucidated an alkane biosynthesis pathway from cyanobacteria [118]. In this pathway, intermediates of fatty acid metabolism are converted to alkanes and alkenes by an acyl-acyl carrier protein reductase and an aldehyde decarbonylase. Heterologous production of C13–C17 mixtures of alkanes and alkenes was achieved in *E. coli* by the expression of this pathway.

Chemicals

While issues such as the recurring oil crisis and global climate change have spurred the development of new fuel alternatives, they have also led to an increased awareness of the world's traditional petroleum-based chemical production processes. Consequently, clean, mild, and safe chemical synthesis processes utilizing renewable resources are highly desirable.

Organic acids

The wide application of organic acids as platform chemicals, along with the few catalytic steps required for their production, has led to intensive investigation into the microbial synthesis of organic acids. One prominent example is the production of lactic acid and its derivatives. Lactic acid is commercially produced by glucose fermentation using *Lactobacillus* species [15]. Additionally, recent studies have reported the production of

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lactic acid from other renewable substrates such as glycerol, cellobiose, and even cellulose [81, 125].

Succinic acid, a compound commonly used as a surfactant, is another widely investigated platform chemical. Succinic esters are precursors to many known petrochemical products (e.g., 1,4-butanediol). Succinate acid can be biochemically produced using bacteria such as *Actinobacillus succinogenes*, *Mannheimia succiniproducens*, as well as recombinant *E. coli* [68, 112]. Lang and coworkers reported the production of succinic acid in recombinant *S. cerevisiae* with an interrupted TCA cycle that resulted from a quadruple gene deletion. This engineered strain can produce succinic acid at a titer of 3.62 g/l with a yield of 0.11 mol/mol glucose [101].

3-Hydroxypropionic acid (3-HPA) has received significant attention mainly due to its applications in the polymer industry. 3-HPA can be produced from glucose or glycerol through various pathways [54]. Park and coworkers achieved production of 3-HPA from glycerol in a recombinant *E. coli* strain that expresses heterologous glycerol dehydratase and aldehyde dehydrogenase with a titer of 0.58 g/l. However, the imbalanced enzyme activity and instability of glycerol dehydratase hampers the efficient production of 3-HPA in the recombinant strains. Further study has shown that the use of an α-ketoglutaric semialdehyde dehydrogenase instead of aldehyde dehydrogenase, along with proper fermentation condition optimization, could improve the 3-HPA production to a titer of 38.7 g/l [103].

D-Glucaric acid, a compound found in fruits, vegetables and mammals, has been investigated for a variety of therapeutic purposes. D-Glucaric acid can be synthesized by the mammalian D-glucuronic acid pathway initiated with D-galactose or D-glucose. Prather and coworkers constructed a recombinant D-glucaric acid producing *E. coli* strain by heterologous expression of the myo-inositol-1-phosphate synthase (Ino1) from *S. cerevisiae* and myoinositol oxygenase (MIOX) from mice with a titer of 0.3 g/l [87]. The activity of MIOX was identified as rate limiting in the pathway, resulting in the accumulation of both myo-inositol and D-glucuronic acid. Co-expressing the urinate dehydrogenase from *Pseudomonas syringae* that facilitates the conversion of D-glucuronic acid into D-glucaric acid improved the production titer to more than 1 g/l. In a follow-up study, synthetic scaffolds were introduced into the recombinant system to help improve the effective concentration of myo-inositol [86]. Specifically, polypeptide scaffolds built from protein–protein interaction domains were used to co-localize three heterologous pathway enzymes involved in D-glucaric acid synthesis in a complex. The synthetic scaffolds significantly increased the specific activity of MIOX and resulted in a recombinant strain with a Wvefold improved D-glucaric acid production titer.

Rare sugars and sugar alcohols

Xylitol is a favorable sugar substitute with low caloric content and anticariogenic properties. The traditional xylitol production method involves chemical or enzymatic hydrogenation of hemicellulosic hydrolysate and extensive puri-Wcation of non-specific reduction products. However, one of the major impurities, L-arabinitol, is an epimer of xylitol, and the subsequent complex impurity separation process ultimately culminates in a prohibitively high cost for xylitol production. To overcome this obstacle, Zhao and coworkers developed a recombinant *E. coli* strain to produce pure xylitol from hemicellulose hydrolysate. First, an engineered aldose reductase was constructed to specifically reduce D-xylose. This engineered enzyme was obtained through rounds of directed evolution on a promiscuous aldose reductase using an in vivo selection method. The resulting enzyme exhibits a 50-fold lower catalytic efficiency toward L-arabinose while maintaining most of its D-xylose activity [91]. Furthermore, the recombinant strain was subjected to extensive metabolic engineering to further improve the selective reduction of D-xylose to xylitol. The resultant engineered *E.*

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coli strain was capable of production of nearly 100% pure xylitol from an equiweight mixture of D-xylose, L-arabinose, and D-glucose [92].

L-Ribose, a rare sugar in nature, is a very important intermediate for the preparation of pharmaceutical, food, and agrochemical products. Woodyer and coworkers developed a new synthetic platform for production of L-ribose involving the use of a unique NAD-dependent mannitol-1-dehydrogenase (MDH). The recombinant *E. coli* strain expressing this enzyme can be used as a whole-cell catalyst for the production of L-ribose from ribitol. As a result, L-ribose productivity of 17.4 g/l day was achieved using this system at 25°C in one-liter fermentation [142]. In a follow-up study, a directed evolution strategy was applied to improve the activity and thermal stability of MDH. The recombinant strain harboring the improved MDH achieved a conversion rate of 46.6% and a productivity of 3.88 g/l day in shake Xasks at 34°C with a overall 19.2-fold improvement. Since MDH can catalyze the interconversion of several polyols and their L-sugar counterparts, the L-ribose production system can be potentially applied in the production of other rare sugars as well [24].

1,3-Propanediol

1,3-Propanediol (1,3-PD) is a platform chemical with applications in the production of plastics, cosmetics, lubricants, and drugs. Production of 1,3-PD was achieved in *E. coli* using either glucose or glycerol as a substrate with a high production titer of 135 and 104 g/l, respectively [93, 129]. *yqhD* from *E. coli* and *dhaB* from *Citrobacter freundii* were cloned into a temperature-inducible vector and introduced into a recombinant *E. coli* strain to enable the heterologous production of 1,3-PD. Through optimization of the cultivation and supplementation of vitamin B12 (a coenzyme required for 1,3-PD production), heterologous production of 1,3-PD with a titer of 43.1 g/l could be achieved in *E. coli* [149]. In a more recent study, introduction of *dhaB1* and *dhaB2* genes from *C. butyrium* and *yqhD* from *E. coli*, along with a novel two-stage fed-batch fermentation strategy, achieved 1,3-PD production with a titer of 104 g/l in *E. coli* using glycerol as a substrate [129]. In addition to *E. coli*, *S. cerevisiae*, a well-known glycerol producer, was also engineered to produce 1,3-PD from glucose. In a recent study, both *dhaB* from *K. pneumonia* and *yqhD* from *E. coli* were introduced into *S. cerevisiae* via the *Agrobacterium tumefaciens* genetic transfer system [102]. It was shown that stable expression of the 1,3-PD production genes can be achieved in *S. cerevisiae*. However, the production titer of 1,3-PD only reached 0.4 g/l due to low glycerol availability.

Vitamins and amino acids

Vitamins and amino acids are important food supplements and microbial production of these compounds has been extensively investigated [17, 30]. Recently, new metabolic engineering tools have been applied to improve the production of vitamins and amino acids. For example, the carbon storage regulator (Csr), a global regulatory system of *E. coli*, was engineered to improve phenylalanine biosynthesis [130]. In a follow-up study, Madhyastha and coworkers explored the effects of *csrA* and *csrD* mutations and *csrB* overexpression on phenylalanine production in *E. coli* NST37 (NST) and discovered that the overexpression of *csrB* led to a significantly greater phenylalanine production than *csrA* and *csrD* mutations. Together with *tktA* overexpression, a phenylalanine production titer of 2.39 g/l was achieved [143].

Improving cellular properties

To achieve a high-level of value-added compound production, a microorganism must exhibit a high tolerance towards any substrate inhibitors and toxic products as well as the currently utilized strict industrial fermentation conditions. One good example for engineering microbial tolerance towards substrate inhibitors is the improvement of lignocellulosic

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hydrolysate tolerance in biofuel producing microorganisms. Lignocellulosic hydrolysate contains a variety of cell-growth inhibitors due to the harsh chemical/enzymatic hydrolysis process. These inhibitors mainly consist of acetic acid, furan derivatives, and phenolic compounds. Different engineering approaches, such as long-course adaptation, genomic library selection, and rational design, have all been applied to improve microbial tolerance and have each yielded different results [14, 44]. In a recent study, Brown and coworkers investigated the hydrolysate inhibitor tolerance of both *Z. mobilis* and *S. cerevisiae* in regards to the expression level of a conserved bacterial member of the Sm-like family of RNA-binding proteins Hfq and its homologue Lsm. Their results indicate that these regulator proteins are very important for pretreatment inhibitor tolerance [146].

Extensive investigation has been carried out to explore the tolerance of Wnal products. The understanding and engineering of microbial ethanol and butanol tolerance is a particularly active and interesting example. For example, Keasling and coworkers examined the transcript, protein, and metabolite levels in *E. coli* to construct a cell-wide view of the *n*-butanol stress response. Their results indicate that butanol stress includes the perturbation of respiratory, oxidative, heat shock, and cell envelope stresses, as well as disrupting general metabolite transport and biosynthesis [110]. Jin and coworkers studied alcohol tolerance in *S. cerevisiae* using transformation of a genomic DNA library and serial subculture into media containing isobutanol. Sequence analysis revealed overexpression of INO1, DOG1, HAL1, or a truncated form of MSN2 in the enriched population and provided a potential target for the understanding and engineering of an alcohol-tolerant phenotype [52].

For industrial microorganisms, resistance to stress is highly desirable due to the simultaneous or sequential combinations of different environmental stressors present in biotechnological processes. The molecular basis of stress resistances is very complicated, making it difficult to engineer stress resistances by rational approaches. However, using evolutionary engineering approaches, engineered strains with multiple-stress resistances are possible. Sauer and coworkers tested various selection procedures in chemostats and batch cultures systematically for a multiple-stress resistant *S. cerevisiae* phenotype. Mutant populations harvested at different time points as well as clones were randomly chosen and grown in batch cultures to be exposed to high-temperature, oxidative, freeze–thaw, and ethanol stresses. A unique high-throughput procedure utilizing 96-well plates combined with a most-probable-number assay was developed for the selection of multi-stress resistant strains. In this research, the best selection strategy to obtain highly improved multiple-stress-resistant yeast was found to be batch selection for freeze–thaw stress. Mutants not only significantly improved in freeze–thaw stress resistance but also in the other stress resistances identified by this strategy. The best isolated clone exhibited a 102-, 89-, 62-, and 1,429-fold increased resistance to freeze–thaw, high-temperature, ethanol, and oxidative stress, respectively [19].

Transport issues

Efficient uptake of a substrate into microbial cells and export of a product outside microbial cells are critical to value-added compound production. Recently, some progress has been made to improve substrate uptake, especially lignocellulose hydrolysate product uptake in biofuel producing microorganisms.

Pentose uptake through sugar transporters is the Wrst step of pentose utilization in *S. cerevisiae*. However, pentose sugars only enter *S. cerevisiae* cells through the hexose uptake system, a system that has two orders of magnitude lower affinity for pentose sugars than hexoses [63]. As a result, pentose uptake in pentose-assimilating yeast strains is very slow and inhibited by the presence of D-glucose in the growth media. To improve D-xylose uptake, heterologous D-xylose transporters were introduced into recombinant *S. cerevisiae* strains.

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Spencer-Martins and coworkers discovered one high affinity D-xylose/D-glucose symporter (GXS1) and one low affinity D-xylose/D-glucose facilitator (GXF1) from *Candida intermedia* and characterized them at the molecular level in *S. cerevisiae* [67]. It was observed that overexpression of the Gxf1 transporter improved fermentation performance in a recombinant D-xylose-utilizing *S. cerevisiae* strain [108]. Similarly, *S. cerevisiae* strains overexpressing heterologous D-xylose transporters from *Arabidopsis thaliana* showed up to 2.5-fold increased D-xylose consumption and 70% increased ethanol production [49]. In addition, overexpression of the D-glucose transporter Sut1 from *Pichia stipitis* was also shown to improve ethanol productivity during D-xylose and D-glucose co-fermentation by a D-xylose-assimilating *S. cerevisiae* strain [56]. However, all of the transporters mentioned above still have a lower affinity for D-xylose when compared to glucose. Recently, two D-xylose-specific transporters from pentose assimilating fungal species *N. crassa* and *P. stipitis* were discovered, heterologously expressed, and characterized in *S. cerevisiae*. Although the overexpression of these two D-xylose-specific transporters failed to improve D-xylose utilization in recombinant *S. cerevisiae* strains, the sequencing of these types of transporters may provide some insight that could eventually lead to the discovery and engineering of highly active pentose-specific sugar transporters [32].

Cellodextrins are glucose polymers with various lengths that cannot be metabolized by ethanol-producing *S. cerevisiae*. Cate and coworkers discovered a group of cellodextrin transporters from hemicellulose-assimilating species *N. crassa* through a microarray study. By introducing the newly discovered cellodextrin transporters along with β-glucosidase into *S. cerevisiae*, cellodextrin-assimilating recombinant strains could be constructed [37]. Follow-up studies have shown that by enabling intracellular cellodextrin utilization, the long-lasting glucose repression that occurs during mixed sugar utilization can be circumvented through the use of cellobiose instead of glucose as the carbon source [75]. Simultaneous and synergistic utilization of cellobiose and xylose could significantly reduce the cost of biomass-based fuel alternatives [45].

Conclusions and future prospects

Numerous impressive accomplishments have been made in the engineering of microbial factories for synthesis of value-added products in the past few years. However, continuous efforts towards exploring new production hosts, creating novel enzymes that catalyze unnatural reactions, and developing more powerful tools for functional genomics and proteomics will be necessary to expand the range of products that can be synthesized by microbial factories. Of special note, innovative synthetic biology approaches for pathway and genome engineering are expected to play an increasingly important role in this effort.

For example, Zhao and coworkers developed a DNA assembler approach for rapid construction and engineering of a biochemical pathway either on a plasmid or on a chromosome in *S. cerevisiae* in a single-step fashion [121]. This approach was further extended for discovery, characterization, and engineering of natural product biosynthetic pathways [120]. In this method, the entire expression vector containing the target biosynthetic pathway and the genetic elements required for DNA maintenance and replication in various hosts is synthesized. Because the DNA fragments to be assembled are completely mobile and amenable to all sorts of sophisticated genetic manipulations accessible to PCR, or can be chemically synthesized de novo with optimized codons, this strategy offers the ultimate versatility and Xexibility in characterizing and engineering a biochemical pathway. More importantly, the recent success in chemical synthesis of entire bacterial genomes implies the possibility of constructing artificial organisms [41–43].

In the future, synthetic biology could become as powerful as synthetic chemistry, and could greatly expand the range of products that can be produced from renewable sources. In particular, a combination of synthetic biology platforms with current protein and metabolic engineering tools is expected to give rise to a new generation of organisms that function as highly robust and programmable biological machines [72, 82].

Acknowledgments

We thank the National Institutes of Health (GM077596), the National Academies Keck Futures Initiative on Synthetic Biology, the Biotechnology Research and Development Consortium (BRDC) (Project 2-4-121), the British Petroleum Energy Biosciences Institute, and the National Science Foundation as part of the Center for Enabling New Technologies through Catalysis (CENTC), CHE-0650456, and the National Research Foundation of Korea (NRF) (220-2009-1-D00033) for Wnancial support. J. Du also acknowledges both the Chia-chen Chu graduate fellowship from the School of Chemical Sciences and the Henry Drickamer Fellowship support from the Department of Chemical and Biomolecular Engineering at the University of Illinois.

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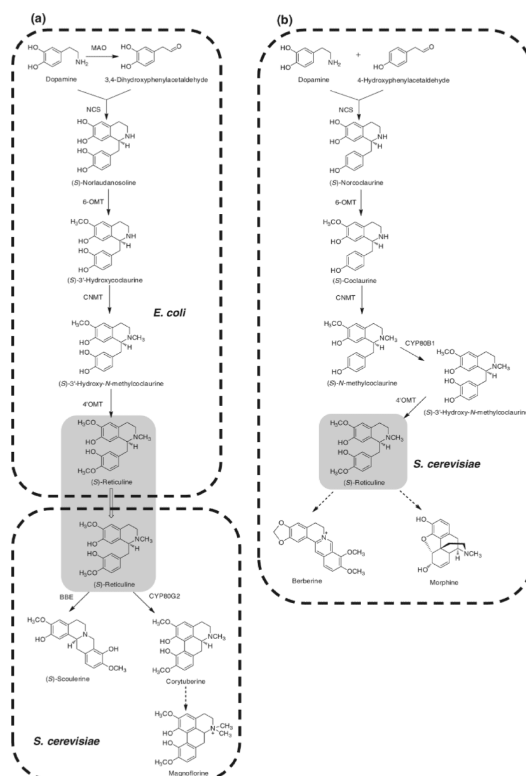

Fig. 1.

Production of benzylisoquinoline alkaloids either by a co-culturing system of *E. coli* and *S. cerevisiae* (**a**) or by *S. cerevisiae* alone (**b**)

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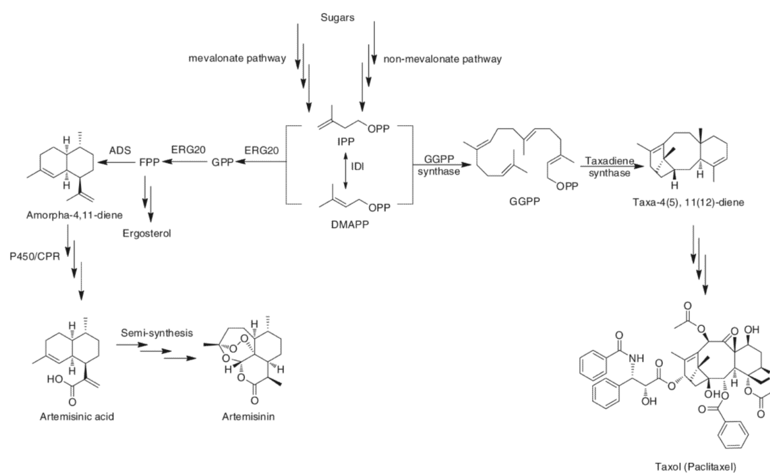

Fig. 2.
Utilizing *S. cerevisiae* to synthesize artemisinic acid (**a**) and taxol (**b**)

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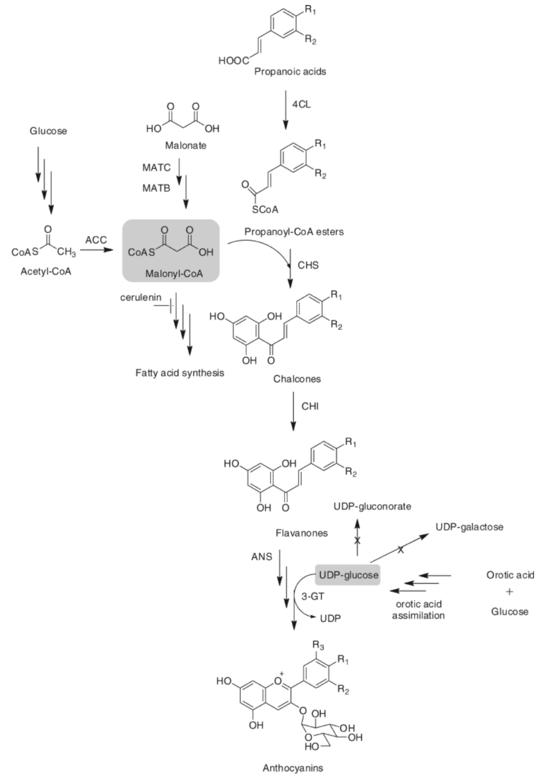

Fig. 3.
Engineered pathways for increased flavanone and anthocyanin biosynthesis in *E. coli*

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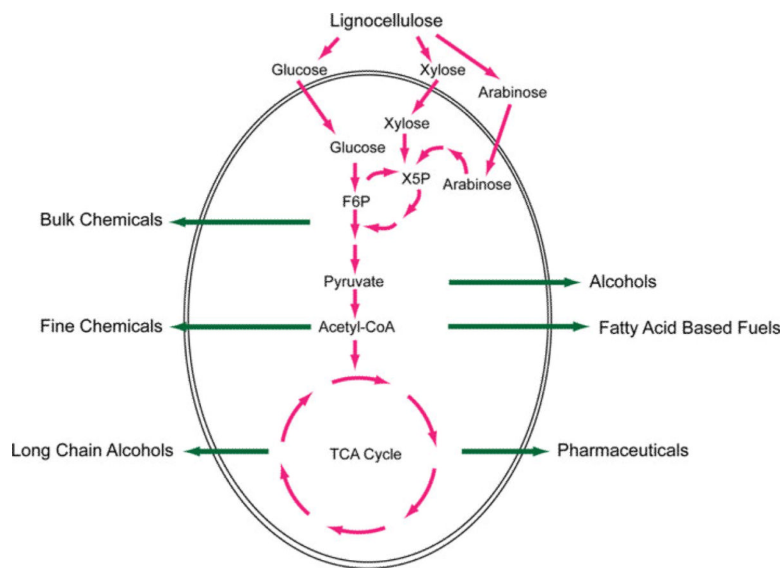

Fig. 4.
Production of fuels and chemicals from lignocelluosic biomass

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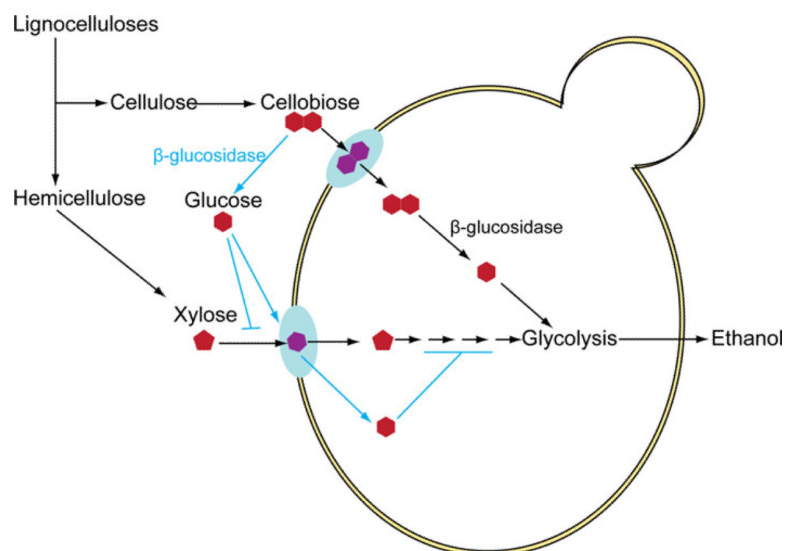

Fig. 5.

Co-fermentation of cellobiose and xylose using recombinant *S. cerevisiae* co-expressing a cellobiose transporter and an intracellular β-glucosidase. This novel approach eliminates the use of exogenously added β-glucosidase and alleviates glucose repression on xylose uptake and utilization (shown in *blue*)