Review

Industrial biotechnology: Tools and applications

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Industrial biotechnology involves the use of enzymes and microorganisms to produce value-added chemicals from renewable sources. Because of its association with reduced energy consumption, greenhouse gas emissions, and waste generation, industrial biotechnology is a rapidly growing field. Here we highlight a variety of important tools for industrial biotechnology, including protein engineering, metabolic engineering, synthetic biology, systems biology, and downstream processing. In addition, we show how these tools have been successfully applied in several case studies, including the production of 1,3-propanediol, lactic acid, and biofuels. It is expected that industrial biotechnology will be increasingly adopted by chemical, pharmaceutical, food, and agricultural industries.

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1 Introduction

Industrial biotechnology, also known as white biotechnology, is the application of modern biotechnology to the sustainable production of chemicals, materials, and fuels from renewable sources, using living cells and/or their enzymes. This field is widely regarded as the third wave of biotechnology, distinct from the first two waves (medical or red biotechnology and agricultural or green biotechnology). Much interest has been generated in this field mainly because industrial biotechnology is often associated with reduced energy consumption, greenhouse gas emissions, and waste generation, and also may enable the paradigm shift from fossil fuel-based to bio-based production of value-added chemicals.

The fundamental force that drives the development and implementation of industrial biotechnology is the market economy, as biotechnology promises highly efficient processes at lower operating and capital expenditures. In addition, political and societal demands for sustainability and environment-friendly industrial production systems, coupled with the depletion of crude oil reserves, and a growing world demand for raw materials and energy, will continue to drive this trend forward [1]. McKinsey & Co., predicted that by 2010, industrial biotechnology will account for 10% of sales within the chemical industry, amounting to US$125 billion in value (http://www.chemie.de/news/e/pdf/news_chemie.de_56388.pdf). In the US, bio-based pharmaceuticals account for the largest share of the biotechnology market followed by bio-ethanol, other bio-based chemicals, and bio-diesel [2]. Other major players in industrial biotechnology include the European Union [3, 4], China, India, and Brazil. In China alone, the value of bio-based chemical products exceeded US$60.5 billion in 2007 [5].

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Abbreviations: ISPR, in situ product removal; MFA, metabolic flux analysis; **1,3-PD**, 1,3-propanediol

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Government policies including tax incentives, mandatory-use regulations, research and development, commercialization support, loan guarantees, and agricultural feedstock support programs have helped fuel the adoption of industrial biotechnology. Moreover, breakthroughs in enzyme engineering, metabolic engineering, synthetic biology, and the expanding "omics" toolbox coupled with computational systems biology, are expected to speed up industrial application of biotechnology. These advances have provided scientists with toolsets to engineer enzymes and whole cells, by expanding the means to identify, understand, and make perturbations to the complex machinery within the microorganisms. Another equally important tool is the advancement in downstream processing technology, which enables translation of laboratory benchtop experiments into economically viable industrial processes.

In this review, we will highlight the advances of a wide variety of biological toolsets for industrial biotechnology, including protein engineering, metabolic engineering, synthetic biology, systems biology (which includes "omics" and *in silico* approaches), as well as downstream processing. In addition, we will show how these toolsets are utilized in several case studies, specifically the production of 1,3-PD, lactic acid, and biofuels.

2 An expanding toolbox for industrial biotechnology

2.1 Protein engineering

One of the most important tools for industrial biotechnology is protein engineering. More often than not, a wild-type enzyme discovered in nature is not suitable for an industrial process. There is a need to engineer and optimize enzyme performance in terms of activity, selectivity on non-natural substrates, thermostability, tolerance toward organic solvents, enantioselectivity, and substrate/product inhibition in order for the enzymatic process to be commercially viable [6].

There are two general approaches for protein engineering: rational design and directed evolution. In rational design, the structure, function, and catalytic mechanism of the protein must be well understood in order to make desired changes *via* site-directed mutagenesis. However, such understanding is lacking for most proteins of interest. In addition, although computational protein design algorithms were developed to predict optimal mutations at specific residue positions in the protein, only limited success has been demonstrated [7–9]. In contrast, the directed evolution approach requires only knowledge of the protein sequence. This approach involves repeated cycles of random mutagenesis and/or gene recombination followed by screening or selection for positive mutants [10–12]. For example, error-prone PCR and site saturation mutagenesis have been used to engineer the enantioselectivity of the cytochrome P450 BM-3 from *Bacillus megaterium* [13]. Iterative site-specific saturation mutagenesis has also been used to alter the ligand-binding specificity of the human estrogen receptor α (hERa) to recognize non-steroidal synthetic compounds [14–16] and xylose-specific xylose reductase for xylitol synthesis [17]. In addition, a family shuffling approach was used to increase the catalytic activity and thermostability of a type III polyketide synthase, PhlD from the soil bacterium *Pseudomonas fluorescens* Pf-5 [18]. A summary of directed evolution techniques is shown in Table 1.

Often, finding an enzyme with desirable properties in a library of mutants generated by directed evolution is akin to looking for a needle in a haystack. Over the past several years, a multitude of screening and/or selection techniques have been developed to isolate the variants of interest. An example of a selection method was described by Boersma *et al.* [19] in the directed evolution of *B. subtilis* lipase A variants with inverted and improved enantioselectivity. The method is based on the use of an *Escherichia coli* aspartate auxotroph, the growth of which is dependent upon hydrolysis of an enantiomerically pure aspartate ester by desired lipase variants. A covalently binding phosphonate ester of the opposite enantiomer was used as a suicide inhibitor to inactivate less enantioselective variants.

Another commonly used method is microtiter plate-based screening. A typical screening procedure in a 96-well microtiter plate format begins with the generation of a library of mutants which are picked and grown in 96-well plates. The proteins of interest are expressed and are often subjected to a high throughput assay based on UV-absorption, fluorescence, or colorimetric methods. Mutants displaying desired characteristics are then verified and sequenced. The best mutant is then selected as the template for the next round of mutagenesis. The process is repeated in an iterative manner until the goal is achieved or no further improvements are possible (Fig. 1). Other screening/selection methods include the agar plate screen, cell-in-droplet screen, cell as microreactor, cell surface display, and *in vitro* compartmentalization, which has been described in earlier reviews [20, 21]. Despite the availability of a wide range of

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Table 1. Summary of the advantages and disadvantages of selected directed evolution methods (adapted with due permission from ref. [129])

Technique	Advantages	Disadvantages
epPCR	Simplicity Tunable mutation rate	Biased mutagenesis
SeSaM	Unbiased mutagenesis Codon randomization possible	2–3 days to perform Several steps, reagents & enzymes required Special primers required Several purification steps involved
RID	Random insertions and deletion Large diversity possible Codon randomization possible	Several steps, reagents & enzymes required Frameshift mutations possible
RAISE	Random insertions and deletion Codon randomization possible	Frameshift mutations possible DNaseI digestion bias
DNA shuffling	Robust, flexible Back-crossing to parent removes non-essential mutations Synergistic/additive mutations can be found	DNaseI digestion bias Biased to crossovers in high homology regions Low crossover rate High percentage of parent
Family shuffling	Exploits natural diversity Accelerated phenotype improvement	DNaseI digestion bias Biased to crossover in high homology regions Need high sequence homology in family Low crossover rate High percentage of parent
RACHITT	No parent genes in shuffled library Higher rate of recombination	Several steps, reagents & enzymes required Recombine genes of low sequence homology Requires synthesis and fragmentation of single-stranded complement DNA
NExT DNA shuffling	Predictable fragmentation pattern	Non-random fragmentation Several steps, reagents & enzymes required Toxic piperidine used
StEP	Simplicity	Need high homology Low crossover rate Need tight control of PCR
CLERY	Not limited by ligation efficiency of gene into vector	Transformants contain more than one mutant, so rescue and retransformation required Long PCR program for reassembly DNaseI digestion bias Background mutation in plasmid possible Limited diversity
ITCHY	Eliminates recombination bias Structural knowledge not needed Completely homology-independent	Limited to two parents One crossover per iteration Significant fraction of progeny out-of-frame Complex, labor-intensive Single crossovers
SCRATCHY	Eliminates recombination bias Structural knowledge not needed Multiple crossovers possible	Limited to two parents Significant fraction of progeny out-of-frame Complex, labor-intensive DNaseI digestion bias

screening or selection tools, their applicability is often specific only to a particular substrate/enzyme combination and much effort is still required to customize and optimize a screening/selection method for different directed evolution experiments.

2.2 Metabolic engineering

An equally important tool for industrial biotechnology is metabolic engineering. By manipulation of enzymatic, transport, and regulatory functions in the cell, metabolic engineering redirects precursor metabolic fluxes, changes protein cellular levels,

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fine-tunes gene expression, and controls gene expression regulation in microorganism hosts such as *E. coli* [22], *Saccharomyces cerevisiae* [23], and actinomycetes [24].

For example, *Corynebacterium glutamicum*, originally a L-glutamic acid-secreting microorganism, was subjected to various genetic modifications to construct strains that can produce amino acids such as lysine, threonine, and isoleucine [25]. Recently, *C. glutamicum* was further engineered to produce L-valine by modulating the expression of genes involved in the biosynthesis of branched-chain amino acids [26]. The final result was a *C. glutamicum* strain that produces 136 mM L-valine in 48 h. Similarly, thermotolerant, methylotrophic bacterium *B. methanolicus* MGA3 was metabolically engineered to improve L-lysine production *via* the overexpression of aspartokinase, by cloning the four-gene aspartate pathway in *B. methanolicus* [27]. Up to 7 g/L of L-lysine was achieved in the engineered *B. methanolicus* compared to only 0.12 g/L in the wild type strain.

Metabolic engineering of microbes to produce large amounts of valuable metabolites that are difficult to extract from their natural sources, and too complex or expensive to produce *via* chemical synthesis, is an attractive option. Taxol® (paclitaxel) is an antimitotic agent used in the treatment of ovarian cancer and metastatic breast cancer, with annual sales revenue of US$1 billion [28]. Paclitaxel was originally extracted and purified from the bark of the yew *Taxus brevifolia* in very low yield, with about 9000 kg of yew bark (3000 trees) required to produce 1 kg of purified paclitaxel. Hence, microbial production of Taxol is an attractive and economic alternative to extraction from plant biomass. An efficient synthesis of taxadiene (an intermediate in Taxol biosynthesis) in yeast was recently developed. By analyzing and manipulating the expression of heterologous genes encoding biosynthetic enzymes from the taxoid biosynthetic pathway and isoprenoid pathway, and incorporating a regulatory factor to inhibit the competitive pathways, a 40-fold increase in taxadiene to 8.7 mg/L as well as significant amounts of precursor geranylgeraniol (33.1 mg/L) was achieved [29]. It is noteworthy that two new tools were recently developed to facilitate metabolic engineering in *S. cerevisiae*. One method is called "DNA assembler," which can be used to rapidly construct a biochemical pathway, a plasmid, or even a microbial genome [30]. The other method is called mutagenic inverted repeat assisted genome engineering (MIRAGE), which can be used to introduce chromosomal mutations in *S. cerevisiae* in a single transformation step [31].

2.3 New developments in synthetic biology tools

While protein and metabolic engineering have led to significant advances in industrial biotechnology, an emerging area of synthetic biology, in which basic genetic parts and modules are integrated into a

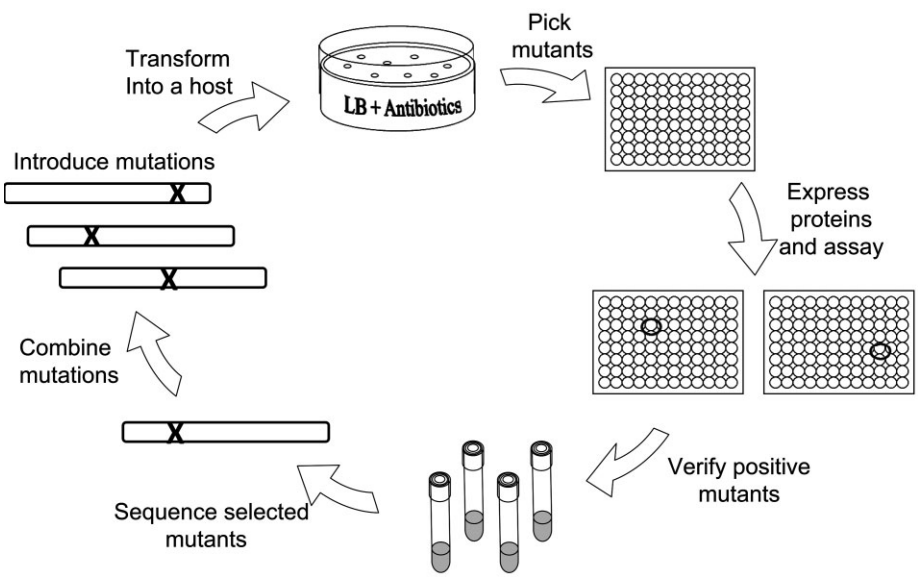

Figure 1. A typical 96-well plate screening procedure in directed evolution includes five main steps: (1) Generation of a library of mutants which are picked and grown in 96-well plates. (2) The proteins are expressed and subjected to a high throughput assay. (3) Positive mutants displaying desired characteristics are verified and sequenced. (4) The best mutant is used as a template for the next round of mutagenesis. (5) This process is repeated iteratively until the directed evolution goal is achieved or no further improvements are made.

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synthetic biological circuit, holds significant promises to the understanding, design, and construction of customized gene expression networks [32].

Scientists are attempting to create *de novo* genomes in synthetic microorganisms which are easier to understand and manipulate compared to those available in nature [33]. A recent example of this approach is the assembly of a synthetic genome of *Mycoplasma genitalium* from chemically synthesized overlapping DNA fragments of 5–7 kb [34, 35]. The synthetic genome contains all the genes of wild type *M. genitalium* except one which was disrupted by an antibiotic marker to prevent pathogenicity and to allow for selection.

Synthetic biology has also been applied to expand the genetic code for the incorporation of unnatural amino acids [36, 37]. In a recent example, a phage display system that allows the incorporation of unnatural amino acids has been utilized in the directed evolution of anti-gp120 antibodies [38]. This work demonstrates that an expanded genetic code can be combined with protein engineering strategies to allow for evolution of unique catalytic properties, binding modes, and structures where the unnatural amino acids contribute to the increase in evolutionary fitness and expand the structure–function range that can possibly be achieved.

Synthetic biology has provided scientists with the ability to design and build synthetic networks at the level of transcription, translation, and signal transduction, by manipulating and stringing together modular biological components such as promoters, repressors, and RNA translational control devices [39]. When combined with metabolic engineering, synthetic biology provides scientists with tools to build synthetic pathways for the production of biofuels, chemicals, and pharmaceuticals [40, 41]. One notable example is the engineering of a synthetic metabolic pathway based on the mevalonate-dependent isoprenoid pathway of *S. cerevisiae* into *E. coli* [42]. Isoprenoid is an important terpenoid precursor for the synthesis of many drugs, including an expensive antimalarial drug that is currently harvested from the rare *Artemisia annua* plant. The isoprenoid system was further modified to construct an artemisinin biosynthetic pathway in yeast [43, 44]. Up to 1 g/L of artemisinic acid can be produced, thus potentially providing a cheaper and reliable alternative source of antimalarial drugs. More examples of successful synthetic biology applications can be found in the case studies that will be discussed in the later section of this review.

2.4 Systems biology: "Omics" and *in silico* approaches

Increased genome sequencing efforts have ushered in a new era of systems biology, in which entire cellular networks are analyzed and optimized for application in the development of strains and bioprocesses. The properties of these complex cellular networks cannot be understood by monitoring individual components alone, but from the integration of non-linear gene, protein, and metabolite interactions across multiple metabolic and regulatory networks *via* computer simulation [45]. Thus, a variety of "omics" sub-disciplines have emerged such as genomics and metagenomics (study of interactions and functional dynamics of whole sets of gene and their products), transcriptomics (genome-wide study of mRNA expression levels), proteomics (analysis of structure and function of proteins and their interactions), metabolomics (measurement of all metabolites to access the complete metabolic response to a stimulus), and fluxomics (study of the complete set of fluxes in a metabolic reaction network). "Omics" approaches provide a greater set of data and a more complete understanding of the cell in various environments, thus complementing the metabolic and protein engineering efforts for strain improvement.

With the availability of whole-genome sequences, it has become possible to reconstruct genome-scale biochemical reaction networks in microorganisms. Over the recent years, genome-scale metabolic reconstructions for *E. coli* K-12 MG1655 [46], *B. subtilis* [47], *Methanosarcina barkeri* [48], and *S. cerevisiae* [49] were reported. "Omics" technologies have also opened the doors to new research areas such as high throughput metabolomics [50], MS for protein measurement [51], and yeast two-hybrid systems.

In silico methods have been used extensively in metabolic flux analysis (MFA). Among the most commonly used approaches is the ^{13}C labeling MFA approach, coupled with NMR or GC-MS [45, 52]. The labeling dynamics of intracellular intermediates is analyzed by solving a high-dimensional set of non-linear differential equations. Nöh *et al.* [53] recently presented a ^{13}C MFA approach using cytosolic metabolite pool sizes and the ^{13}C labeling data from an *E. coli* fed-batch experiment. A computational flux analysis tool 13CFLUX/INST was used to determine the intracellular fluxes based on a complex carbon labeling network model.

In another approach, Henry *et al.* [54] proposed a thermodynamics-based MFA (TMFA) which integrates thermodynamic data and constraints into a constraints-based metabolic model, such that the

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model produces only flux distributions that are thermodynamically feasible, and provides data on the free energy change of reactions and the range of metabolite activities, in addition to reaction fluxes. This approach was applied in the analysis of the thermodynamically feasible ranges for the fluxes and Gibbs free energy changes of the reactions and activities of the metabolites in the genome-scale metabolic model of *E. coli*.

By comparing the transcriptomes of the wild type *C. glutamicum* strain and its isogenic derivatives using a DNA microarray, novel genes, NCgl0855 (putatively encoding a methyltransferase) and the *amtA-ocd-soxA* operon, that could improve the production of lysine were identified and overexpressed. Total lysine production was found to have increased by about 40% [55]. In order to understand the factors that are involved in the high level secretion of a recombinant protein, Gasser *et al.* [56] analyzed the differential transcriptome of a *Pichia pastoris* strain overexpressing human trypsinogen *versus* that of a non-expressing strain. Six novel secretion helper factors were identified, namely Bfr2 and Bmh2 (involved in protein transport), the chaperones Ssa4 and Sse1, the vacuolar ATPase subunit Cup5, and Kin2 (a protein kinase connected to exocytosis). These helper factors were also demonstrated to increase both specific production rates and the volumetric productivity of an antibody fragment up to 2.5-fold in fed-batch fermentations of *P. pastoris*.

By combining rational metabolic engineering, transcriptome profiling, and an *in silico* gene knockout simulation, Lee and coworkers [57] have successfully engineered an *E. coli* strain to produce L-valine at a high yield of 0.378 g/g glucose. All known negative regulatory mechanisms, including feedback inhibition and transcriptional attenuation regulations, were removed by site-directed mutagenesis. Competing pathways were removed by gene knockout and the operon for L-valine biosynthesis was overexpressed. By comparative transcriptome profiling, an important regulatory circuit of the leucine responsive protein (Lrp), and L-valine exporter encoded by the *ygaZH* gene, was identified and amplified. Based on the *in silico* genome-scale metabolic simulation, a triple-knockout mutant strain was identified to further improve the L-valine production rate. In a subsequent paper by the same group, a similar approach coupled with an *in silico* flux response analysis was used to engineer an *E. coli* strain to produce L-threonine with a yield of 0.393 g/g glucose [58].

Although the combined "omics" approaches and *in silico* analyses have resulted in several successful examples of systems metabolic engineering, there is still much more information embedded in large-scale genome-wide data and computational simulation results that are yet to be fully explored.

2.5 Tools for downstream bioprocessing

The scale-up of enzyme-catalyzed reactions from the laboratory benchtop to industrial scale is an expansive discipline. It involves different areas such as sterilization, rheology, mixing, agitator design, enzyme immobilization, fluidization, heat transfer, mass transfer, separation and purification, surface phenomena, hydrodynamics, modeling, and instrumentation and process control. The majority of bioprocesses are batch-wise, although continuous and semi-continuous bioreactors are also used, depending on the type of bioprocess. Table 2 compares the batch and continuous bioreactors. Typical bioreactors include stirred-tank bioreactors [59] and airlift reactor systems [60].

Product recovery and purification is often the major cost in downstream bioprocessing [61]. Among the commonly used separation processes are extraction by distillation or liquid–liquid extraction, chromatographic methods (adsorption), and membrane separation [62]. In thermodynamically unfavorable reactions, equilibrium conversion limits the achievable product concentration. In

Table 2. Comparison between batch and continuous bioreactors

	Batch bioreactor	Continuous bioreactor
Advantages	Reduced risk of contamination	High productivity
	Lower capital investment for same bioreactor volume	Reproducible and consistent product quality due to constant operating parameters
	More flexibility in varying bioprocess/product	Reduced labor expense, due to automation
		Suitable for system investigation and analysis
		Higher degree of control in growth rates, biomass concentration, and secondary metabolite production
Disadvantages	Low productivity	Susceptible to contamination or organism mutation
	Higher costs for labor and/or process control	Minimal flexibility in bioprocess
		Higher investment costs in control and automation equipment

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addition, many biocatalytic reactions, which convert high concentrations of non-natural substrates, are limited by the product, which may be inhibitory or toxic to the biocatalyst. However, the use of *in situ* product removal (ISPR) can help resolve this issue *via* the direct removal of product while the reaction is progressing [61, 63].

In a recent example, *in situ* substrate feeding and product removal (SFPR) based on the use of adsorbent resin was successfully applied to a preparative scale Baeyer–Villiger biooxidation reaction using recombinant *E. coli* in a bubble column [64]. The substrate and product, which are stored on the resins, can be separated from the cell broth at any time during the biotransformation process, and the whole cells can be easily replaced by a fresh batch. The enantiopure product was obtained in 75 to 80% yield. A stirred tank reactor (STR) with ISPR (STR-ISPR) was also developed for the production of the sodium salt of an a-keto acid, 4-methylthio-2-oxobutyric acid (MTOB), which avoids the unwanted conversion of MTOB to 3-methylthiopropionic acid (MTPA). The reaction setup involved the co-immobilization of D-amino acid oxidase (DAAO) and catalase onto Eupergit C in the reactor and ISPR by coupling Amberlite IRA-400 column. A yield of 75% with 95% product purity was obtained [65].

Besides protein engineering approaches, protein immobilization is often the solution to issues of enzyme instability in industrial processes. Immobilization can also optimize the enzyme dispersion in hydrophobic organic media by preventing the aggregation of the hydrophilic protein particles. Immobilized enzymes can be employed in different solvents, at extremes of pH and temperature, and at high substrate concentrations. Moreover, immobilization allows the enzyme to be recycled, making it suitable for continuous processes. Different approaches to enzyme immobilization have been demonstrated, including adsorption *via* hydrophobic or hydrophilic interactions, ionic interactions, covalent binding to solid supports, cross-linking of enzymes, and encapsulation [66]. Examples of application of enzyme immobilization at the industrial level are the production of 6-amino-penicillanic acid [67] and the conversion of cephalosporin C into α-keto-adipoyl-7-amino-cephalosporanic acid [68]. Another recent example is the reversible immobilization of *Candida rugosa* lipase on fibrous polymer-grafted and sulfonated beads [69]. The beads have an adsorption capacity of 44.7 mg protein/g beads and can be regenerated with less than 10% capacity loss over six cycles of adsorption/desorption.

3 Case studies

3.1 1,3-propanediol (1,3-PD)

1,3-PD has a variety of applications in solvents, adhesives, laminates, resins, detergents, and cosmetics. Since 1995, commercial interest in 1,3-PD has grown significantly because Shell (Netherlands) and DuPont (US) commercialized a new 1,3-PD-based polyester poly(propylene terephthalate) with properties (good resilience, stain resistance, low static generation, *etc.*) appropriate for fiber and textile applications [70]. 1,3-PD is mainly manufactured by chemical synthesis, requiring expensive catalysts, high temperature and pressure, and a high level of safety measures. When DuPont took over the Degussa (Germany) chemical process of manufacturing 1,3-PD, competition from the Shell process led DuPont to invest more research effort into development of an economically feasible and sustainable bioprocess for the production of 1,3-PD.

A wide range of microorganisms, including those belonging to the Clostridiaceae and Enterobacteriaceae families, are known to ferment glycerol to 1,3-PD [71]. Within the Clostridiaceae family, the best known producer of 1,3-PD is *Clostridium butyricum* followed by acetone/butane producers *C. acetobutyricum*, *C. pasteurianum*, and *C. beijerinckii* [72–74]. An engineered strain of *C. acetobutylicum* DG1(pSPD5), containing the 1,3-PD pathway from *C. butyricum* VPI 3266 on the pSPD5 plasmid, was demonstrated to convert glycerol to 1,3-PD at a volumetric productivity of 3 g/L-h and a titer of 788 mM in an anaerobic continuous culture, which is almost a two-fold improvement when compared to *C. butyricum* [75, 76]. Furthermore, in a fed-batch culture with the engineered *C. acetobutylicum*, up to 1104 mM of 1,3-PD could be obtained.

Meanwhile, in the Enterobacteriaceae family, *Klebsiella pneumoniae* [77] and *Citrobacter freundii* [78] are known to convert glycerol to 1,3-PD. By overexpressing the glycerol dehydrogenase and 1,3-PD oxidoreductase enzymes in a recombinant *K. pneumoniae*, Zhao *et al.* [79] investigated the significance of these enzymes on the conversion of glycerol into 1,3-PD in a resting cell system under micro-aerobic conditions. A yield of 222 mM and a conversion ratio of 59.8% (mol/mol) were obtained. In another study, the metabolic network of glycerol metabolism in *K. pneumoniae* was extended, and elementary flux modes (EFM) analysis incorporating oxygen regulatory systems was carried out for 1,3-PD production, by comparing the metabolic networks under aerobic and anaerobic conditions.

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Flux distribution and the effect of the pentose phosphate pathway (PPP) and transhydrogenase on 1,3-PD production, under different aeration conditions, were also investigated [80].

In a collaboration between DuPont and Genencor International (US), metabolic engineering was used to design and build an *E. coli* K12 strain that converts D-glucose to 1,3-PD directly [81–84]. The engineered strain depends on a heterologous carbon pathway that diverts carbon from dihydroxyacetone phosphate (DHAP), a major artery in central carbon metabolism, to 1,3-PD (Fig. 2) [85]. The carbon pathway involves glycerol 3-phosphate dehydrogenase (*dar1*) and glycerol 3-phosphate phosphatase (*gpp2*) genes from *S. cerevisiae* to produce glycerol from DHAP. Glycerol is further converted to 3-hydroxypropionaldehyde by utilizing the glycerol dehydratase (*dhaB1*, *dhaB2*, *dhaB3*) and its reactivating factors (*dhaBX*, *orfX*) obtained from *K. pneumoniae* [81, 83]. Fed batch fermentation results showed that the presence of strains utilizing *yqhD* (which encodes the 1,3-PD oxidoreductase isoenzyme, an NADP-dependent dehydrogenase from wild type *E. coli*) produced 1,3-PD titers of approximately 130 g/L, which are higher than identical strains utilizing *dhaT* (which encodes for 1,3-PD). Glycerol kinase (*glpK*) and glycerol dehydrogenase (*gldA*) genes were also deleted to prevent glycerol from being metabolized as a carbon source [82]. The two main changes to the metabolic pathways in *E. coli* are the replacement of the phosphoenolpyruvate (PEP)-dependent glucose phosphorylation system with ATP-dependent phosphorylation and the downregulation of glyceraldehyde 3-phosphate dehydrogenase (*gap*). The final result is a metabolically engineered *E. coli* strain that produces 1,3-PD at a rate of 3.5 g/L-h, a titer of 135 g/L and a weight yield of 51% in D-glucose fed-batch 10 L fermentations [85]. Commercial manufacture of the biologically derived 1,3-PD is currently being carried out by DuPont Tate and Lyle BioProducts, LLC.

In a more recent example, *E. coli* K12 was engineered to convert glycerol to 1,3-PD by constructing a novel 1,3-PD operon of three genes (*dhaB1* and *dhaB2* from *C. butyricum,* and *yqhD* from wild type *E. coli*) tandemly arrayed under the control of a temperature-sensitive promoter in the vector pBV220 [86]. The 40 h process consists of two stages, a high-cell-density fermentation step at 30°C, followed by a second stage in which glycerol is rapidly converted to 1,3-PD following a temperature shift from 30 to 42°C. An overall yield and productivity of 104.4 g/L and 2.61 g/L-h was achieved with the conversion rate of glycerol to 1,3-PD reaching 90.2% (g/g).

Researchers have also attempted to engineer *S. cerevisiae* for 1,3-PD production due to the various advantages of yeast as a biocatalyst in fermentations utilizing biomass hydrolysates [23]. Rao *et al.* [87] recently engineered *S. cerevisiae* by integrating genes *dhaB* from *K. pneumoniae* and *yqhD* from *E. coli* into the chromosome of *S. cerevisiae* by *Agrobacterium tumefaciens*-mediated transformation. The 1,3-PD yield is low, at only about 0.4 g/L. Further metabolic engineering work will be required to increase the yield. Other 1,3-PD producing species that have been investigated include Lactobacilli (*e.g. Lactobacillus brevis* and *L. buchneri* [88]) and thermophilic microorganisms (*e.g. Caloramator viterbensis* [89]).

Downstream processing and product recovery of 1,3-PD involves three main steps: (i) removal of microbial cells; (ii) removal of impurities and separation of 1,3-PD from the fermentation broth; and (iii) final purification of 1,3-PD by vacuum distilla-

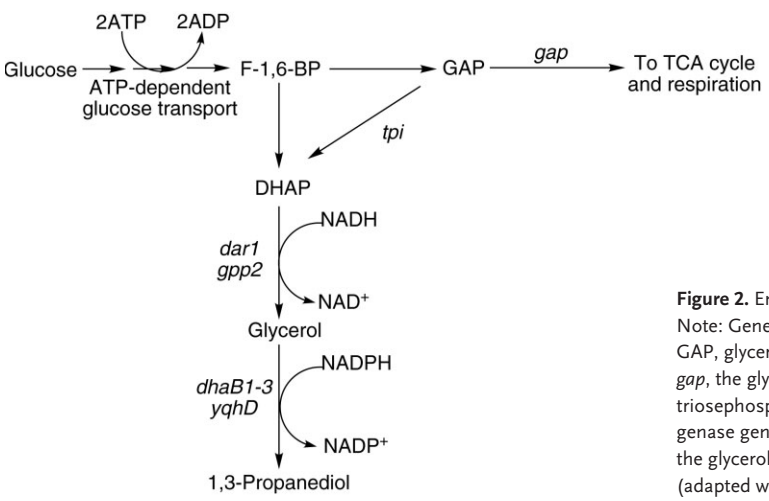

Figure 2. Engineering metabolic pathways from d-glucose to 1,3-PD. Note: Genes have been italicized. F-1,6-BP, fructose-1,6-biphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; *gap*, the glyceraldehyde 3-phosphate dehydrogenase gene; *tpi*, the triosephosphate isomerase gene; *dar1*, the glycerol 3-phosphate dehydrogenase gene; *gpp2*, the glycerol 3-phosphate phosphatase gene; *dhaB1–3*, the glycerol dehydratase gene; *yqhD*, the putative oxidoreductase gene (adapted with due permission from ref. [85]).

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tion or LC. These methods have been reviewed previously [90].

3.2 Lactic acid

Worldwide production of lactic acid (also known as 2-hydroxypropanoic acid) exceeds 100 000 metric tons/year [91]. Much of the increase in demand for lactic acid is attributed to two emerging products, polylactic acid for biodegradable plastics and the environmentally friendly solvent ethyl lactate. Lactic acid can also be applied in food, cosmetics, tanning industry, and as an intermediate in pharmaceutical processes.

Traditionally, *Lactobacillus* strains were utilized in the production of D-(-) or L-(+)-lactic acid. However, these lactic acid bacteria have shortcomings including requirement for amino acids or complex nutrients such as sugarcane juice, cornsteep liquor or whey, as well as poor ability to utilize pentoses for growth [92]. Therefore, other biocatalysts, especially engineered *E. coli* strains, were developed to produce D- or L-lactic acid. These modified *E. coli* derivatives were also shown to overcome the inhibitory properties of high lactic acid concentrations [93].

E. coli K011 was engineered to ferment glucose or sucrose to produce D-lactate by deleting genes encoding competing pathways. Over 1 M D-lactate (optical purity >99.5%) was achieved with a maximum volumetric productivity of 75 mM/h in LB media with 10% w/v sugar [94]. Subsequently, further improvements were made to the *E. coli* B strain SZ132 which fermented 12% w/v glucose to 1.2 M D-lactate in mineral salts medium. However, chiral purity declined from 99.5 to 95% [95]. Further metabolic engineering and evolution enabled the construction of *E. coli* strains which produced optically pure D- and L-lactate (>99.9%). By deleting the methylglyoxal synthase gene (*msgA*) and selecting for improved lactate productivity and cell yield by evolutionary engineering, the TG114 strain was isolated and found to produce optically pure D-lactate with high productivity (Fig. 3). The D-lactate strain can be reengineered to produce primarily L-lactate by replacing the native D-lactate dehydrogenase gene (*ldhA*) with the L-lactate dehydrogenase gene (*ldhL*) from *Pediococcus acidilactici*. Highly optically pure D- and L-lactate with a yield of >95% and a titer of >100 g/L in 48 h were obtained [96]. In another recent example, Portnoy *et al.* [97] created an *E. coli* K12 MG1655 strain which ferments glucose to D-lactic acid (yield 80% w/w) under aerobic conditions, by knocking out three terminal cytochrome oxidases (*cydAB*, *cyoABCD*, and *cbdAB*).

C. glutamicum is known to produce organic acids such as L-lactic, succinic, and acetic acids from glucose in mineral salts medium, under anaerobic conditions [98]. By expressing the *ldhA*-encoding genes from *E. coli* and *L. delbrueckii* in *C. glutamicum* D*ldhA* strains, Okino *et al.* [99] constructed an engineered *C. glutamicum* that can produce up to 120 g/L (1336 mM) of D-lactic acid with >99.9% optical purity in mineral salts medium within 30 h [99]. In another example, *P. stipitis* was GM to express the L-lactate dehydrogenase (LDH) from *L. helveticus*. A lactate yield of 0.58 g/g on xylose and 0.44 g/g on glucose are reported [100]. A *L. buchneri* strain NRRL B-30929 was also demonstrated to produce lactate as the main fermentation product from xylose and/or glucose [101]. Other biocatalysts developed to produce optically pure lactic acid isomers include *Kluyveromyces* [102], *Saccharomyces* [103, 104], and *Rhizopus* [105]. Further optimization of lactic acid fermentation and downstream processing has been described previously [91, 106].

3.3 Biofuels

Depleting petroleum supply, soaring fuel costs, and increasing environmental deterioration are critical challenges facing the world. These concerns have motivated the development and production of renewable biomass-derived biofuels such as bioethanol, biobutanol, and biodiesel. Bioethanol, derived mainly from sugarcane (Brazil) and corn (US), was introduced in the 1970s as an additive or complete replacement for petroleum-derived transportation fuels [107]. In 2008, over 17 billion

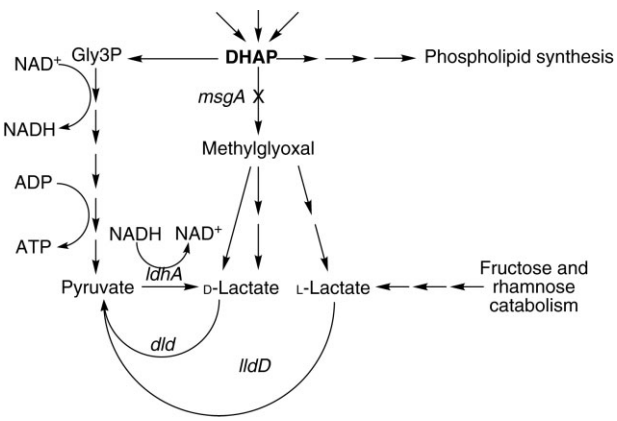

Figure 3. Metabolic engineering for production of enantiopure lactic acid. Notes: Genes have been italicized. Gly3P, glycerol-3-phosphate; *msgA*, the methylglyoxal synthase gene; *ldhA*, the D-lactate dehydrogenase A; *lldD*, the L-lactate dehydrogenase gene; *dld*, the D-lactate dehydrogenase gene. Multiple steps are indicated by consecutive arrows (adapted with due permission from ref. [96]).

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gallons of bioethanol was produced worldwide (http://www.ethanolrfa.org/resource/facts/trade/). However, despite its immense success, bioethanol has some drawbacks, such as low energy density, high vapor pressure, and corrosion issues, thus preventing its widespread use in the existing fuel infrastructure. This has led to an increasing interest in microbially produced butanol as an alternative gasoline substitute. Butanol's lower hygroscopicity allows compatibility with existing fuel infrastructure, higher energy density, and lower vapor pressure compared to ethanol.

Production of *n*-butanol, utilizing various species of *Clostridium* has been well studied [108]. Recent studies also demonstrated acetone-butanol-ethanol (ABE) production by *C. beijerinckii* using acid and enzyme hydrolyzed corn fiber [109] and wheat straw hydrolysate [110], respectively. Using *C. pasteurianum* ATCC 6013, crude glycerol generated during biodiesel production was converted to butanol, 1,3-PD, and ethanol [111]. Unfortunately, the complex physiology and lack of genetic tools for engineering *Clostridia* present difficulties in further improving the strain via metabolic engineering for optimal *n*-butanol production [92].

Due to the limitation of *Clostridia*, focus was shifted to well-characterized hosts such as *E. coli* and *S. cerevisiae* for biobutanol production. Using metabolic engineering approaches, the Liao group successfully engineered a recombinant *E. coli* strain that produces *n*-butanol, using the *n*-butanol production pathway from *C. acetobutylicum*. A set of essential genes (*thl*, *hbd*, *crt*, *bcd*, *etfAB*, *adhE2*) from *C. acetobutylicum* were cloned and expressed in *E. coli*, using a two-plasmid system, resulting in an initial *n*-butanol production at 14 mg/L. The pathway was optimized further by replacing the *C. acetobutylicum thl* gene with the *E. coli atoB* gene, leading to a threefold increase in *n*-butanol production. By deleting the native *E. coli* pathways that compete with the *n*-butanol pathway for acetyl-CoA and NADH, the *n*-butanol production was improved by more than two-fold. The highest titer of *n*-butanol produced by the engineered strain is 552 mg/L in rich medium [112].

In another strategy, keto acid intermediates, generated by amino acid biosynthesis, were converted to higher alcohols (C4 to C8) by expressing broad-substrate-range keto acid decarboxylase and alcohol dehydrogenase in *E. coli* [113]. The production and specificity of the desired alcohols were further improved by modifying the *E. coli* metabolic pathways to increase the production of the specific 2-keto acid and reduce by-product formation. For increased isobutanol production, the native *ilvIHCD* operon was overexpressed to enhance 2-ketoisovalerate biosynthesis. In addition, genes that led to by-product formation (*adhE*, *ldhA*, *frdAB*, *fnr*, and *pta*) were knocked out. The gene *alsS* from *B. substilis*, which has a higher affinity for pyruvate, was used to replace the *E. coli ilvIH* gene, and *pflB* was deleted to decrease further competition for pyruvate. By combining overexpressions and metabolic modifications, the engineered *E. coli* was able to produce isobutanol at a titer of 22 g/L, with a yield of 0.35 g isobutanol/g glucose [113]. Using a systematic approach, Shen and Liao [114] further improved the *n*-butanol and *n*-propanol co-production in *E. coli* through deregulation of amino acid biosynthesis and elimination of competing pathways. A production titer of 2 g/L with nearly 1:1 ratio of *n*-butanol and *n*-propanol was achieved by the engineered strain.

In a rational protein design approach, Zhang *et al.* [115] expanded branched-chain amino acid pathways in *E. coli* to produce non-natural longer chain keto acids and alcohols (>C5) by engineering the chain elongation activity of 2-isopropylmalate synthase and altering the substrate specificity of downstream enzymes. In another study, directed evolution was also applied to the citramalate synthase from *Methanococcus jannaschii*, which directly converts pyruvate to 2-ketobutyrate, thus providing the shortest keto-acid mediated pathway for producing *n*-propanol and *n*-butanol [116]. The best citramalate synthase variant showed enhanced specific activity over a wide temperature range and was insensitive to feedback inhibition by isoleucine, thus resulting in 9- and 22-fold higher production levels of *n*-propanol and *n*-butanol, respectively, compared to the strain expressing the wild type citramalate synthase gene. By expressing the six synthetic genes of *C. acetobutylicum* (*thiL*, *hbd*, *crt*, *bcd-etfB-etfA*, and *adhe*) in *E. coli*, about 1.2 g/L *n*-butanol production, with 100 mg/L butyrate as a byproduct, was achieved [92].

S. cerevisiae, the current industrial strain for producing ethanol and a well-characterized organism, has been demonstrated to have tolerance to *n*-butanol [117], thus making it a suitable host strain for *n*-butanol production. The Keasling group recently demonstrated *n*-butanol production of up to 2.5 mg/L in *S. cerevisiae* using galactose as a sole carbon source. Isozymes from a variety of organisms including *S. cerevisiae*, *E. coli*, *C. beijerinckii*, *Streptomyces collinus*, and *Ralstonia eutropha* were explored, and the best *n*-butanol-producing strain was found to consist of the *C. beijerinckii* 3-hydroxybutyryl-CoA dehydrogenase and the acetoacetyl-CoA transferase from *S. cerevisiae* or *E. coli* [118].

Biodiesel is prepared from triglycerides or free fatty acids by transesterification with short chain

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alcohols. Feedstock for biodiesel production includes vegetable oils and animal fats such as soybean oils, rapeseed oils, palm oils, and waste cooking oils. In order to meet the increasing demand for biodiesel, much attention has been given to microbial-derived biodiesel. Microbial oils can be used for biodiesel production and are produced by oleaginous microorganisms such as yeast, fungi, bacteria, and autotrophic microalgae, as reviewed previously [119]. Microbial oils are advantageous over the plant- and animal-derived oils because they are not limited by geographical and seasonal restrictions.

Kalscheuer *et al.* [120] engineered an *E. coli* strain to produce fatty acid ethyl esters (FAEE) *via* heterologous expression of the *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase, and the acyltransferase from *Acinetobacter baylyi* ADP1. Lu *et al.* [121] also engineered an *E. coli* strain to synthesize about 2.5 g/L of total fatty acids with a linear production of 0.024 g/h/g dry cell mass. This was accomplished by knock out of the endogenous *fadD* gene (which encodes an acyl-CoA synthetase) to block fatty acid degradation, heterologous expression of a plant thioesterase, and overexpression of acetyl-CoA carboxylase and an endogenous thioesterase.

Alkali-catalyzed transesterification is widely used for the commercial production of biodiesel. However, drawbacks of this method include energy intensiveness and difficulty of glycerol recovery, removal of alkaline catalyst from the product, and treatment of the highly alkaline waste water [122]. Biocatalysis approaches offer advantages over conventional methods, especially since the glycerol byproduct can be easily separated without any expensive or complex processes. The use of lipases for the production of biodiesel has been well studied [123]. Lipase-producing whole cells of *Rhizopus oryzae* (ROL), immobilized onto biomass support particles (BSPs), produced biodiesel from non-edible oil obtained from the seeds of *Jatropha curca*. The ROL activity was also shown to be higher than the commercially available lipase Novozym 435 [124]. In a follow-up study, immobilized recombinant cells of *Aspergillus oryzae*, expressing a lipase gene from *Fusarium heterosporum*, was used for enzymatic biodiesel production. The methyl ester content attained by *A. oryzae* was also demonstrated to be higher than that of *R. oryzae* [125]. In another study, recombinant *E. coli* expressing a lipase gene from *Proteus* sp. was applied as a biocatalyst in the transesterification process for biodiesel production. The permeabilized *E. coli* also demonstrated a conversion of close to 100% after a 12 h reaction at an optimal temperature of 15°C [126]. Salis *et al.* [127] explored the use of different support materials, including polypropylene (Accurel), polymethacrylate (Sepabeads EC-EP), silica (SBA-15), and an organosilicate (MSE), on the loading and enzymatic activity of the immobilized *Pseudomonas fluorescens* lipase used for biodiesel synthesis. The use of yeast and fungal whole cells in bioethanol and biodiesel production was reviewed previously [123].

4 Concluding remarks

In this review, we have described the recent advances in various aspects of industrial biotechnology, including protein engineering, metabolic engineering, "omics" based analytic tools, computational modeling tools, and the engineering of downstream bioprocesses, as well as several case studies. Ultimately, the success of industrial biotechnology depends on the economics of specific processes. Dwindling fossil fuel reserves and their rising cost, global warming, feedstock prices, government policies, consumer awareness, and further technological advancement are among the factors which would greatly influence the growth of industrial biotechnology. With the increased availability of genetic information and an expanding toolbox to manipulate metabolic pathways and engineer designer bugs, an increasing number of processes in the chemical and pharmaceutical industry will be biotechnologically driven.

Companies such as GlaxoSmithKline, Lonza, Degussa, Codexis, Verenium, DSM, Genencor, DuPont, Bristol-Myers Squibb, and Pfizer have made large investments in biotechnology research and development as they realize that the application of biotechnology in industrial production could translate into higher competitiveness, lower manufacturing cost, and lower capital expenditures, while significantly reducing their environmental footprint [128]. In addition, the adoption of industrial biotechnology will stimulate market growth with the increasing commercialization of more catalytic processes, and the discovery of new chemicals and drugs through the identification of new enzymatic routes.

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