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REVIEW

Advances in bioprocess for efficient biomanufacture

Genlin Zhang,[a,b]† Feng Qi,[a,c]† Haiyang Jia[a], Changling Zou[d] and Chun Li [a*]

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Bioprocess uses cost-effective renewable resources as feedstock to produce various products with less energy consumption under mild conditions. The technical progress in the modification of biocatalysts including enzymes and cells has significantly improved their efficiencies. In this article, the strategies involving molecular, cellular and community levels for improving various bioprocesses are reviewed with specific examples presented. To modify of enzyme molecule to better fit biomanufacture, semi-rational design of enzyme molecules and intensification of substrate channelling have been proved as effective methods. Progress in metabolic engineering and synthetic biology has promoted engineering the intelligent microbial cell factories. Through modularization of non-native synthesis pathways, microbial cell factories can be optimized at whole cellular levels, and consequently achieved the efficient production of pharmaceuticals, biofuels, chemical compounds as well as natural products. The use of synergistic interactions in microbial community would provide an opportunity to engineer more complex and robust functions. In addition, several special technologies including bioprocess under microgravity conditions and nanobiotechnology provide novel ways for developing new applied areas and opportunity to improve bioprocess efficiency.

Introduction

Energy conservation, waste minimization, safety and health issues have led to global concern for sustainable development. Process improvement for efficient production of various products has thus garnered increased attention for this regard. It is not surprising that scientific and technological advances in bioprocess have created a mindset for a bright future, because it offers substantial advantages over conventional chemical process.[1] Bioprocess uses biocatalysts including living cells or enzymes to produce desired products, which also includes upstream process such as the pretreatment of lignocellulosic biomass for biorefinery and medium development although no living cells or enzymes are involved with these processes.[2-4] In general, bioprocess uses cost-effective renewable resources as feedstock with less energy consumption under mild conditions,[5-6] and consequently decreases dependence on non-renewable fossil resources, and in the meantime reduces pollution associated with the corresponding industrial process.

Since the observation of microbes by Robert Hooke and Antoni van Leeuwenhoek, bioprocess has been studied and practiced for centuries by manufacturing industries to produce brewages, baked foods, pharmaceuticals, fuels, chemicals, and so on.[3,7-8] Although microbial cultures or enzymes discovered from various sources or mutated by conventional techniques had led to the development of many industrial biocatalysts,[1,9] the challenges for them are limited types of cells or enzymes and substrates as well as the instability of the biocatalysts for practical applications.[4] Recently, various approaches like enzyme engineering and metabolic engineering derived from molecular biology have been rapidly and extensively used to improve biocatalysts, even develop new ones to better fit the requirements for biomanufacture.[9-10] And also, advances in synthetic biology enable researches to redesign microbial cells, and thus greatly expand their potential applications in bioprocess. Moreover, bioprocess under special environments such as microgravity is being developed for providing insights for efficient biomanufacture.[11-13] Therefore, this review focuses on the strategies involving molecular, cellular and community levels for improving the existing bioprocesses, with specific highlight of progress achieved and challenges ahead. Fig.1 illustrates an overview of main technologies and bioprocesses for efficient biomanufacture.

Modification of enzyme molecules to better fit biomanufacture

Biocatalysis has reached present industrial level with the development of molecular biology methods.[4] The exclusive properties of enzyme, such as high turnover frequencies, amazing selectivities and enhanced reaction rate under relatively mild reaction conditions compared with homogeneous and heterogeneous catalysis, received much attention of chemists.[4,14] Enzyme catalysis has thus been widely applied in industrial and synthetic fields, particularly

[a] School of Life Sciences, Beijing Institute of Technology, Beijing 100081, China. E-mail: lichun@bit.edu.cn
[b] Key Laboratory for Green Processing of Chemical Engineering of Xinjiang Bingtuan, Shihezi University, Shihezi 832000, China.
[c] College of Life Sciences/Engineering Research Center of Industrial Microbiology, Fujian Normal University, Fuzhou 350117, China.
[d] School of Physics, Beijing Institute of Technology, Beijing 100081, China.
† Authors contributed equally.

with pharmaceutical innovations, for environmentally friendly alternatives to traditional chemical synthesis. However, natural enzymes are often unable to function in non-natural conditions.[15] Previously, enzyme immobilization has been developed to implement long-term operational stability and re-use of the enzymes.[16-17] Medium engineering, probably the most interesting achievement is the discovery of enhanced enzymatic performance in ionic liquids, is also used to improve operational and thermal stabilities of enzymes or altered substrate specificities, [18,] e.g. our research group employed a hydrophobic 1-butyl-3-methylimidazolium hexafluoro-phosphate ([BMIM]PF6) as the reaction medium to perform glycyrrhizin hydrolysis by recombinant β-d-glucuronidase which demonstrated significantly enhanced bond-selectivity.[2] However, the shortcomings of natural enzymes were not yet fully overcome. The main challenges are still how to increase the substrate availability, stability and activity of enzymes, and how to optimize the efficiency when multi-step enzymatic reactions are necessary.

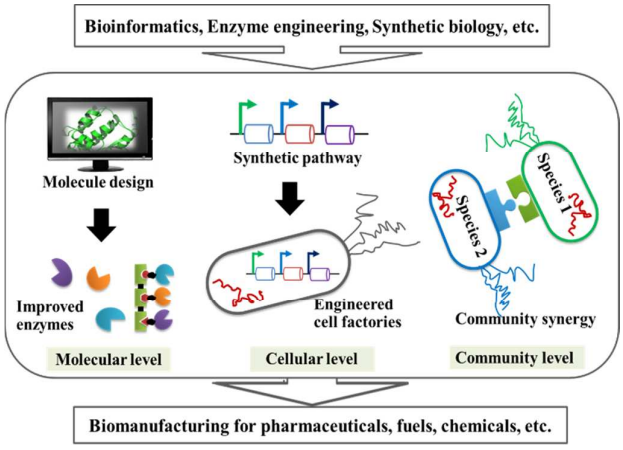

Fig. 1 Overview of main technologies and bioprocesses for efficient biomanufacture. Bioprocess uses enzymes, cells and even microbial community as biocatalysts to produce desired products. At molecular, cell and community levels, various technologies have been developed to assist design and optimize bioprocess.

Improvement of enzyme properties through molecular design and modification

In recent decade, most advances in bioinformatics and experimental technologies have dramatically promoted the redesign of natural enzymes. For example, based on the RCSB PDB design of the molecule of the month can be achieved.[19] The accumulation of experimental data and a growing number of protein sequences and structures enrich the database, allowing rational, non-rational or semi-rational design of enzyme molecules for acquiring desirable qualities.[9,20-21]

Rational design of enzyme molecules requires an in-depth understanding of the connection between the structures of enzymes and their functions. Guided by the structural information, amino acids may be accurately targeted and changed to improve the functions of enzymes, and even create novel enzymes by structurally mimicking native enzymes, making this strategy the easiest and fastest approach for enzyme molecular modifications. For example, Ugwumba *et al*. modified the naturally occurring tyrosine amino acid of bacterial phosphotriesterase with unnatural L-(7-hydroxycoumarin-4-yl) ethylglycine at position 309, which realized the hydrolysis of toxic organophosphate phosphotriester pesticides with the 11-fold increase of catalytic efficiency.[22] The method of incorporating unnatural amino acids into proteins to form artificial enzymes with improved properties, even new catalytic functions opens a way for the rational design of enzymes. However, in most instances, the application of this strategy is still limited due to the absence of necessary information with molecular structures and catalytic functions. In contrast, non-rational design based on directed evolution is widely used for engineering enzyme by simulating natural selection process to evolve enzymes. A critical step for non-rational design is to create a specific mutant library, which is extremely labor-intensive with the screening process for desired mutants.[15] As a result, semi-rational enzyme design has been developed for a compromise between the rational and non-rational strategies, in which one or few amino acids are identified as the target(s) for improving enzyme performance through structure-guided rational analysis or directed evolution.[15,20] For instance, phenylacetone monooxygenase modified by semi-rational design for catalytic asymmetric ketones Baeyer-Villiger (BV) reaction with the formation of chiral esters or lactones, an attractive transformation in organic chemistry, and the mutants obtained granted an unusually high activity and enantioselectivity in the oxidative kinetic resolution of a variety of 2-aryl and 2-alkylcyclohex-anones which cannot be performed by the wild-type enzyme.[20] Through the semi-rational design, a more specific and much smaller library was created, which significantly reduced the screening work compared to non-rational techniques with a library capacity of 10^3-10^6 mutants.[23] Observing that semi-rational design strategy generates a smarter library, shortens research cycle and reduces expenses. Thus it would be particularly favorable when high throughput screening method is not available.

Optimization of enzymatic efficiency by intensifying substrate channelling

Nature has evolved a kind of enzymes in a cascade fashion to catalyse the chemical reactions. Such multi-step reactions in living cells are frequently performed by multi-enzyme complexes to maintain high local concentrations of intermediates for enhanced reaction rates, so-called substrate channeling.[24-25] Inspired by natural enzyme complexes (e.g. bacterial micro-compartment, machinery evolved for signal processing in metazoan cells) [26-28], researchers keep an eye on the construction of static enzyme complexes and are trying to artificially produce drugs or chemicals.[29-30] Currently, two ways including construction of fusion proteins and synthetic scaffolds are developed to facilitate substrate channelling.

Fusion proteins, the simplest approach to achieve substrate channelling, construct a chimeric enzyme complex by combining two or more different enzyme genes as illustrated in Fig.2 A. Zhou *et al*. engineered the active sites of labdadienyl/copalyl diphosphate synthase (SmCPS) and a kaurenesynthase from *Salvia miltiorrhiza* for enhanced metabolic flux channeling to miltiradiene biosynthesis in yeast,

which significantly improved miltiradiene production.[29] Based on the fact that natural proteins or nucleic acid-protein interactions (aptamer or single-stranded DNA, ssDNA) are used as scaffolds to build functional multi-enzyme complexes, artificial scaffolds have been developed to construct multi-enzyme complex performing multi-step enzymatic catalysis process (Fig.2 B and C). For example, non-catalytic eukaryotic protein–protein interaction domains (GBD, SH3 and PDZ) serve as a scaffold to assemble an enzymatic pathway. By co-expressed GBD, SH3 and PDZ domains as a synthetic scaffold, three enzymes of the mevalonate pathway AtoB, HMGS, and HMGR were co-localized by domain–ligand interactions and thereby achieved 77-fold improvement in mevalonate titer.[28] Supplemented with cellulolytic enzymes to hydrolyze pretreated biomass to glucose, an enzyme complex assembling cellobiose phosphorylase and potato alpha-glucan phosphorylase by synthetic protein miniscaffoldin successfully synthesized starch from glucose in the one-pot enzymatic process.[25] RNA and DNA can also be used as scaffolds to form special complex. By designing and assembling multi-dimensional RNA structures as scaffolds, the engineered RNA modules were assembled into the distinct protein-docking sites for hydrogen producing pathway and used to control the spatial organization.[31] Fu *et al.* employed DNA as a scaffold for assembling glucose oxidase and horseradish peroxidase, resulting 15-times higher enzymatic activities with the 10 nm inter-enzyme complex, compared to the control.[32] Multi-enzyme assemblies may mimic the metabolic channelling processes as they occur in nature, especially for the application of complex matrices with diffusion limitation. The assembly of multi enzymes has been proved to implement some complicated reactions. In addition, the assembly of multi-enzyme complexes can avoid the interactions between the various enzymes and promote reaction rates. As a result, not only is an environment-friendly biosynthesis with engineering enzymes or enzyme catalytic process developed, but most importantly improved yields for pharmaceuticals, biofuels and other bulk compounds can be expected. However, using DNA or RNA scaffolds are still too costly due to imcomplete knowledge and available methods for studying the factors such as enzyme ratio, linker length and orientation on activity of multi-enzyme complexes.[31-32]

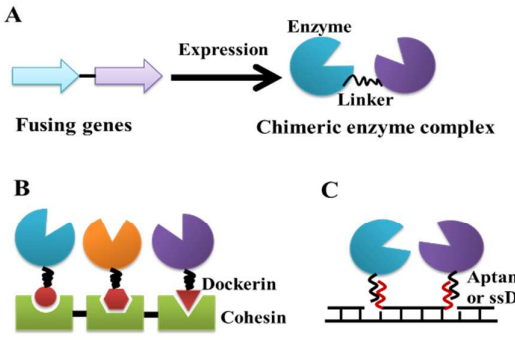

Fig. 2 Construction of multi-enzyme complexes. (A) Chimeric enzyme complex by fusing genes. (B) Multi-enzyme complex yielded by protein scaffold consisting of cohesin and scaffoldin units. (C) Multi-enzyme complex based on nucleic acid protein interactions either by DNA-directed assembly or affinity interaction of enzyme with its aptamer.

Engineering microbial cells for biomanufacture

Many advantages such as fast growth rate with microbial cells and associated environment-friendly processes drive scientists and engineers to use them as production hosts. In the 19th and 20th centuries, microorganisms had mainly been used to produce fundamental products mainly for brewages and other food usage such as ethanol, acetic acid, lactic acid, citric acid and so on. Within the past 20 years, progress in metabolic engineering and synthetic biology has enabled to engineer microbes, and even design cell factory as illustrated in Fig. 3. Various efficient tools have been developed for these purposes, i.e. enlarging the spectrum of feedstock, developing new metabolic routes to produce non-native products, optimizing robust and responsive genetic control systems for the desired pathways and debottlenecking the constructed pathway.[33] Furthermore, microbial cell factories based on the optimization of cellular metabolic levels have achieved numerous biorefinery products such as pharmaceuticals, biofuels and chemical compounds, which are partly summarized in Table 1.

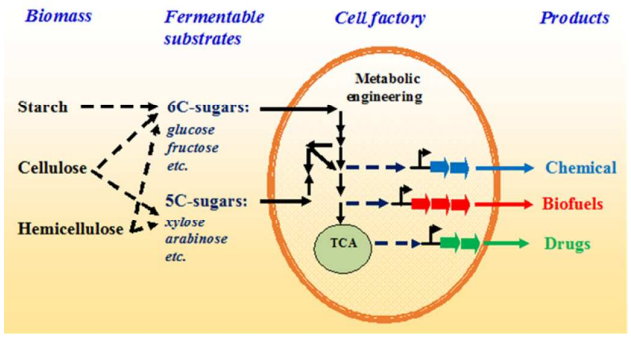

Fig. 3 Design and production of bio-based products by microbial metabolic engineering and cell factory.

Table 1 Typical applications of cell factory

Product		Engineered Cell factory	Yield	Ref.
Pharmaceuticals	Artemisinic acid	*S. cerevisiae*	25 g/L	7
	Taxadiene	*E. coli*	1 g/L	34
Fuels	Alkanes alkenes	*E. coli*	up 4mg/L	5
	Fatty-acid ethyl esters	*E. coli*	71mg/L	3
	Pinene	*E. coli*	1.50 mg/L	3
Chemicals	3-methyl-3-butenol	*E. coli*	55 mg/L	8
	3-methyl-2-butenol	*E. coli*	up 30mg/L	8

Biopharmaceuticals

Natural products particularly active pharmaceutical ingredients are generally too complex to be chemically synthesized, which leads to low production yield and high cost. Fortunately, microbial cell factory provides a substitute for effective synthetic methods. Several drug candidates were recently achieved by genetically manipulating the biosynthetic machinery involved in the assembly of these natural products.[7,34] The most inspiring case is the production of artemisinic acid that is used as a precursor of antimalarial drug (artemisinin) in the engineered yeast. Limited supply of artemisinin from *Artemisia annua* (Sweet wormwood) and the difficulties in its chemical synthesis result in the high price. Using *S. cerevisiae* as the artemisinic acid producing cell factory, Paddon *et al*. introduced the complete biosynthetic pathway of artemisinic acid into the yeast cells, followed by an effective regulation of the pathway through metabolic engineering including the up-regulation of the supply of the precursor FPP and in the meantime decreasing its consumption for sterols production by overexpressing a global transcription factor UPC2-1 that regulates the biosynthesis of sterols, which finally achieved a fermentation titer of 25 g/L artemisinin.[7] Another excellent example is to overproduce precursor of the potent anticancer drug taxol in *E. coli*. Using a multivariate-modular approach to engineering taxadiene (the first committed taxol intermediate) pathway in *E. coli*, the titer of taxadiene was increased 15,000-fold with a high concentration of 1 g/L.[34] Except for introduction of exogenous pathway, through improving the thermo-tolerance of host *S. cerevisiae* we increased the production of a triterpenoid β-amyrin 28.1%.[35] This strategy also greatly reduced the cooling costs and minimized microbial contamination during the fermentation. It is thus observed that the optimization of an efficient route to natural medicine biosynthesis is capable of providing potential commercial production of microbial pharmaceutical ingredients.

Biofuels

Although the change from fossil fuels to renewable alternative biomass might be achieved by chemical or thermo-chemical routes, their development and large scale application are still limited because of expensive catalytic process and intensive energy consumption. In contrast, most organisms have the ability to carry out unique chemical transformation under proper conditions, which could be explored for biofuels production.[36] Recent advances in metabolic engineering and cell factory have also focused on engineering microorganisms for producing biofuels through fatty acid or isoprenoid pathways. Based on intermediates of fatty acid metabolism, Schirmer *et al*. changed alkane biosynthesis pathway consisting an acyl-acyl carrier protein reductase and aldehyde decarbonylase derived from *Cyanobacteria* into *E. coli*, and the modified cells successfully converted renewable carbohydrate to C_{13}-C_{17} mixtures of alkanes and alkenes as the major constituents of gasoline, diesel and jet fuel.[5] Utilizing the fatty-acid ethyl esters production pathway, the engineered *E. coli* strain not only grew with plant biomass as single carbon source but also directly synthesized fatty-acid ethyl esters, a typical biodiesel product from plant oils.[3] Likewise, the introduction of the pinene synthesis pathway as well as cellulose and hemicelluloses utilizing pathways into *E. coli* resulted in the direct conversion of switch grass to 1.5 mg/L of monoterpene pinene, an immediate chemical precursor to a potential jet fuel.[3] With the advanced tools of metabolic engineering and synthetic biology, it is possible to produce more biofuel candidates. Nevertheless, as the commodity materials, the production costs of biofuels are now still higher than refining petroleum, limiting their competitive advantage, and thus extra control methods such as optimization of triglyceride synthesis pathways and modification of hosts need to be further established.

Bio-based materials and chemicals

Microbial cell factories are also employed for the production of bio-based chemicals. Although some of them such as amino acids, organic acids, vitamins as well as polymers can be produced through chemical methods, their production through microbial fermentations is more economically competitive. For example, biopolymers not only decrease demand for petroleum-based products, but also are biodegradable. Polyhydroxybutyrate-valerate (PHBV) is a co-polymer of poly-(β-hydroxybutyrate) produced by engineered *E. coli* from monomer 3-hydroxybutyrate and 3-hydro-xyvalerate, which is expected to be a well-developed alternative to petroleum-based products.[6] In addition, some chemicals with new structures and functions could be synthesized by metabolically engineered cells. By verifying two novel pathways to convert isopentenyl diphosphate into 3-methyl-3-butenol, 3-methyl-2-butenol and 3-methylbutanol, Chou and Keasling engineered *E. coli* for the production of these five-carbon alcohols.[8] Our previous works successfully achieved direct xylitol production from xylan by engineering xylitol pathway in *S.cerevisiae*, and use of transcriptional regulation of pathway leaded to yield increased by 1.7 folds.[37]

High yields and productivities are crucial for microbial cell factories producing biopharmaceuticals, fuels and chemicals. Besides various efficient tools previous mentioned, optimization of precursor supply, fine regulation of pathway at different levels, and modification of host have been proved as the efficient approaches for engineering microbial cell factories. Although reliable data derived from functional-genomics, proteome, metabolomics and effective bioinformatics tools (e.g. BLAST, ClustalW, SynBioSS) have driven the cost-efficient production of some products (e.g. propanediols, butanediol, succinic acid),[38] the yields of most bio-based products are still limited.

Mixed culture strategy based on microbial community synergy

Although many products are produced by pure culture, some secondary products such as antibiotics and enzymes are

produced through mixed culture.[39-40] This fermentation way can carry out both utilizing solid-state fermentation and submerged culture technologies, of which the submerged culture has been actively studied thanks to its advantages of better monitoring and operation. In a mixed culture system, microorganisms interact in both space and time characterized by their community synergy through which mass and energy are utilized sequentially, and cofactor circulation is established for mutual benefits with the production of metabolites that cannot be obtained by pure culture.[41-42] Synergy and cooperation also can reduce metabolic burden on single species (Fig. 3). For example, the growth of lactic acid bacteria in the mutualistic system of lactic acid bacteria and propionic acid bacteria is often stimulated by propionic acid bacteria through lowering lactate accumulation.[43] Sometimes, altruism behavior may be activated for maintaining better survival and higher productivity when bacterial community faces stressful conditions. Tanouchi *et al.* verified that some cells trigger the cell death program and release stress-relieving substances that increase the survival chances of other cells.[44] Fig. 4 summarizes the mechanisms of cooperative interspecies interactions and altruistic death.

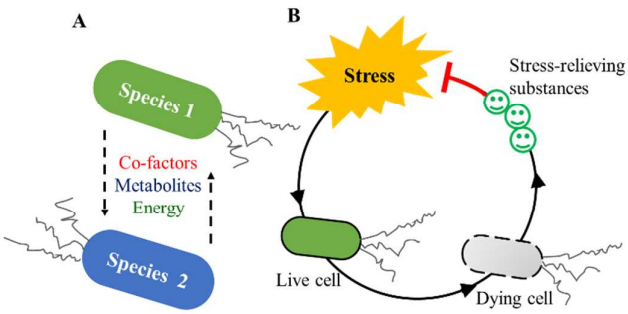

Fig. 4 Schematic of cooperative interspecies interactions and altruistic death. (A) Cooperative interspecies interact by cross-feeding of co-factors, metabolites or energy. (B) Altruistic death helps survivors by dying cells release the stress-relieving substances to reduce environmental stress.

Industrial production of vitamin C is another excellent case for submerged mixed culture. In the 1960's, two-step vitamin C fermentation process was originally developed in China, in which *Ketogulonicigenium vulgare* and *Bacillus sp*. were used to synthesize the vitamin C precursor 2-keto-L-gulonic acid.[45] *Bacillus sp*. was demonstrated to strengthen the oxidation and energy generation of *K. vulgare* through providing metabolites such as amino acids and purines and increase 2-keto-L-gulonic acid production by improving *K. vulgare*'s resistance to reactive oxygen species.[46-47] The insights into cooperative mechanism between strains have already activated the research of combining related pathway into one strain for one-step vitamin C production process.[48]

Contribution of the special technologies to bioprocess

Bioprocess under microgravity condition

Studies under special environments demonstrated that bioprocess would be affected significantly.[11,49] As an extreme and unique environment, microgravity presents a novel condition to optimize bioprocess because of its significant effect on numerous cellular functions. However, the lacking of mechanistic understanding on the response of cell to changed gravity conditions has limited their applications in bioprocess. Simulated microgravity (SMG) that is more applicable and feasible to the study provides a methodology for exploring its applications in bioprocess. The SMG environment can be created by a High Aspect Ratio Vessel (HARV) when the device is rotated horizontally with the axis of the vessel perpendicular to the gravitational vector, as illustrated in Fig. 5.

So far, several studies have demonstrated that cell growth, gene expression and metabolites production can be affected when microbial cells were cultured under microgravity or SMG conditions.[12] We utilized SMG to study its effect on improving the production of recombinant proteins, and verified its effectiveness in both prokaryotic and eukaryotic hosts.[11,13] The transcriptomic analysis illustrated that SMG changed the transcription levels of genes related to methanol metabolism and protein transportation or secretion family, which possibly contributed to the enhanced protein production.[12] These valuable information further help us find more novel helper factors to rationally engineer hosts for normal fermentation.[50]

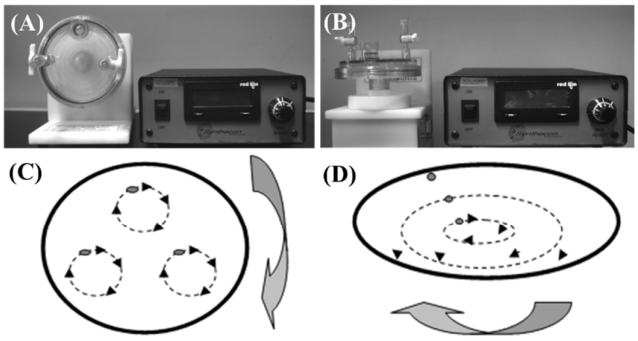

Fig. 5 Photos of the HARV system used to generate SMG (A) and NG (B) conditions, respectively. At the back of HARV there is a gas-permeable membrane for the exchange of O_2 and CO_2. The tracks of the cells maintained in (C) LSMMG and (D) NG conditions corresponding to (A) and (B), respectively. Cells in the medium would continually suspend under SMG.

The production of secondary metabolites, however, may be different. Demain and Fang scrutinized that both MccB17 and β-lactam production under SMG conditions were lower,[51] but the production of gramicidin by *Bacillus brevis* remained unaffected, indicating that microbes were affected differently in their physiology and metabolism by SMG.[11] Therefore, mechanism through which cells sense SMG conditions and how they convert these mechanical signals into molecular and biochemical responses need to be investigated.

Application of nanobiotechnology

The intersection of biotechnology and material science has brought new opportunities for bioprocess. DNA and protein can be directly used as nanomaterials for analysing intracellular metabolites or drug delivery.[52-54] The combination of nucleic acids and carbon nanotubes has also shown practical applications in biosensors.[55] More importantly, nanoscaled enzyme catalysts would expand the application of enzyme catalysis and improve enzyme property. Compared to traditional methods for enzyme immobilization, the use of nanomaterials was effective for simplifying the purification of enzyme, increasing target enzyme load and enhancing the efficiency of enzyme. For example, our research group employed highly porous graphene/γ-Fe_2O_3 hybrid aero-gels for immobilizing β-glucuronidase, achieving one-step magnetic adsorption and avoiding the multi-step recovery process.[56] Using aptamer-modified magnetic beads simultaneously purified and immobilized β-glucuronidase from the complex cell lysates without any other treatment.[57] Nanoscaled enzyme catalysts also can decrease mass-transfer resistance, and improve thermal stability, activity and tolerance of polar solvents. Magnetic enzyme nanogel prolonged the lipase half-life of 14 times at high temperature.[58] Hybrid organic–inorganic laccase nanoflowers showed 650% increase in activity compared with free laccase in solution.[59] The multiple advantages of nanobiocatalysis make us believe that it will have well promise for catalyzing complex chemical and biochemical reactions.

Conclusions and Prospective

Scientific and technological advances in enzymes and cells have made bioprocess more significant than ever for sustainable development. New tools of engineering enzymes and cells have improved production efficiencies with various bioprocesses, and thus created new applications both in laboratory research and on industrial scales as well. The increased ability to modify enzymes and engineer cell factory will bring more opportunities for bioprocess with less energy consumption, minimized wastes, and more safety and health, which are highlighted below:

(1) Several strategies are being adopted to improve the industrial applicability of enzymes, of which design and modification enzyme molecules is more effective. Multi-step enzymatic catalysis process can be appreciably improved by mimicking metabolic channelling through constructing artificial multi-enzyme complexes.

(2) Development of metabolic engineering and synthetic biology enables the engineering of non-native synthesis pathways in microbial cells and regulating cell factories at whole cellular metabolic levels, which makes microbial process being extensively explored for more efficient production of pharmaceuticals, biofuels, and bio-based materials and chemicals. But more efforts are still required to seek for new pathways and to optimize the robustness of hosts.

(3) Understanding the synergistic effect of interspecies in microbial community has advanced engineering their functions for mixed culture. With the potential advantages in reducing metabolic burden on single species and increasing its survival, mixed culture is promising for producing products that cannot be effectively produced by individual microorganism.

(4) Bioprocesses under microgravity condition and nanobiotechnology have opened the novel research areas with a potential for a buildup of knowledge and developing new technologies. Nevertheless, more investigation on the response mechanisms of biocatalysts under microgravity conditions is needed.

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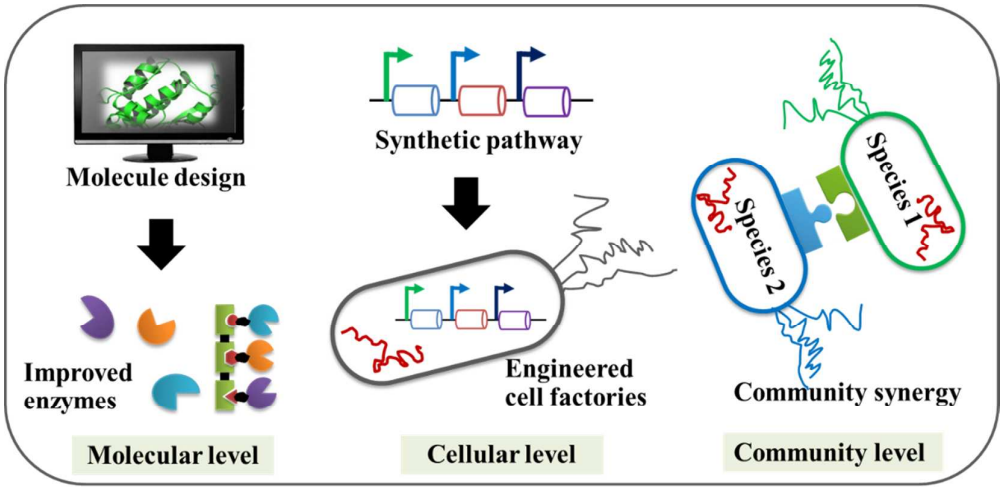

The strategies involving molecular, cellular and community levels for improving various bioprocesses are reviewed with specific examples presented.