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Abstract

With the development of metabolic engineering, employment of a selected microbial host for accommodation of a designed biosynthetic pathway to produce a target compound has achieved tremendous success in the past several decades. Yet, increasing requirements for sophisticated microbial biosynthesis call for establishment and application of more advanced metabolic engineering methodologies. Recently, important progress has been made towards employing more than one engineered microbial strains to constitute synthetic co-cultures and modularizing the biosynthetic labor between the co-culture members in order to improve bioproduction performance. This emerging approach, referred to as modular co-culture engineering in this review, presents a valuable opportunity for expanding the scope of the broad field of metabolic engineering. We highlight representative research accomplishments using this approach, especially those utilizing metabolic engineering tools for microbial co-culture manipulation. Key benefits and major challenges associated with modular co-culture engineering are also presented and discussed.

Keywords

Modular co-culture engineering, microbial co-cultures, pathway modularization, microbial biosynthesis

1. Introduction

The development of metabolic engineering has enabled the biosynthesis of a wide range of value-added chemicals through utilizing various microorganisms as biocatalysts (Woolston et al., 2013). High-efficiency bioproduction can be achieved by exploiting various cellular resources such as precursors, cofactors, energy and so forth, to support a designed biosynthetic pathway. Conventionally, this is accomplished by assembly of the target biosynthetic pathway in the context of a single microbial strain. The resulting cell population with homogeneous genetic and metabolic settings is referred to as a mono-culture. Although, substantial success using microbial mono-cultures has been documented in the past, this methodology is challenged by the increasing requirements for fulfilling complicated biosynthetic tasks and achieving higher efficiency for bioproduction.

To this end, employment of microbial co-cultures or synthetic consortia has been widely studied to overcome the technical barriers associated with microbial mono-cultures. In fact, there has been a history of using microbial co-cultures for applications such as efficient utilization of complex substrates and complicated molecule biosynthesis (Brenner et al., 2008; Hays et al., 2015; Sabra et al., 2010; Song et al., 2014). Recently, there has emerged a new co-culture-based engineering approach which aims to manipulate a target biosynthetic pathway in the context of a mixed culture composed of multiple microbial strains. Distinct from previous co-culture studies,

this approach divides a complete biosynthetic pathway into separate serial modules, and uses selected microbial strains to accommodate individual modules for achieving designed biosynthesis. Such methodology, with strong features of modularity, is herein referred to as modular co-culture engineering in this review.

Modular co-culture engineering is a new and yet viable option for sophisticated engineering of biosynthetic pathways. In fact, recent developments of metabolic engineering and synthetic biology encourage the adoption of modular or spatial organization for design, construction and control of a target biosynthetic pathway for improved biosynthesis performance (Agapakis et al., 2012). In this regard, modular co-culture engineering offers a great platform to pursue desired modularity for bioproduction purposes. On the other hand, new strategies for advanced manipulation of artificial microbial co-cultures are being developed, providing a rich toolbox for accommodation of engineered biosynthetic pathways in co-culture systems (Jagmann and Philipp, 2014). This review summarizes recent progress in employment of modular co-culture engineering for microbial biosynthesis and pathway optimization, and in particular highlights the reports that used metabolic engineering approaches for development of microbial strains for building co-culture systems. Notably, utilization of engineered co-cultures without pathway division, and use of separated sequential cultures for pathway modularization are not included in this review.

2. Benefits of modular co-culture engineering

The combination of pathway modularization and employment of multiple microbial strains grants modular co-culture engineering a unique characteristic and perspective to tackle issues that are hard to address using conventional approaches. Compared with mono-culture engineering, modular co-culture engineering has several important benefits for building high-

efficiency bioproduction processes, which are depicted in **Fig. 1**. It should be noted that, all of these benefits have been exploited by reported research detailed in the following section. Also, although only two constituent strains are involved in **Fig. 1** for illustration purposes, modular co-culture engineering encompasses involvement of more than two co-culture members, or polycultures.

One primary benefit of using modular co-culture engineering is that, relative to a mono-culture, it enables reduced metabolic burden on each constituent strain and thus helps improve the overall bioproduction/bioconversion performance. A long and complicated pathway often involves a large number of biosynthetic genes whose overexpression can excessively consume cellular resources and leads to overwhelming metabolic burden on a single host strain. As a result, the host strain often suffers from impaired growth and/or poor biosynthetic behavior (Wu et al., 2016). Modular co-culture engineering recruits more than one microbial strains (of the same or different species) to share the overall metabolic burden and thus goes beyond the metabolic capacity limit of one single strain (**Fig. 1a**). Since the metabolic stress on each strain is reduced, cell fitness is improved correspondingly, which consequently benefits the biosynthesis performance. This effect is even more pronounced when the target biosynthetic pathway is particularly long or certain pathway genes' expression imposes outstanding metabolic stress on the host.

Second, the employment of multiple strains provides diversified cellular environments for functional expression of different pathway genes. A biosynthetic pathway may contain a variety of enzymes whose biochemical properties and requirements for effective expression vary to a large extent. One single strain can only provide a uniform cellular environment that may not be suitable for the expression of all pathway genes. In comparison, the utilization of multiple strains

offers the needed diversity of cellular environments for meeting the need of different pathway enzymes (**Fig. 1a**). For example, when a biosynthetic pathway is composed of prokaryotic and eukaryotic enzymes, a combination of bacterial and yeast hosts would be highly advantageous over using either host alone for achieving the biosynthesis. In fact, multi-species co-cultures have been extensively adapted to harness the joint biosynthetic capabilities of all involved species, exemplified by taxol precursor biosynthesis by *E. coli-S. cerevisiae* co-cultures (Zhou et al., 2015).

Third, modular co-culture engineering reduces the undesired interference of different pathway modules. In fact, certain byproducts of one pathway module, e.g. reactive oxygen species, can be detrimental to the activity of other pathway modules (Artsatbanov et al., 2012). Spatial segregation of these modules in separate strains can greatly reduce, if not eliminate, the adverse influence between them (**Fig. 1a**), as demonstrated by a representative study for fuel desulfurization (Martinez et al., 2016). However, it should be noted that the co-culture approach cannot address the issue when the different pathway modules interact with each other through molecules that can travel freely across the cell membrane. Nonetheless, modular compartmentalization offers a new effective way to limit negative cross-talk between pathway modules and thus to improve the biosynthesis performance. The three advantages summarized above are illustrated in **Fig. 1a**.

Fourth, modular co-culture engineering offers the flexibility to balance the biosynthetic strength between pathway modules. For mono-culture engineering, the relative expression level of different pathway modules is generally optimized by varying the promoter strength, gene copy number, ribosomal binding site, etc. (Jones et al., 2015; Zhao et al., 2015). On top of these techniques, modular co-culture engineering provides another straightforward means to

111 manipulate the relative strength of the pathway modules by changing the strain-to-strain ratio
112 (Jones, J. A., 2016) (**Fig. 1b**). For example, insufficient supply or excessive accumulation of a
113 pathway intermediate can be readily addressed by increasing the relative population size of the
114 upstream or downstream strain, respectively. The tuning of a strain subpopulation can be
115 achieved by changing the inoculation ratio, e.g. flavonoid production (Jones et al., 2016), or by
116 addition of the needed strains in the middle of the cultivation process, e.g. n-butanol production
117 (Saini et al., 2015). Detailed discussion for the representative studies is provided in the following
118 section.

119 Fifth, modular co-culture engineering is capable of high-efficiency utilization of complex
120 materials containing multiple active substrates, as shown in **Fig. 1b**. In fact, consumption of
121 complex biosynthetic substrates can be achieved by engineering all of the involved substrate
122 uptake pathways in one single strain or co-cultivation of microbial strains with different substrate
123 preferences (traditional co-culture engineering). In contrast, modular co-culture engineering
124 offers a unique benefit of enabling smart allocation of appropriate substrates for supporting
125 different biosynthetic pathway modules. For example, one specialized co-culture member can be
126 responsible for using glycolysis to make a key pathway intermediate from glucose, while the
127 other co-culture member can be dedicated to converting xylose to other needed intermediates via
128 the engineered pentose phosphate pathway. Such an advantage allows for proper and rational
129 utilization of different substrates based on the need of the target biosynthetic pathway. In
130 addition, the modular nature of modular co-culture engineering also enables flexible utilization
131 of various substrates at different combinations (i.e. strains individually engineered for uptake of
132 different substrates can be co-cultured together at desired combinations to consume substrates
133 with varying compositions).

Last, but not least, modular co-culture engineering supports plug-and-play biosynthesis of various target products. The co-culture members are engineered to specifically satisfy the need of accommodated pathway modules only, rather than the entire pathway. As such, the co-cultures can be easily programmed for new target biosynthetic pathways by re-organization or addition of the involved pathway modules/strains that have been pre-optimized for a specific part of the biosynthesis. As shown in **Fig. 1b**, a variety of products can be produced from the same upstream module by simply swapping the downstream modules. This intrinsic advantage of implementing modular design is well in line with the concept of modularity in synthetic biology and holds the potential of extensive applications in metabolic engineering.

3. Recent progress in modular co-culture engineering

Encouraged by the benefits and engineering opportunities associated with modular co-culture engineering, there have been extensive research interest and efforts in implementation of this approach. **Table 1** summarizes recent progress in utilizing modular co-culture engineering for production of various products. In particular, microbial co-cultures comprising metabolically engineered strains of the same and different species are discussed, respectively. In spite of many technical barriers, these pioneering studies explored the potential of modular co-culture engineering and paved the way for its further application in the broad field of metabolic engineering.

3.1 Co-cultures comprising strains of the same species

Engineering two microbial strains of the same species to construct a robust co-culture system has been utilized for bioproduction of a wealth of value-added chemicals. Not

surprisingly, *E. coli* is the most popular species used in this type of co-culture, largely due to the readily available metabolic and process engineering tools for effective manipulation of the involved strains. For example, Shin et al developed an *E. coli* binary culture for direct conversion of hemicellulose to ethanol (Shin et al., 2010). Specifically, one *E. coli* strain was engineered for hydrolysis of hemicellulose to xylooligosacharides, which was further metabolized by the other *E. coli* strain overexpressing xylooligosacharide-utilizing enzymes. This co-culture system distributed the metabolic burden between two strains that were dedicated for extracellular and intracellular enzyme expression, respectively. Importantly, a transporter of the intermediate xyloside was functionally expressed to ensure the desired assimilation of xyloside into the downstream strain. The combined efforts in this study resulted in a significant ethanol production improvement over the mono-culture approach.

E. coli-*E. coli* co-cultures have also been utilized to improve the production titer and yield of muconic acid (Zhang et al., 2015b). In particular, two *E. coli* strains were constructed individually to accommodate different pathway modules, which helped reduce the overwhelming metabolic stress on each strain. This co-culture strategy also enabled effective regulation of the endogenous upstream pathway and expression of the challenging heterologous enzymes in two distinct cellular metabolic backgrounds, respectively. Moreover, the use of two separate strains allowed for simultaneous uptake of two major lignocellulosic sugars, glucose and xylose, with significantly higher efficiency than the mono-culture approach. For rational use of sugar mixtures, xylose was mainly devoted to the pentose phosphate pathway to produce a critical pathway intermediate in the upstream strain, whereas glucose was used for the expression of the downstream heterologous enzymes for converting the intermediate. A membrane-bound transporter was also engineered to enhance the mass transfer of the pathway intermediate

between the upstream and downstream strains. The co-culture showed improved bioproduction behavior in a fed-batch bioreactor, showing the potential of modular co-culture engineering in large scale production. Moreover, the modularization of the pathway allowed for straightforward swapping of the involved pathway module and achieved biosynthesis of 4-hydroxybenzoic acid in a plug-and-play fashion.

A more recent study showed that engineering an *E. coli-E. coli* co-culture is a superior strategy for conversion of a single carbon source, glycerol, to muconic acid (Zhang et al., 2015a). The findings of this study indicate that lessening metabolic stress by using multiple strains to accommodate an entire biosynthetic pathway plays a key role in improving bioproduction performance. In the meantime, it was shown that the subpopulation ratio between the constituent strains fluctuated to a large extent, largely due to the growth competition for the same carbon source. The difficulty in controlling microbial co-culture composition presented a challenge for further production improvement.

3-amino-benzoic acid heterologous biosynthesis has also been achieved through co-cultivation of two engineered *E. coli* strains (Zhang et al., 2016). In this report, a fixed *E. coli* strain was used to contain the upstream pathway module, whereas 10 *E. coli* strains with distinct genotypes were evaluated for accommodation of the downstream pathway module. The employment of modular co-culture engineering allowed for rapid screening of the downstream strains to identify the best candidate host for supporting the involved pathway module. Such an effort resulted in a 15-fold production improvement over the control mono-culture, highlighting the flexibility and power of modular co-culture engineering for selecting the right microbial strain for biosynthesis optimization.

Saini et al. constructed an *E. coli-E. coli* co-culture system for bioproduction of n-butanol (Saini et al., 2015). In this study, the complete n-butanol biosynthetic pathway was divided into butyrate-producing and butyrate-conversion modules, each of which was incorporated into an engineered *E. coli* strain. This system took advantage of the modular co-culture engineering approach to alleviate metabolic stress and maintain desired pathway redox balance. Notably, acetate, a pathway byproduct that travels freely across the cell membrane, was recycled between the upstream and downstream *E. coli* strains to facilitate butyrate and butyryl-CoA interconversion. Optimization of the inoculation ratio and adapting periodic addition of the downstream strain were found to balance the upstream and downstream biosynthetic strength, and led to the production of 5.5 g/L n-butanol, 2-fold higher than achieved by the reference mono-culture. In another study, the same group successfully expanded the application of the *E. coli-E. coli* co-culture system for production of n-butanol by using a more challenging substrate, renewable cellulose hydrolysate (Saini et al., 2016).

Willrodt et al. adapted a mixed culture of the resting cells of two *E. coli* strains for biosynthesis of perillyl acetate (Willrodt et al., 2016). It was first found that coupling limonene synthesis and oxyfunctionalization in one single *E. coli* strain was highly ineffective and resulted sub-optimal production of perillyl acetate. In comparison, recruiting two separate strains for limonene formation and hydroxylation significantly improve the perillyl acetate concentration and specific yield. This was largely due to flexibility to match the production rate of different biosynthetic modules by changing the mixing ratio of accommodating cells and the possibility to select optimal conditions for expression of individual enzymes.

Modular co-culture engineering is also a robust approach for producing chemicals with complex structure. Jones et al. reported co-cultivation of two metabolically engineered *E. coli*

strains for biosynthesis of value-added flavonoids (Jones et al., 2016). Specifically, the complex target biosynthetic pathway leading from phenylpropanoic acids to flavan-3-ols was divided into the malonyl-CoA-dependent upstream module (phenylpropanoic acids to flavanones) and the NADPH-dependent downstream module (flavanones to flavan-3-ols). This modular design not only allowed for division of the overwhelming metabolic burden, but enabled the individual optimization of the pathway intermediate supply and the pathway co-factor provision in separate strains. Moreover, the co-culture setup facilitated straightforward screening of four *E. coli* strains for their cross compatibility, and the best combination was determined for optimal production performance. The effects of induction point, inoculation ratio, carbon source, and induction temperature were systematically investigated, upon which an empirical scaled-Gaussian model was developed to describe the co-culture production behaviors. Further production optimization based on the model prediction was performed and resulted in an impressive 970-fold flavonoids production improvement over the mono-culture approach.

It is noteworthy that *E. coli-E. coli* co-cultures containing parallel pathways have also been developed for microbial biosynthesis. For example, Eiteman et al. engineered two strains of *E. coli* and cultured them together for parallel conversion of xylose and glucose to lactic acid (Eiteman et al., 2009). Bokinsky et al. constructed co-cultures comprising highly engineered *E. coli* strains expressing enzymes for cellulosic substrate utilization and biofuel biosynthesis, and simultaneously converted different substrates of pretreated plant biomass to desired biofuels (Bokinsky et al., 2011). Although these studies didn't deploy serial pathway modularization for biosynthesis as highlighted in this review, their achievements offer important insight and reference for modular engineering using same-species co-cultures.

In addition to *E. coli-E. coli* co-cultures, engineered co-cultures of *P. putida* strains have also been adapted to achieving metabolic engineering goals. In fact, the advantage of spatial segregation of modular co-culture engineering was well exemplified by a recent report on petroleum desulfurization *P. putida-P. putida* co-cultures (Martinez et al., 2016). For the desulfurization pathway, it has been found that a key pathway intermediate 2-(2-hydroxybiphenyl) sulfonate (HBPS) was an inhibitor of upstream pathway enzymes, resulting in poor biodesulfurization performance. To address this challenge, Martinez et al. designed and engineered a consortium comprising the resting cells of two engineered *P. putida* strains harboring the biosynthetic modules upstream and downstream to HBPS, respectively. By this rational design, the inhibitory intermediate HBPS was separated from the upstream enzymes contained by the corresponding strain. Through optimization of the mixing ration of the constituent cells, the conversion of substrate dibenzothiophene *(*DBT) to the desired 2-hydroxybiphenyl (2HBP) product was improved significantly, compared with the mono-culture strategy. The modular design also enabled the straightforward re-programming of the co-culture system to achieve high-efficiency biosynthesis of intermediate 2-(2-hydroxybiphenyl) sulfonate (HBPS) from DBT.

3.2 Co-cultures comprising strains of different species

Employment of two different microbial species takes advantage of biosynthetic or bioconversion capabilities of both species, and has been extensively studied in the past. In fact, a wide range of value-added chemicals, including hydrogen, methane, acetic acid, lactic acid, polyhydroxyalkanoates, acidic polysaccharide, carotenoid, have been successfully produced by engineering artificial cultures containing different microbes (Bader et al., 2010; Jagmann and Philipp, 2014; Taniguchi et al., 1998). These studies split a target bioconversion pathway into

independent modules, and relied on the natural competence of the selected microbial species to conduct the bioconversion for each corresponding module. However, most such efforts implemented pathway modularization based on the intrinsic biosynthetic capability of the involved microbial species, and mainly used process engineering approaches to manipulate the microbial co-cultures, as metabolically engineering the involved microbes is highly challenging.

Recent studies on modular co-culture engineering focused more on designed modification of the microbial metabolism to not only enhance the activity of corresponding pathway modules, but also manipulate the co-existence between the co-culture members. Zhou et al. engineered a co-culture composed of *S. cerevisiae* and *E. coli* for improving the production of oxygenated isoprenoids (Zhou et al., 2015). The constructed co-culture combined the advantages of the metabolically engineered bacterial MEP pathway for the key pathway intermediate provision as well as the yeast's robust machinery for functional expression of required eukaryotic pathway genes. Notably, reactive oxygen species produced by one pathway module could inhibit the activity of the other module. As such, the spatial segregation of these pathway modules in separate strains was adapted to prevent the undesired interference and helped improve the production behavior. In addition, a mutualistic interaction between the co-culture members was established to overcome the challenge of imbalanced growth of the two different microbial species. Systematic engineering of the co-culture system finally led to production of 33 mg/L oxygenated taxanes, in contrast to no detectable biosynthesis by the mono-culture of either *E. coli* or *S. cerevisiae* harboring the same pathway. In the same study, the bacterium-yeast co-cultures were also successfully engineered for producing tanshinone precursors and functionalized sesquiterpenes.

Minami et al. reported the biosynthesis of plant benzylisoquinoline alkaloids using combination cultures of *E. coli* and *S. cerevisiae* (Minami et al., 2008). In this study, a recombinant *E. coli* was constructed to express required enzymes to convert the substrate dopamine to the key intermediate reticuline, whereas the heterologous pathway enzymes were functionally expressed in *S. cerevisiae* for making target alkaloids from reticuline. When the engineered microbes were combined together in a co-culture, 7.2 mg/L of magnoflorine product was produced within 72 h. Furthermore, the modularization of the pathway allowed for flexible re-programming of the co-culture system for production of a different product scoulerine by engineering the corresponding enzymes in the downstream *S. cerevisiae* strain.

Bayer et al. integrated of a methyl halide biosynthetic pathway into an *A. fermentans* and *S. cerevisiae* co-culture (Bayer et al., 2009). 89 methyl halide transferases were first screened, and the best candidate was identified and functionally expressed in engineered *S. cerevisiae* to produce methyl iodide. The methyl-halide-producing *S. cerevisiae* was then co-cultured with the ethanol/acetate-producing bacterium *A. fermentans* to accommodate the biosynthetic pathway leading from biomass to final methyl halide products. Importantly, the co-culture was designed to be symbiotic for improved stability. Specifically, *S. cerevisiae* relied on *A. fermentans* for providing ethanol and acetate for carbon and energy use through hydrolysis of raw biomass materials, whereas *A. fermentans* was dependent on *S. cerevisiae* for consumption of toxic intermediates. The constructed co-culture was shown capable of achieving methyl halide production from various biomass materials.

Koizumi et al. engineered a three-component co-culture system for biosynthesis of 5'-diphospho-galactose (UDP-Gal) and globotriose (Koizumi et al., 1998). Specifically, metabolically engineered *C. ammoniagenes* was utilized to produce uridine triphosphate (UTP)

from precursor orotic acid. In the meantime, an *E. coli* strain was genetically modified to overexpress the needed enzymes to biosynthesize UDP-Gal from UTP and exogenous galactose. Importantly, when another *E. coli* strain expressing a heterologous globotriose biosynthetic enzyme was added as the third co-culture member, the co-culture system was able to produce globotriose using orotic acid, galactose, and lactose as the starting substances. As each engineered strain was responsible for one specific module, this co-culture system was equipped with enlarged metabolic capacity for reconstitution of a complex pathway. The engineered co-culture system was successfully cultivated in a bioreactor to achieve large scale production of globotriose.

In a recent study, an engineered *G. oxydans* and *K. vulgare* co-culture was developed for one-step 2-keto-l-gulonic acid (2-KGA) production from D-sorbitol (Wang et al., 2016). Specifically, two sequential pathway modules are separately incorporated into *G. oxydans* and *K. vulgare* to achieve D-sorbitol-to-sorbose and sorbose-to-2KGA conversion, respectively. *G. oxydans* was also metabolically engineered to reduce its competition against *K. vulgare* for sorbose. Co-cultivation of these two microbes with high bioconversion capabilities generated a simplified one-step bioproduction process whose performance was comparable to the traditional two-step process. Importantly, the metabolomics analysis was performed to gain insight for dynamic metabolic interaction of the constituent strains in the constructed co-culture system.

Interestingly, modular co-culture engineering overlaps with current efforts in consolidated bioprocess in lignocellulosic biomass utilization. For example, bioethanol production from cellulose by a co-culture of *C. phytofermentans* and *S. cerevisiae* has been reported (Zuroff et al., 2013). The complete pathway was divided into cellulose hydrolysis and ethanol production modules, which were integrated into *C. phytofermentans* and *S. cerevisiae*, respectively. The

connection of the separated pathway modules were facilitated by expression of intermediate cellodextrin transporters in the downstream *S. cerevisiae* strain. Moreover, β-glucosidase was engineered in *S. cerevisiae* for intracellular cellodextrin conversion. The engineered co-culture achieved 22 g/L ethanol from 100 g/L α-cellulose. It should be noted that, different from this study, the majority of current consolidated bioprocess studies for bioethanol production do not implement serial pathway modularization but rather use parallel pathways/strains to construct microbial co-cultures for high-efficiency utilization of complex substrates (Chen, 2011).

Co-cultures comprising two different microbial species were also used for organic compound degradation. Gilbert et al. constructed a consortium containing metabolically modified *E. coli* and *P. putida* for biodegradation of organophosphorus insecticide parathion (Gilbert et al., 2003). Break-down of starting molecule parathion into intermediate *p*-nitrophenol and further mineralization of this undesired intermediate were conducted by the two separate strains expressing corresponding pathway enzymes, respectively. Consolidating of engineered *E. coli* and *P. putida* in one culture enabled effective hydrolysis of 500 μM parathion and removal of the intermediate *p*-nitrophenol within 52 h. Based on a similar approach, *E. coli*-*Ochrobactrum* co-cultures expressing a modularized bioconversion pathway were also constructed to achieve high efficiency removal of methyl parathion (Li et al., 2008; Zhang et al., 2008). It is noteworthy that microbial communities have been widely used in waste water treatment and organic compound degradation in the past several decades (Narihiro et al., 2007; Nielsen et al., 2012). Most of the microbial communities involved a large number of species that are evolved to live together as a whole and hard to culture otherwise. Compared with engineered microbial co-cultures with rational design, the metabolic pathways or network inside these microbial communities are also much more complex, and community structures are more

difficult to understand and engineer. As such, manipulation of such communities often relies on process engineering, rather than metabolic engineering approaches, making a recognizable difference with modular co-culture engineering discussed in this review. Similarly, although methane production by microbial communities has been extensively studied, implementation of modular co-culture engineering in this context is critically challenging, as functional interactions of methogenic populations still need to be better understood and limited tools are available for metabolically engineering involved community members to achieve effective pathway modularization and optimization (Chaudhary et al., 2013; Ferry, 2011).

In spite of significant advances for modular co-culture engineering, there is a pressing need for development of theoretical models to guide rational design, operation and control of modularized co-cultures in the future. Some progress has been made in this direction. Minty et al. developed a synthetic microbial consortium of *Trichoderma reesei* and *E. coli* for lignocellulosic biomass conversion to isobutanol (Minty et al., 2013). Specifically, *T. reesei* produced cellulases for extracellularly hydrolyzing cellulose to soluble saccharides, which were then used as substrates for both microbes' growth and isobutanol production by *E. coli*. Importantly, an ordinary differential equation model consisting of 50 parameters was developed to describe and predict the behavior of the co-culture. The sophisticated model allowed for the identification of key parameters for production improvement as well as in-depth analysis of co-culture stability, marking a significant progress in development of applicable modelling for modular co-culture engineering.

In summary, previous efforts in modular co-culture engineering have generated an array of biochemicals with improved production performance. These products can be categorized into different groups, including alcohols, organic acids, aromatics, flavonoids, alkaloids, isoprenoids,

polysaccharides etc. Notably, many other families of biochemicals hold potential of high-efficiency production by modular co-culture engineering. For example, complex natural products involving long biosynthetic pathways and amino acids with regulated pathways are considered promising candidates for application of modular co-culture engineering to overcome the barriers associated mono-culture engineering.

4. Challenges and potential solutions

Although there exists appealing benefits and opportunities for utilization of modular co-culture engineering, advances and development of this emerging approach need to address several critical challenges. One primary challenge is how to maintain the co-existence of the constituent strains in the co-culture systems. Unlike the natural consortia which exist and operate for the purpose of survival of all involved members, the artificial co-cultures are operated in order to optimize the production of specific products. As such, the growth of the involved co-culture members may not necessarily be compatible, often resulting in competition between the co-culture members for growth resources. In addition, the growth rates of microbial strains, especially for those of different species, vary to a large extent. As a result, co-cultivation of these species under a uniform growth condition can easily lead to the outgrowth of one species over the others.

Optimization of the growth conditions, such as pH, temperature and dissolved oxygen level, can only partially overcome the difficulty for growth coordination. To this end, employment of microbial strains derived from the same species can be an applicable strategy, as the involved strains require similar growth conditions and possess similar intrinsic growth rates. However, the general applicability of such same-species co-cultures is limited, as many biosynthesis processes require mixed biosynthesis capabilities from two different microbial species. An alternative

strategy is engineering the co-culture members for growth on separate carbon source to reduce the growth competition and thus improve the growth compatibility. On the other hand, different designed interaction modes between co-culture members, including mutualism, commensalism, parasitism, have been extensively studied, offering important insight for modular co-culture engineering design (Jagmann and Philipp, 2014; Johns et al., 2016; Song et al., 2014).

Beyond co-existence, stabilization of sub-population ratio between the co-culture members constitutes another major challenge. In fact, the interaction between the co-culture members is highly dynamic and balanced growth is remarkably hard to maintain. As a result, the co-culture population composition can fluctuate to a large extent due to subtle growth environmental perturbations, greatly reducing the reproducibility of modular co-culture engineering studies. On the other hand, from the perspective of production optimization, stabilizing the strain-to-strain ratio at desired values is the key to successful balancing between different biosynthetic modules throughout the co-cultivation process. In this regard, recent research has attempted to vary the inoculation ratio between the co-culture members as a major means of regulation, although the involved strains' relative sub-population sizes were often found to fluctuate or change unfavorably soon after inoculation. Besides, mutualistic growth has also been studied for maintaining desired population composition of the engineered co-cultures (Bayer et al., 2009; Kerner et al., 2012; Mee et al., 2014; Shou et al., 2007; Zhou et al., 2015). Moreover, synthetic biology tools, such as quorum sensing, are being developed for manipulating cell-cell communication through signaling mechanisms, which holds strong application potential for growth and metabolic pathway coordination between the co-culture members in the future (Bacchus and Fussenegger, 2013; Marchand and Collins, 2013; Pai et al., 2009).

Efforts have also been made to develop mathematical models to instruct the manipulation of the co-culture population composition. For example, aided by a computational model, Kerner et al. established a symbiotic circuit to regulate the growth rate and composition of a synthetic consortium consisting of two *E.coli* auxotrophs. The model was further utilized to generate design space for programming the constructed co-culture and in certain cases helped achieve desired growth rates or strain ratios (Kerner et al., 2012). Chiu et al. constructed a comprehensive computational framework to investigate the metabolic capacity and dynamics of a two-species co-culture system, and identified important principles for instructing the co-culture design (Chiu et al., 2014). Chen et al. established a complex mathematical model for construction of a synthetic consortium capable of regulating gene expression through signaling mechanism. Importantly, genetic oscillations were engineered to occur between the two constituent *E. coli* strains, which resulted in successful co-culture population manipulation with designed patterns (Chen et al., 2015). In spite of above experimental and theoretical research progress, modular co-culture engineering still lacks reliable and well established techniques for manipulating the co-culture population composition within a desired operation window. The difficulty of overcoming this problem increases exponentially as more constituent strains are involved in the co-culture system.

Another critical issue for modular co-culture engineering is the mass transfer of the key pathway intermediates. In fact, the proceeding of a biosynthetic process in a modularized co-culture is dependent on relocation of the pathway intermediate produced from the upstream strain to the downstream strain where it can be further converted the product. However, a wide range of biosynthetic intermediates, such as various CoA species and phosphorylated metabolites, have limited mobility for exportation and importation across the cell membrane. As a result, they

451 can hardly be used as linking molecules for different pathway modules contained by separate
452 cells. Therefore, the division of a complete biosynthetic pathway should be carefully designed to
453 ensure the intermediates connecting different modules can travel between co-culture members
454 for the completion of the entire pathway. In fact, a number of families of compounds, such as
455 organic acids, alcohols, simple sugars, amino acids, certain flavonoids and alkaloids, can be
456 transported by microbes relatively easily and thus serve as potential candidates for modular co-
457 culture engineering intermediates. In addition, appropriate membrane-bound metabolite
458 transporters should be engineered to enhance the secretion and/or assimilation of the pathway
459 intermediates in the favored direction (Lee et al., 2012; Zhou et al., 2012). To this end, the
460 exemplified studies in the previous section demonstrate the applicable strategies to tackle the
461 mass transfer issue for modular co-culture engineering.

462 **5. Conclusion**

463 Modular co-culture engineering is an emerging approach for microbial biosynthesis. It
464 harnesses the metabolic power and resources of multiple microbial strains to meet the
465 requirement of a target biosynthetic pathway. It offers a viable option to share the undesired
466 metabolic burden between different microbial strains and go beyond the limit of the metabolic
467 capacity of a single microbial strain. In addition, it presents a new perspective to address the
468 issues of functional expression of various proteins, efficient utilization of complex substrates
469 with varied compositions, rapid reconstitution of new biosynthesis pathways, and other
470 unprecedented challenges. As such, modular co-culture engineering holds the prospect for wide
471 application in the broad field of metabolic engineering.

472 As an emerging research area, modular co-culture engineering is still under development.
473 Many of the above benefits and advantages can in theory be amplified when more microbial

strains are used in the co-culture system. However, the difficulty of maintaining a stable and reliable co-culture system increases with the number of the constituent strains. In fact, most of the recent reports in modular co-culture engineering focus on employment of only two constituent strains in order to achieve their engineering goals. However, as modular co-culture engineering progresses, it is anticipated that co-cultures consisting of more specialized members, or polycultures, will be developed and utilized for meeting the demand of more complicated biosynthesis or bioconversion tasks in the future. To this end, substantial efforts in delicate co-culture design, construction, control and modelling, are required for advancing modular co-culture engineering. Furthermore, effective regulation of dynamic interaction between the co-culture members in artificial microbial co-cultures will benefit from mimicking natural consortia as well as the development of synthetic biology and metabolic engineering tools.

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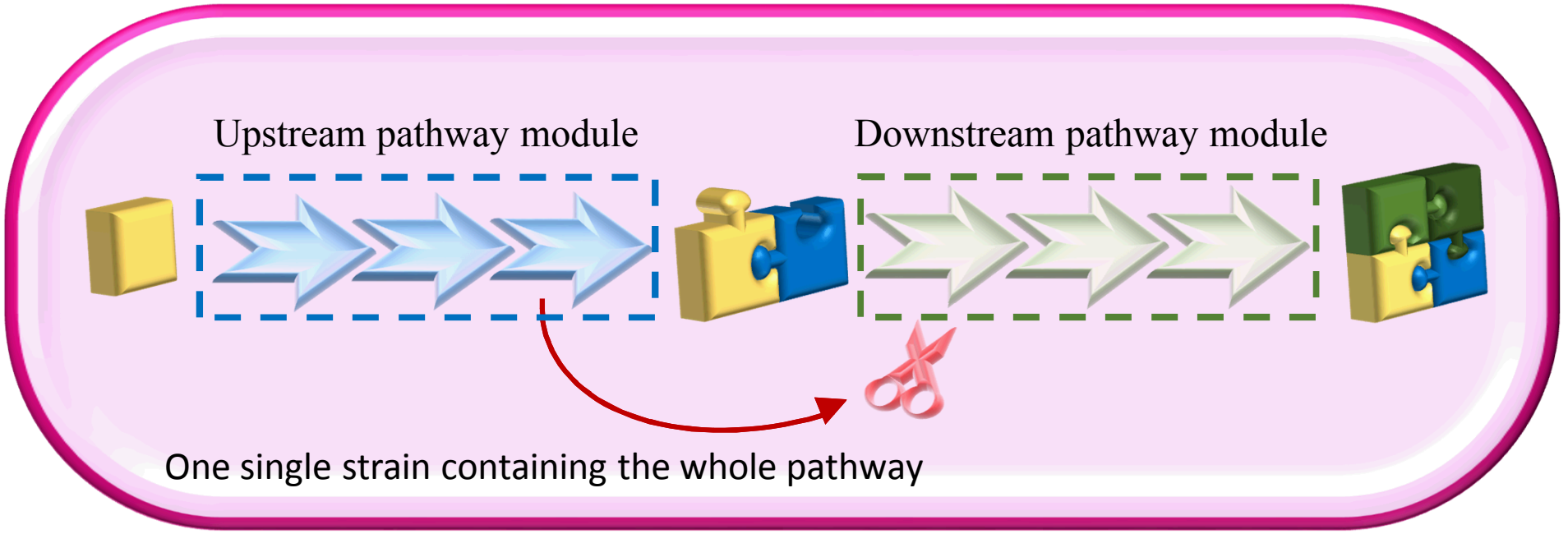
Figure 1. Schematic illustration of the design and benefits of modular co-culture engineering. (a) A biosynthetic pathway established in a single microbial strain (mono-culture) is divided into the upstream and downstream modules. Each module is accommodated in an independent host strain which undertakes less biosynthesis labor and reduced metabolic stress (fewer arrows in each strain). Individual strains offer better cellular environments to match the requirements of the specific pathway modules (matching colors between the pathway modules and the background accommodating strains). Spatial segregation prevents the undesired interference between the upstream module and the downstream module (represented by the red scissors). (b) The high biosynthetic strength of the upstream module (large arrows) can be matched by recruiting more downstream cells with low biosynthetic strength (small arrows). Different substrates of a complex raw material can be utilized separately by the upstream and downstream strains for

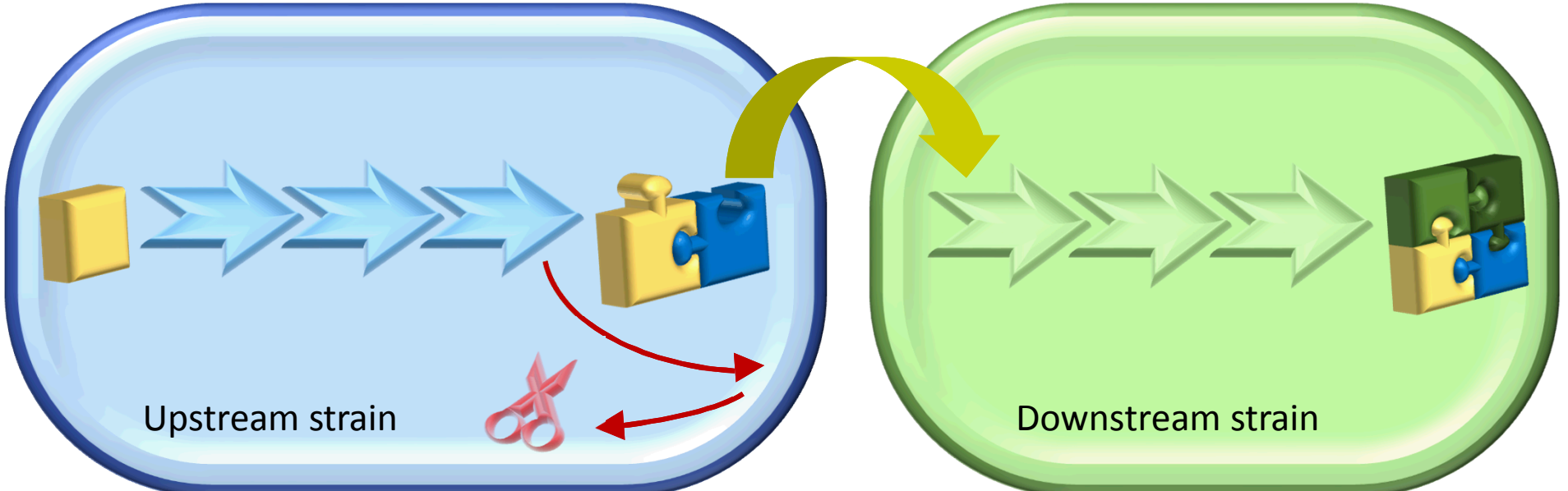
629 improved bioconversion efficiency. The swapping of the downstream module allows for
630 production of a variety of new products in a plug-and-play manner.

631

632

(a)


Mono-culture
Upstream pathway module
Downstream pathway module
One single strain containing the whole pathway


Co-culture
Upstream strain
Downstream strain

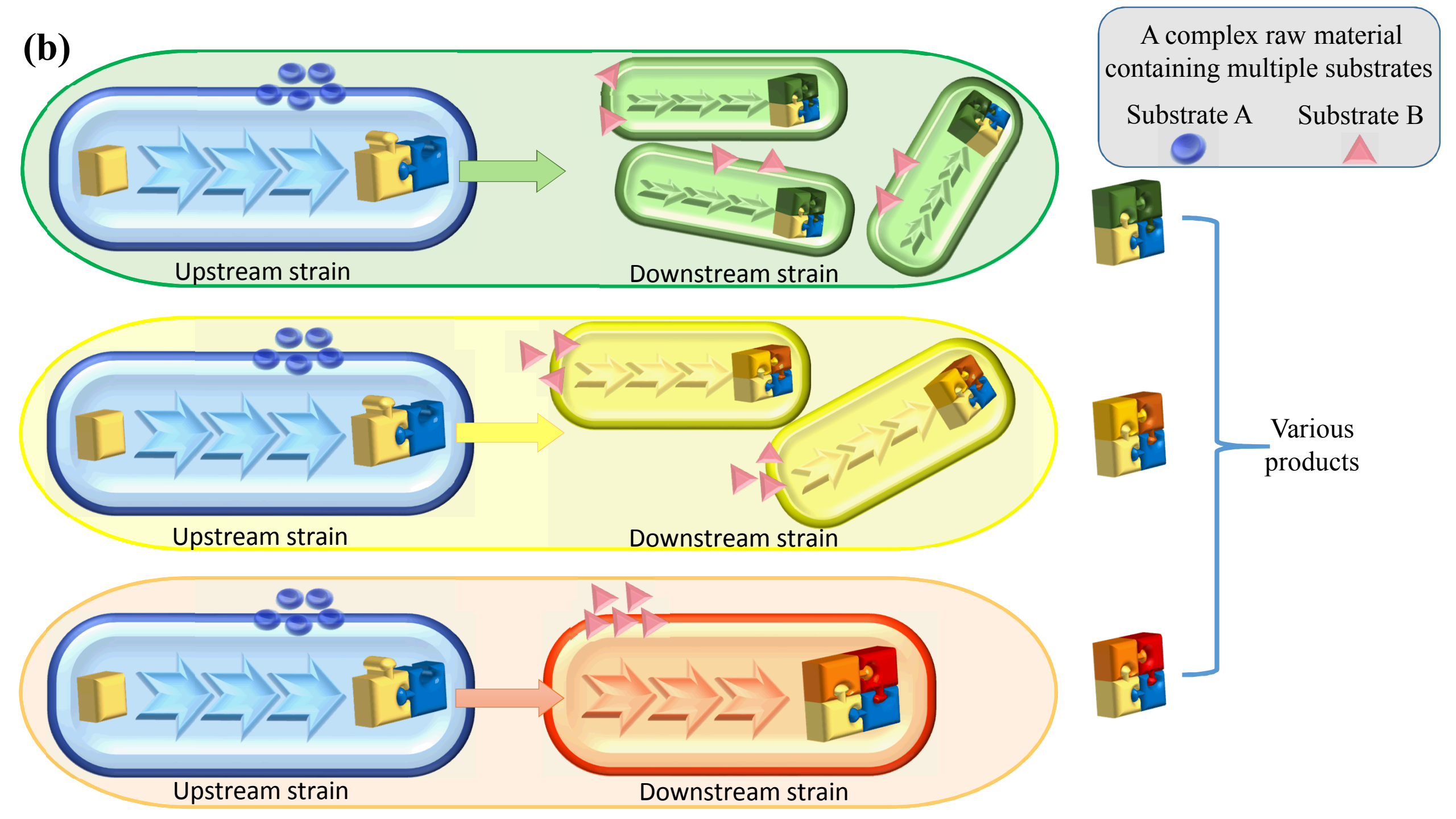

(b)
A complex raw material containing multiple substrates
Substrate A
Substrate B
Upstream strain
Downstream strain
Upstream strain
Downstream strain
Upstream strain
Downstream strain
Various products

Table 1 Summary of recent progress in modular co-culture engineering

Product/ Process	Co-culture composition	Achievement	Reference
Ethanol	*E. coli-E. coli*	1.45-fold higher yield than mono-culture	(Shin et al., 2010)
Muconic acid	*E. coli-E. coli*	19-fold titer improvement on glucose/xylose mixture 14-fold titer improvement on glycerol	(Zhang et al., 2015b) (Zhang et al., 2015a)
4-hydroxy benzoic acid	*E .coli-E. coli*	8.6-fold titer improvement	(Zhang et al., 2015b)
3-amino benzoic acid	*E. coli-E. coli*	15-fold titer improvement	(Zhang et al., 2016)
n-Butanol	*E. coli-E. coli*	2-fold titer improvement on glucose 75% titer improvement on cellulose hydrolysate	(Saini et al., 2015) (Saini et al., 2016)
Perillyl acetate	*E. coli-E. coli*	12-fold higher production of perillyl acetate	(Willrodt et al., 2015)
Flavonoid	*E. coli-E. coli*	970-fold titer improvement over mono-culture	(Jones et al., 2016)
Dibenzothiophene desulfurization	*P. putida-P. putida*	~50% dibenzothiophene degradation	(Martinez et al., 2016)
Oxygenated isoprenoids	*E. coli-S. cerevisiae*	33 mg/L vs non-detected by mono-culture	(Zhou et al., 2015)
Benzylisoquinoline alkaloids	*E. coli-S. cerevisiae*	7.2 mg/L magnoflorine and 8.3 mg/L Scoulerine	(Minami et al., 2008)
Methyl iodide	*A. fermentans-S. cerevisiae*	Yield >10 mg/(L•h) on various biomass	(Bayer et al., 2009)
Globotriose	*C. ammoniagenes-E. coli-E. coli*	188 g/L globotriose in 36 h	(Koizumi et al., 1998)
2-keto-l-gulonic	*G. oxydans -K.*	Concentration of 2-KGA: 76.6 g/L	(Wang et al., 2016)

acid	*vulgare*	Yield of 2-KGA: 89.7 % of the theoretical yield	
Ethanol	*C. phytofermentans* - *S. cerevisiae*	2.4-fold titer improvement	(Zuroff et al., 2013)
Parathion degradation	*E. coli-P. putida*	Full degradation of 500 µM parathion and the intermediate	(Gilbert et al., 2003)
Isobutanol	*T. reesei-E. coli*	1.88 g/L; 62% of the theoretical yield	(Minty et al., 2013)