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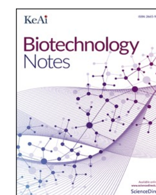

Synthetic microbes and biocatalyst designs in Thailand

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ABSTRACT

Furthering the development of the field of synthetic biology in Thailand is included in the Thai government's Bio-Circular-Green (BCG) economic policy. The BCG model has increased collaborations between government, academia and private sectors with the specific aim of increasing the value of bioindustries *via* sustainable approaches. This article provides a critical review of current academic research related to synthetic biology conducted in Thailand during the last decade including genetic manipulation, metabolic engineering, cofactor enhancement to produce valuable chemicals, and analysis of synthetic cells using systems biology. Work was grouped according to a Design-Build-Test-Learn cycle. Technical areas directly supporting development of synthetic biology for BCG in the future such as enzyme catalysis, enzyme engineering and systems biology related to culture conditions are also discussed. Key activities towards development of synthetic biology in Thailand are also discussed.

1. Introduction

Thailand's economy depends largely on agricultural activities which produce diverse crops. The country is a major producer of staple crops such as rice, cassava, rubber, sugarcane and oil palm as well as various fruits and orchids. Although the majority of the agricultural sector has been operated under small and family-owned operations in the past, there has been a gradual shift towards larger and corporate-own farms. Operation of contract farming where certain crops are grown with recommended sets of fertilizers and pesticides has been widely practiced throughout the country. While this allows boosting of crop productivity per land area,[1] the industry faces problems of pesticide overusage and dependency on imported chemical fertilizers. Pesticide usage causes contamination of pesticide toxins in food products and the environment. Imported chemical pesticides also cause economic burdens for poor farmers and decrease the richness of soil microbes.[2–4]

Although Thailand has more than 55% of its area covered by agricultural land,[5] the income earned from the agricultural sector is only 8.7% of the total gross domestic product (GDP).[6] This is mainly because the country produces mostly low value products such as grain, starch, sugar and vegetable oil. As the land area for farming cannot be expanded beyond the current state due to urban utilization, the overall agricultural productivity in Thailand has reached its limit. The only way to increase the earning value from this sector is to create new modes of producing high value products rather than relying on production of commodities. Therefore, Thailand urgently needs to support a new industrial agriculture model to promote sustainability and the country's competitiveness. This would increase the overall national income and improve the quality of people's lives while ensuring sustainable usage of natural resources and reducing adverse environmental effects.

To drive the country towards a knowledge-based and value-added economy, the Thai government has initiated the Bio-Circular-Green (BCG) policy as a national agenda in 2021 to support the country's sustainable growth and competitiveness and drive towards Sustainable Development Goals (SDGs). The BCG model leverages the large amount of biomass produced from bioindustry and relies upon the country's high biodiversity (ranked eighth in the world with the highest biodiversity index in Southeast Asia). The model supports a self-sufficient economy at the local and national levels by applying biotechnologies and innovations to four focused sectors: increasing the economic value of food and agricultural products; developing technologies to support biomedicine, pharmaceuticals and wellness; promoting production of bioenergy from biomass and biowaste, of biomaterials and biochemicals *via* biorefinery approaches; promoting tourism and a creative economy

Abbreviations: BCG, Bio-Circular-Green; GDP, Gross Domestic Product; SDGs, Sustainable Development Goals; ASEAN, Association of Southeast Asian Nations; STI, National Science Technology and Innovation.

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particularly in small cities. The overall goal of the policy is to ultimately increase the domestic GDP by 2025.[7] In addition, the government has implemented four driving force strategies to enable and support the BCG framework i.e. supporting research and development, establishing infrastructure and national science databanks, promoting the collaboration of public and private sectors, and implementing government regulatory/policy and incentives for investors (Fig. 1). These actions have resulted in drawing more investments to bioindustry – especially in the food and pharma sectors.[8] The Thai government has set the goal to draw new investments into the BCG model to reach 23 billion US dollars in 2026 with 30% of this investment coming from the private sector.[7] The country aspires to be among the leaders of the ten-member ASEAN (Association of South East Asian Nations) union with the BCG economy and seeks to be in the ASEAN Bio Hub.

Synthetic biology and biotechnology are thus important technologies to support the BCG model mentioned above. These technologies can lead to discovery and innovations which increase productivity and the value of the overall bioindustry in Thailand.[9] Synthetic biology is a multi-disciplinary field of science and engineering research that generally aims to design organisms or biomolecules to exhibit new functions or properties not naturally found. The discipline has a wide range of operation ranging from creation of artificial life, molecular circuits, new metabolic pathways, metabolically engineered microbes or new biocatalysts to support production of specific compounds for various novel detection technologies or therapeutic purposes.[10] Primary research related to metabolic engineering typically creates synthetic pathways or microbes to convert inexpensive feedstocks to valuable compounds such as precursors or monomers of bioplastics and biomaterials as well as specialty compounds such as food additives, fragrances or active pharmaceutical ingredients (API).[11] Therefore, synthetic biology is well aligned with the BCG model to support the Thai bioeconomy and enable the country's transformation towards sustainability. To boost research in synthetic biology in the country, around $26 million of public research funds have been allocated from the National Science Technology and Innovation (STI) Policy Office fund to support 261 BCG projects in 2021.[8] Endeavors to promote synthetic biology including establishment of the Thailand synthetic biology consortium and arrangement of public seminars and workshops supported by the government, national research centers, and academic and private sectors during 2020–2022. These workshops were aimed at creating greater

Fig. 1. Overview of the Thai Bio-Circular-Green (BCG) economy policy. The policy supports sustainable development goals.

awareness in the general public and in the industrial and academic communities of the impact of synthetic biology.

To this end, it seems that major industrial sectors in Thailand are aware of potential impact of synthetic biology on Thai bioindustry. However, comprehensive knowledge of current technologies developed in academia which forms an important foundation to support the competitiveness of technology readiness is lacking. Therefore, we conducted searches of scientific publications during the past 10 years (dating from 2012 to around middle of 2022) with keywords "synthetic biology", "biosynthesis" and "biocatalysis" published by authors with addresses in Thailand (Fig. 2A). As we did not find substantial developments in synthetic biology related to medical science in Thailand, we thus only focused on work which can promote bioindustry. Titles and abstracts of articles found were manually inspected to assure that their content is relevant to work related to the keywords used. To enlist only work in which the main activities were done in the country, we further selected work with corresponding authors with addresses in Thailand (Fig. 2B).

The review content covers research on utilization of genetic manipulation or metabolic engineering to produce valuable chemicals, systems biology approaches for analysis of synthetic cells, construction of cofactor enhancing systems to enhance biochemical production, and development of new tools and educational programs for promoting better understanding of synthetic biology. In addition, development of research in enzyme engineering, novel reaction cascades and systems biology approaches to increase the competitiveness of the synthetic biology research field are also included. The last section discusses current and future perspectives of synthetic biology in Thailand.

2. Overview of current synthetic biology status in Thailand

Synthetic biology is generally defined as work employing combined principles of the Design-Build-Test-Learn cycle to create biomolecular systems to accomplish specific tasks. Based on the literature search mentioned above, only a few projects in Thailand reported work resulting from a full cycle of Design-Build-Test-Learn methodology (Fig. 2A); we thus can conclude that the current work status of this field in Thailand is still in its infancy. However, we are aware of many ongoing projects conducted and expect to see significant research output in the near future i.e. 3-5 years. Although most of the published articles have not yet fully employed a full cycle of Design-Build-Test-Learn to accomplish their goals, various research groups also used metabolic engineering and genetic manipulation strategies to improve performance of bacteria, microalgae, fungus and yeast to produce desired compounds such as polyhydroxyalkanoates (PHAs), D-(−)-lactic acid, 2,3-butanediol, succinic acid, alkane, neurotransmitter, anti-inflammatory reagents and specialty chemicals. For testing and analysis, metabolomics and transcriptomics have been employed to analyze the systems constructed. We also observed interesting developments in tool and device designs for synthetic biology. In addition, when combined with work related to biosynthesis and biocatalysis, we observed considerable contributions in advancing biocatalyst design i.e. work in the fields of enzyme engineering and biocatalysis. Construction of robust and efficient biocatalysts are an important foundation for synthetic biology research because these engineered enzymes would enable metabolically engineered cells to perform their tasks with feasible techno-economics. We thus mapped output of work described into the Design-Build-Test-Learn cycle as shown in Fig. 3. Details of work progress for each topic are discussed in the next section.

As Thailand produces various commodity crops, industries routinely use processes to convert fresh produce into final products which can be preserved and stored for long distance transportation or export. For example, cassava needs to be converted into various forms of starch and oil palm requires milling processes to extract oil. These processes generate huge amounts of biomass and agricultural waste which are of low value. Therefore, most of the research related to synthetic biology in Thailand aims to convert low-value feedstocks such as sugar, palm oil or agricultural wastes such as lignocellulosic biomass into high-value compounds. Our research group also works on several projects aimed at converting low value feedstocks into valuable biochemicals.

3. Research related to development of synthetic biology in Thailand

3.1. Genetic manipulation and metabolic engineering of organisms for production of valuable chemicals

Research under this topic contributes to capability building under the Design and Build modules to produce valuable products (Fig. 3, Table 1). There are four categories of products in this topic including monomers of biobased plastics, specialty compounds, long chain-polyunsaturated fatty acid (LC-PUFA) and their derivatives, and bioactive compounds.

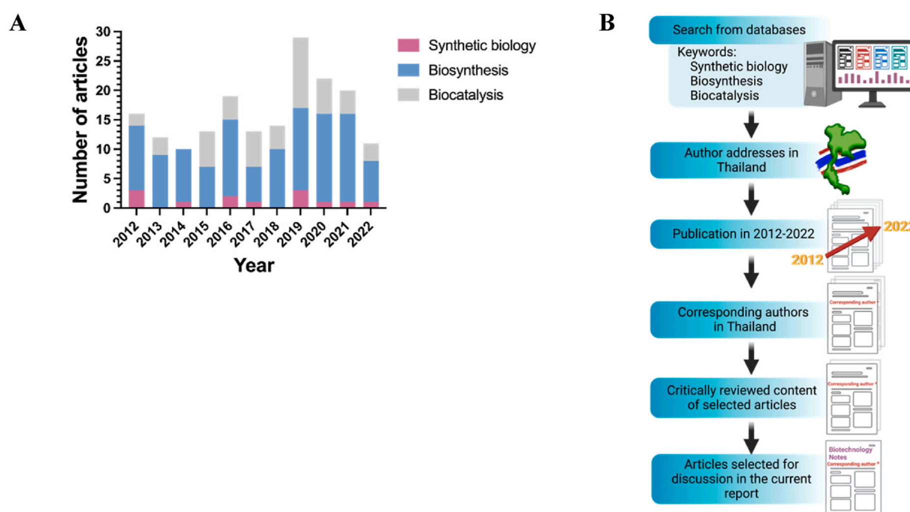

Fig. 2. Number of articles in synthetic biology, biosynthesis and biocatalysis fields since 2012 to 2022 (A). Methodology for selecting work discussed in the current report (B).

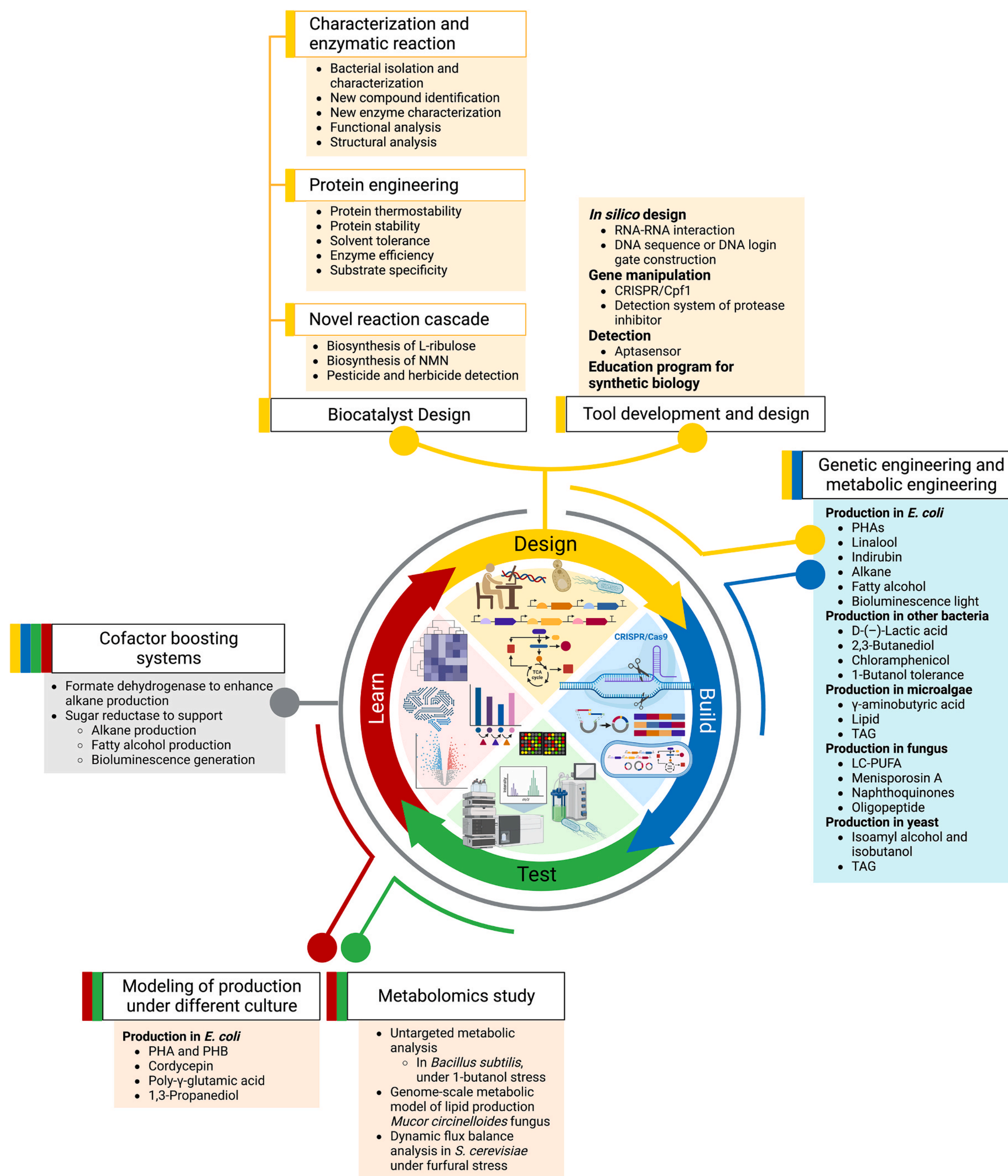

Fig. 3. Overview of current research related to the synthetic biology platform (Design-Build-Test-Learn) in Thailand.

3.1.1. Monomers of bio-based plastics (PHA, PHB, D-(−)-lactic acid and succinic acid) and commodity chemicals (2,3-butanediol)

Polyhydroxyalkanoate (PHA) monomers are the top products found in research under this area. PHA can be classified based on chain-length into three groups including short-chain-length (SCL) PHAs (3–5 carbon atoms) which are strong but rather inflexible, medium-chain-length (MCL) PHAs (6–14 carbon atoms) which are highly flexible but have low stretchability, and copolymers of SCL and MCL PHAs which have advantages of both SCL and MCL properties. Most of the research projects in Thailand which aimed to support production of bio-based plastics used *Escherichia coli* (*E. coli*) as a host for gene manipulation.

To produce SCL-MCL PHA copolymers, *E. coli* BL21 (DE3) was engineered using plasmid systems to overexpress relevant genes for production of PHAs. The recombinant *E. coli* produced the copolymers with various monomers ranging from 4 to 10 carbon atoms such as 3-hydroxybutyrate (3HB), 3-hydroxyhexanoate (3HH), 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD). The culture was carried out using carbon sources such as glycerol (by-product of biodiesel production)[12] and dodecanoate (an abundant component in palm oil mill effluent from the palm oil industry).[13] The system could produce PHA at a level of 3.2% of the dried cell weight (DCW).[14] In a different host, *Bacillus licheniformis* M2-12 was sequentially mutated by UV and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine to obtain the strain with a higher growth rate, biomass and PHA content (with various compositions of 3HB, 3-hydroxyvalerate and 3HH) than that of wild-type. In this case, 3% of palm oil mill effluent was also tested as a substrate for production of PHA and the product was obtained at a 16.23 g/L and 88.64% yield of DCW (Table 1).[15]

Table 1
Genetic engineering and metabolic engineering research in Thailand.

Host organism	Substrate	Product	Gene manipulation	Scale	Ref.
Production in *E. coli*					
E. coli BL21(DE3)	Glycerol with dodecanoate	PHA as 3HB,3HH, 3HO and 3HD with different production efficiencies	Overexpression of genes involved in SCL-MCL PHA copolymers biosynthesis using pETDuet-*phaABCs* and pCDFDuet-*phaJs* plasmids	300 mL Batch cultivation	14
E. coli JM109	Glucose	PHB	Overexpression of genes for PHB production using pColdTF- $phaABC_{A\text{-}04}$ plasmid	2 L Batch cultivation	16
E. coli XL1-Blue	Glucose	PHB	Overexpression of $phaABC_{A\text{-}04}$ gene in pColdTF- $phaABC_{A\text{-}04}$ plasmid	2 L Fed-batch cultivation	17
E. coli XL1-Blue	Glucose	PHB	Overexpression of $phaABC_{A\text{-}04}$ gene in pBAD/Thio-TOPO-$phaABC_{A\text{-}04}$ plasmid	2 L Fed-batch cultivation	
E. coli TOP10	Glucose	PHB	Overexpression of $phaABC_{A\text{-}04}$ gene in pBAD/Thio-TOPO-$phaCAB_{A\text{-}04}$ plasmid	100 mL Batch cultivation	
E. coli KJ122	Sucrose Sugarcane molasses	Succinic acid	Overexpression of *cscKBA* genes (*cscK*, fructokinase encoding gene, *cscB*, a sucrose:H^+ symporter encoding gene and *cscA*, invertase encoding gene) in pKJSUC-24T plasmid	350 mL in Anaerobic bottles 7.5 L Batch fermentation	20
E. coli YYC912	Glucose	Linalool	Overexpression of *gpps* and *lis* genes for linalool production in pSB1AT31 plasmid with a Tet promoter and terminator	In 250 Shake flask	23
E. coli	Indole and isatin	Indirubin	Overexpression of *tmoABCDEF* genes in pKSR12 plasmid	1 mL	24
E. coli	Long-chain fatty acid	Alkane	Overexpression of *Xafdh* genes in a plasmid for conversion of formate by-product to NAD(P)H to enhance alkane production	1 mL	46
Production in other bacteria					
Cupriavidus sp. KKU38	Glucose	PHA	–	100 mL Batch cultivation	19
Bacillus licheniformis PHAs-007 (M2-12)	3% Palm oil mill effluent	PHA	Mutation using UV and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine	–	15
Klebsiella oxytoca KMS004–91T	Glucose	D-(−)-Lactic acid	Deletion of *frdABCD* and *pflB*	2.5 L Fed-batch fermentation	21
	Cassava starch	D-(−)-Lactic acid	Deletion of *frdABCD* and *pflB*	2.5 L Fed-batch fermentation	
Klebsiella oxytoca KMS005–73T	Glucose	2,3-Butanediol	Deletion of *adhE* (alcohol dehydrogenase), *ackA-pta* (acetate kinase A-phosphotransacetylase) and *ldhA* (lactate dehydrogenase A)	1 L Fed-batch fermentation	22
Streptromyces venezuelae ATCC 10712	GI medium containing glycerol, glucose, nutrient broth, K_2HPO_4, yeast extract and L-isoleucine	Chloramphenicol	Overexpression of two rate-limiting step enzymes in the shikimate pathway i.e. *aroB* and *aroK* in pIJ86 plasmid with a strong constitutive promoter	In 500 mL shake flask	37
Bacillus subtilis	Spizizen minimal medium	1-Butanol tolerance strain	Disruption of *mprF* gene in lysyl-phosphatidylglycerols synthesis by homologous recombination	20 mL	40
Lactobacillus plantarum	Glucose	Protein secretion of β-mannanase and chitosanase	Overexpression of genes encoding β-mannanase and chitosanase with their native signal peptides and inducible expression	3 L	83
Production in microalgae					
Synechocystis sp. PCC 6803	Pyruvate	γ-Aminobutyric acid	Knocking out of *gad* and *kgd* genes encoding for glutamate decarboxylase and α-ketoglutarate decarboxylase genes by plasmid construction for homologous recombination	–	26
Synechocystis sp. PCC 6803	BG_{11} medium	Lipid	Overexpression of acyl–acyl carrier protein synthetase gene	100 mL	32
Neochloris oleaginous	Bold's basal medium	TAG	Overexpression of gene encoding diacylglycerol acyltransferase 2	150 mL	31
Production in fungus					
Aspergillus oryzae	Fatty acid	γ-Linolenic acid and dihomo-γ-linolenic acid	Co-overexpression of codon-optimized Δ^6-desaturase and Δ^6-elongase genes from *pythium* sp., and diacylglycerol acyltransferase 2 gene from *Mortierella alpina*	50 mL	27, 29
Aspergillus oryzae	Liquid complex medium	Increase in TAG and ergosterol	Disruption of α-1,3-glucan synthase 1 and chitin synthase B genes in *Aspergillus oryzae*	50 mL	28
Aspergillus oryzae	Menisporopsin A production medium	Menisporosin A	Heterologous expression of polyketide synthase genes	25 mL	34
Ophiocordyceps sp. BCC1869	Potato dextrose broth	Naphthoquinones	Overexpression of MFS transporter gene with a strong constitutive promoter	50 mL	35
Aspergillus oryzae	Semisynthetic medium	Oligopeptide	Replacing the promoter with a constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase for ACV synthetase and deletion of pyruvate decarboxylase gene (competitive pathway) by CRISPR/Cas9	50 mL	36
Production in yeast					
Pichia pastoris	Glucose	Isobutanol and isopentanol	1. Overexpression of endogenous genes involved in valine and leucine biosynthesis pathways	10 mL	38

(*continued on next page*)

Table 1 (*continued*)

Host organism	Substrate	Product	Gene manipulation	Scale	Ref.
			2. Deletion of pyruvate decarboxylase gene (competitive pathway) by CRISPR/Cas9 3. Expression of heterologous genes of the keto acid degradation pathway		
Pichia pastoris	Glucose	Isopentanol	Overexpression of genes involved in valine and leucine biosynthesis	10 mL	39
Saccharomyces cerevisiae	Unsaturated and saturated fatty acids	TAG	Heterologous expression of codon-optimized diacylglycerol acyltransferase gene from *Mortierella alpina* in plasmid	–	30

Systems for production of polyhydroxybutyrate (PHB), a polyester of PHA which is biodegradable, were constructed in *Cupriavidus necator* strain A-04 using the plasmid system overexpressing *phaABC*$_{A\text{-}04}$ with a trigger factor, a ribosome-associated bacterial chaperone which helps protein folding, under the control of arabinose-inducible promoter (araBAD). In another system, *E. coli* JM109 was also tested for PHB production using a cold-shock inducible promoter and glucose (which can be obtained from sugarcane or starch) as a main carbon source.[16,17] As Thailand is one of the world's major cassava producers,[18] the engineered *Cupriavidus* sp. KKU38 was tested for PHA production using starch industrial wastewater as a carbon source, resulting in a yield of 73.88% PHA content or 3.02 g/L of PHA, with the majority of the product existing as a homopolymer of PHB.[19]

In addition to producing bioplastics in microbes, various projects aimed to directly produce monomers of bio-based plastics and commodity chemicals such D-(−)-lactic acid, 2,3-butanediol and succinic acid. D-(−)-Lactic acid is used as a monomer for polylactic acid, a biodegradable plastic. 2,3-Butanediol is used as an anti-freeze, chemical precursors, fuels and pharmaceutical agents. Succinic acid is used as a precursor for pharmaceuticals, food, biodegradable plastics and green solvents.[20] To increase D-(−)-lactic acid production, the genome of the gram-negative bacterium *Klebsiella oxytoca* was engineered by deleting competitive pathways for the compound production. However, the engineered strain could not increase D-(−)-lactic acid production or the growth rate due to substrate inhibition effects. After improving the growth rate *via* media adjustment, fed-batch fermentation of this engineered cell produced 133 g/L and 128 g/L of D-(−)-lactic acid when using glucose or cassava starch pretreated with α-amylase and glucoamylase as substrates, respectively.[21] Enhancement of 2,3-butanediol production in *K. oxytoca* KMS005 was also carried out by eliminating formation of by-products such as acetonin, lactate and formate by removing genes encoding enzymes in competitive pathways. Cassava starch, sugarcane molasses and maltodextrin were used as substrates for the engineered cells. The fed-batch fermentation resulted in 0.49 g of 2, 3-butanediol/g glucose with 2,3-butanediol concentration of 117.4 g/L.[22] For succinic acid production, metabolic evolution and metabolic engineering of *E. coli* KJ122 were performed by overexpressing genes encoding fructokinase, sucrose:H^+ symporter and invertase from *E. coli* KO11 in the recombinant plasmid.[20] The strain showed an increase in succinic acid production to 53.4 and 55.8 g/L using sucrose and sugarcane molasses as substrates, respectively.

3.1.2. Specialty compounds (linalool, indirubin and γ-aminobutyric acid)

E. coli was also used for production of specialty compounds such as linalool and indirubin. Linalool is a specialty terpenoid used as a fragrance and additive in household products,[23] while indirubin is an anticancer agent.[24,25] Linalool (0.55 mM) could be produced from D-glucose in *E. coli* YYC912 harboring the recombinant BioBrick plasmids containing geranyl pyrophosphate synthase (GPPS) and linalool synthase (LIS) within 96 h.[23] For indirubin production, *E. coli* EPI300-T1 containing the recombinant plasmid overexpressing toluene 4-monooxygenase genes from *Pseudomonas* sp. M4 was used for production of indirubin using tryptophan medium and indole as a substrate in a two-phase culture system with mixed aqueous and organic phases of dioctyl phthalate.[24]

In another host system, a cyanobacterium *Synechocystis* sp. PCC 6803 was engineered for production of γ-aminobutyric acid (GABA), an inhibitory neurotransmitter and dietary supplement. Competing pathways to the GABA production were eliminated from *Synechocystis* genome. However, the engineered microbe did not result in increased levels of GABA production because upregulation of genes related to the GABA degradation pathway was induced instead.[26]

3.1.3. Long chain-polyunsaturated fatty acid (LC-PUFA) and their derivatives

For production of long chain-polyunsaturated fatty acid (LC-PUFA) and other derivatives of fatty acids, *Aspergillus oryzae* was used as a host to overexpress two Δ^6-desaturase and Δ^6-elongase genes from *pythium* sp. and two genes from *Mortierella alpina* encoding diacylglycerol acyltransferases for production of γ-linolenic acid and dihomo-γ-linolenic acid, which both are anti-proliferation and anti-inflammation agents, from fatty acids.[27] Additionally, the genes important for cell wall biosynthesis in *A. oryzae* were deleted in order to change the cellular morphology to allow easy transfer of oxygen and nutrients during cultivation, thus leading to an increase in total lipid production. The engineered *A. oryzae* showed 53% (w/w) and 70% (w/w) increased levels of lipid content and triacylglycerol (TAG) production yield, respectively, giving production yields of 198 mg/L for γ-linolenic acid and 166.5 mg/L for dihomo-γ-linolenic acid from D-glucose as a carbon source.[28,29] For TAG production from the esterification of unsaturated and saturated fatty acids, the diacylglycerol acyltransferase gene from *Mortierella alpina* (*Ma*DGAT2) was heterologously expressed in *Saccharomyces cerevisiae* strain which lacks neutral lipid synthesis.[30] In microalgae, lipid production was also improved by overexpression of endogenous DGAT2 in *Neochloris oleoabundans*. For *Synechocystis* sp. PCC 6803, overexpression of genes involved in fatty acid synthesis, deletion of genes in phospholipid hydrolysis, and deletion of genes in alkane synthesis resulted in a higher level of lipid production than wild-type.[31,32]

3.1.4. Bioactive compounds (menisporopsin A, naphthoquinone, oligopeptides and chloramphenicol)

Production of bioactive compounds including menisporopsin A, naphthoquinone, oligopeptides and chloramphenicol were also explored using metabolically engineered cells. Menisporopsin A exhibits antimalarial and antimycobacterial properties, and also displays cytotoxic activities towards breast cancer and nasopharyngeal carcinoma cell lines. The compound could be produced using a set of genes encoding polyketide synthases overexpressed in *A. oryzae*.[33,34]

To increase production of naphthoquinone, a compound with antibacterial, anti-malarial and insecticidal activities, a gene encoding a major facilitator superfamily (MFS) transporter was overexpressed in the plasmid system under a strong constitutive promoter in the fungus *Ophiocordyceps* sp. BCC1869. This increased efflux of naphthoquinones out of the cells and decreased the cellular toxicity of the compound. The engineered cells could tolerate increased levels of naphthoquinone derivatives across a range of 20- to 2300-fold compared to the wild-type.[35]

For production of oligopeptides used as food additives and

pharmaceuticals, genes encoding for non-ribosomal peptide synthases were overexpressed in *A. oryzae*, and the native promoter of glyceraldehyde-3-phosphate dehydrogenase was replaced with a constitutive promoter to increase the production of the tripeptide L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) from D-glucose. These genetic manipulations were done using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 endonuclease (CRISPR/Cas9) method which resulted in increased ACV titers of 137.39 mg/L.[36]

In addition to fungus, *Streptomyces venezuelae* ATCC 10712 was engineered to produce chloramphenicol, a widely used antibiotic by overexpression of genes encoding the rate-limiting step enzymes in the Shikimate pathway to increase chloramphenicol production. Overexpression of the individual genes of *aroB* and *aroK* encoding dehydroquinate synthase and shikimate kinase increased production of choramphenicol by 2.2- and 2.5-fold as compared to that of the wild-type strain, respectively, which might be due to the increase of chorismic acid, an intermediate for the production of choramphenicol.[37]

3.1.5. Medium chain alcohols (isobutanol and isoamyl alcohol)

For production of medium chain alcohols such as isobutanol and isoamyl alcohol (3-methyl-1-butanol), *Pichia pastoris* was engineered to produce these compounds from D-glucose and glycerol using three strategies. First, overexpression of endogenous genes in the valine and leucine biosynthesis pathways was carried out using plasmid systems to increase the levels of 2-ketoisocaproate, a key intermediate for isobutanol and isoamyl alcohol production. Second, to decrease production of ethanol, a by-product from the competitive pathway, the gene pyruvate decarboxylase was deleted from *P. pastoris* genome using CRISPR/Cas9. Third, genes encoding enzymes in the keto acid degradation pathway were overexpressed to decrease the flux through competitive pathways.[38] For isobutanol production, a similar approach of increasing valine and leucine biosynthesis, and keto acid degradation pathways was carried out, resulting in an engineered cell which produced a 43-fold higher isobutanol yield than wild-type.[39] In addition, the gram-positive *Bacillus subtilis* was engineered to produce 1-butanol by disrupting genes involved in membrane lipid biosynthesis to increase 1-butanol tolerance.[40]

3.2. Analysis of synthetic cell performance using metabolomic analyses

In addition to simply measuring product yield, metabolomic analysis provides comprehensive information for comparison of cellular metabolites of native and engineered cells. The data obtained can provide insightful analytic and predictions for further improvement (Test-Learn modules). Typical studies of metabolomics found in this area include untargeted metabolomics, analysis of metabolites associated with relevant metabolic pathways, metabolic interaction networks and metabolic flux (Table 2). This information is also important for understanding cellular biochemistry and physiology in general.

To the best of our knowledge, not many untargeted metabolomic analyses were carried out with metabolically engineered cells constructed in Thailand. We are only aware of a few research projects reporting untargeted metabolomics. *Bacillus subtilis* engineered for production of 1-butanol was examined for cell membrane lipid compositions under 1-butanol stress in combination with transcriptional analysis and cell morphology assessment.[41] For a genome-scale metabolic model (GEM), the fungus *Mucor circinelloides*, which was engineered for production of lipid, was investigated in comparison to the native cell. GEM is a computational tool using databases of gene and protein expression data to characterize metabolic behaviors and build metabolic networks. The results showed that the engineered strain had higher lipid accumulation and higher fatty acid biosynthesis than the native cells.[42,43] *Saccharomyces cerevisiae* which was engineered to produce furfural was analyzed to determine the dynamic flux balance under furfural stress during fermentation to understand the intracellular and extracellular metabolic flux profiles.[44] Recently, untargeted metabolomics was used to identify metabolites associated with engineered cells containing the cofactor enhancing system.[45]

Table 2
Metabolomics studies in Thailand.

Organism	Aim	Analysis	Ref.
E. coli	Enhance fatty alcohol production by sugar reductase system	Untargeted metabolomic analysis identified mechanisms behind the 3-fold increase in fatty alcohol production compared to the system without sugar reductase	45
E. coli	Enhance alkane production by sugar reductase system	Untargeted metabolomic analysis identified mechanisms behind the 1.6-fold increase in tridecane production compared to the system without sugar reductase	45
E. coli	Enhance bioluminescence light generation by sugar reductase system	Untargeted metabolomic analysis identified mechanisms of increased light brightness and prolonged light production compared to the system without sugar reductase	45
Bacillus subtilis	Increase cell viability under 1-butanol stress	Untargeted metabolomic analysis of the cells under 1-butanol stress revealed the change of membrane lipid components resulted in upregulation of diglucosyldiacylglycerol, phosphatidylethanolamine and phosphatidylserine with downregulation of lysyl-phosphatidylglycerol and diacylglycerol	41
Mucor circinelloides	Enhance lipid production	Genome-scale metabolic model study to reveal the increase of lipid metabolism and fatty acid biosynthesis in a lipid over-producing strain.	42, 43
Saccharomyces cerevisiae	Study of flux balance under furfural stress	Dynamic flux balance analysis during fermentation to reveal the interactions between intracellular and extracellular metabolic flux profiles.	44

3.3. Use of the design-build-Test-Learn cycle to enhance production of cofactors and cell fitness for production of valuable biochemicals

Unlike the manipulation of deleting and overexpressing genes directly involved with the main production pathway, our group has shown that addition of genes involved in by-product elimination and cofactor enhancing systems *in vivo* is an important strategy for enhancing production of valuable biochemicals by more than 2-3-fold and improving cell viability. For the engineered *E. coli* which could be used to convert long chain fatty acids to alkanes *via* the reaction of acid reductase and aldehyde deformylating oxygenase (ADO) in which formic acid is obtained as a by-product, the overexpression of formate dehydrogenase (FDH) in the metabolically engineered cells can convert formate to CO_2 and increase the yield of alkane production from 35% to 50%. The addition of FDH helps through a two-pronged approach: by removing unwanted acid to maintain the cellular pH at a neutral range for much longer (maintaining cell stability) and also to regenerate NAD(P)H which is required for reduction of ferredoxin, a co-substrate in the reactions of ADO.[46]

Recently, we employed a full cycle of the synthetic biology platform (Design-Build-Test-Learn model) to demonstrate that enhancement of a pool of sugar phosphates can increase the cellular synthesis of various common cofactors according to the cell demand. We tested the validity of this system with three types of metabolically engineered cells to

produce fatty alcohol, bioluminescence and alkane. The results showed that products of all three systems were indeed enhanced by at least 2–4-fold. Using untargeted metabolomic analysis, we found that this methodology could increase the levels of cofactors according to specific cellular demand i.e. only cofactors relevant to specific cellular requirements were enhanced.[45] We believe that this strategy should be useful as a generic protocol to enhance the performance of *in vivo* biocatalysts in general (Table 2).

3.4. Development of new tools and designs for synthetic biology

On-going research in Thailand regarding development of tools for synthetic biology include *in silico* platform designs, tools for genetic manipulation, and *in vivo* and *in vitro* biodetection tools (Table 3). As RNA-RNA interactions are crucial for gene regulation, the *in silico* tool iDoRNA was used to design RNA-RNA interacting sequences for controlling RNA-RNA interactions. The main purpose of this tool was to monitor gene responses to DNA damage and cell death i.e. in agriculture to control the color of flowers and in biomedicine for cancer therapy. The key principle of this tool relies on interactions between small interfering RNA and their targets.[47–49] Another *in silico* tool developed is based on a computational approach to select the most efficient DNA sequence out of three short DNA strands suitable for DNA logic gate construction. The methods in principle can generate all feasible sequences and select those with the highest probability of success for construction. The main application of the DNA logic gate was aimed towards disease diagnosis.[50]

For development of tools for gene manipulation *in vivo*, the CRISPR/Cpf1 system was developed for gene disruption in *Aspergillus aculeatus* TBRC 277. Cpf1 from *Francisella tularensis* subsp. *Novicida* U112 is a type V and alternative class II nuclease which can specifically induce double-strand breaks and further introduce an endogenous non-homologous end-joining (NHEJ) DNA repair. The system was claimed to be capable of introducing genes into specific locations for gene insertions and deletions in the *A. aculeatus* genome.[51]

For development of *in vivo* detection tools, *E. coli* strain MC4100 was engineered as a tool for detecting protease inhibitors. The system was constructed as a potential model for screening of protease inhibitors for HIV therapy.[52] The engineered *E. coli* had three devices incorporated, including a protein blocking device (protein inhibitor), a protease activity detector device (β-lactamase and translocating signal from cytoplasm to periplasm), and protease generator device (HIV protease). This system was tested for its ability to inhibit the activity of HIV protease.

An additional detection tool includes the use of an aptasensor as non-natural naringenin-responsive riboswitches in combination with fluorescence assays *in vivo* for specific detection of flavonoids and chalcone compounds. This work reported the specificities and properties of six different riboswitches incorporated in *E. coli* which can detect 27 different types of flavonoids and chalcones. The outcome of this study provides a guideline for the future design of ligand and riboswitch structures to obtain specific binding for detection of compounds from synthetic biology.[53]

Table 3
Development of tools and public awareness in Thailand.

Tool	Description	Ref.
Design of RNA-RNA interactions	Development of the iDoRNA tool to design RNA-RNA interacting sequences for RNA-RNA interactions by designing each domain using stochastic and genetic algorithms after breaking down RNA structure to interacting domains	47–49
Design of DNA logic gate construction	Development of computational methods to select efficient DNA sequences for DNA logic gate construction	50
CRISPR/Cpf1 gene manipulation	Development of the CRISPR/Cpf1 system for gene disruption in *Aspergillus aculeatus* TBRC 277	51
Detection system of protease inhibition	Development of a biodetecting machine in synthetic *E. coli* to inhibit proteases	52
Aptasensor form detection	Detection of flavonoids and chalcone compounds by naringenin-responsive riboswitches	53
Education program for synthetic biology	A science education workshop to promote awareness in synthetic biology among high school students	54

3.5. Educational program to enhance the understanding of synthetic biology for students

Another interesting angle of development is an educational workshop to promote science, technology, engineering, art, and mathematics (STEAM) in biology. The workshop was designed and implemented with high school students in Thailand. The program consisted of three parts including bio-digital, synthetic biology and biomimicry. It was conducted by motivating talks and laboratory challenges to encourage students to discuss and gather ideas. These types of workshops can promote and inspire students to be interested in bioengineering and synthetic biology in the future.[54]

4. Competitiveness in the areas important for future development of synthetic biology in Thailand

As the performance of individual enzymes or biocatalysts is important for engineered cells to perform the desired functions, the ability to design biocatalysts would be advantageous for synthetic engineers to put together efficient pathways or systems for production of valuable compounds. During recent years, researchers in Thailand have published cutting edge work within the enzyme catalysis and enzyme engineering areas, particularly showcasing the use of rational and mechanistic approaches to perform enzyme engineering.[55,56] Research on biocatalysis or *in vitro* cascade reactions are also important for testing cell-free *de novo* pathways for performing the desired reactions. These *de novo* pathways will then be tested *in vitro* for application *in vivo* and should be of advantage when they are implemented in synthetic cells in the future. In-depth understanding of enzymatic reactions which can lead to *de novo* or unnatural enzymatic activities is also important because this can help to expand biocatalytic toolboxes for synthetic biology (Table 4). Another interesting area in which Thai researchers have demonstrated their capability is the use of systems biology to analyze the metabolic output of native and engineered cells for generating products under different culture conditions (Table 5). One can imagine that similar analyses can be done in the future with different genetically engineered cells.

4.1. Enzyme engineering

There are generally two main reasons for performing enzyme engineering. One is to improve protein stability such as thermostability and solvent tolerance. Another is to enhance enzyme efficiency and expand the breadth of substrate utilization. For thermostability enhancement, various *in silico* or computational approaches are now available which predict specific amino acids positions that can be changed in order to increase the robustness of the enzymes (for more details please see recent reviews by Phintha et al. Chem Catalysis, 2022). Recent work from our group has shown that use of the FireProt software can predict residues leading to successful engineering to increase the stability of various enzymes. A melting temperature (T_m) of a flavin reductase (C_1) which can be used to generate free reduced flavin for various flavin-dependent monooxygenases can be increased by 2.6–5.6 °C with catalytic efficiency enhancement of 2–3.5-fold compared to the wild-type enzyme.[57] In another example, FireProt predicted information was coupled with site-saturated mutagenesis to increase the thermostability of a flavin-dependent monooxygenase HadA by 72- and 160-fold at 50

Table 4
Competitiveness in the areas important for future development of synthetic biology in Thailand.

Protein Engineering

Enzyme	Function	Improvement	Cause of improvement	Ref.
Flavin reductase (C_1)	Regeneration of reduced flavin mononucleotide ($FMNH^-$) for FMN-dependent enzymes	Increase of thermostability, catalytic efficiency and solvent tolerance	Stabilization of hydrophobic interaction	57
HadA	Degradation of herbicides and pesticides to non-toxic phenolic compounds	Increase of thermostability and activity	Increase of aromatic hydrocarbon interactions between residues	58
ThaI	Catalysis of halogenation reaction of tryptophan	Increase of thermostability, enzyme activity, pH tolerance and substrate utilization scope	Increase of hydrophobic interactions in the tunnel connecting two active sites Rearrangement of water network in the tunnel	56
Xylanase	Xylan degradation	Increase of thermostability	Increase of hydrophobic interactions and disulfide bonds to stabilize loops near the N-terminus and substrate binding site	61
		Increase of catalytic efficiency	Increase of H-bonds in active sites to stabilize key catalytic residues and β-sheet plate Removal of carbohydrate-binding domain	62

Novel reaction cascade

Function	Substrate	Enzymes for cascade reaction	Ref.
L-Ribulose biosynthesis	L-Arabinose	One-pot cascade reaction of P2O(T169G) and xylose reductase biocatalysts with auxiliary systems of FDH and catalase	63
NMN Biosynthesis	Xylose	1. D-Ribose generation in medium from engineered Δ*tktA* Δ*tktB* Δ*ptsG* *E. coli* MG1655 2. Using D-ribose in medium as substrate for NMN biosynthesis using ribose kinase phosphoribosyl pyrophosphate synthetase and nicotinamide phosphoribosyltransferase 3. Auxiliary systems using polyphosphate kinase II for ATP-regeneration and pyrophosphatase to remove pyrophosphate, a feedback inhibitor	64
Pesticide and herbicide detection	Herbicides and pesticides	One-pot chemo-enzymatic cascade reaction of HadA dehalogenase with auxiliary enzymes to convert pesticides to D-luciferin	59

Characterizations of enzymatic reactions

Production	Organism	Type	Function	Ref.
MCL-PHA	*Pseudomonas* sp. ASC2 from soil	Screening, isolation, and identification	3-Hydroxy-5-*cis*-dodecanoate as one of the PHAs used as a softener in biomaterial and food industries	84
Tricyclic polyketide azaphilone derivatives	Fungal *Dothideomyctete* sp.	Identification of new compounds	Antimicrobial and cytotoxic activities	85
Xyrrolin	*Xylaria* sp. *BCC 1067*	New cytotoxic hybrid polyketide and non-ribosomal peptide pyrroline	Anti-oral cavity and anti-cancer	86
Alkaline trehalose synthase	*Pseudomonas monteilii*	New enzyme	Biocatalyst for trehalose production from maltose	65
Elongase	Fungal *Pythium* sp. BCC53698	New enzyme	Biocatalyst for elongation ofthen Δ^6-18 carbon atom of desaturated fatty acid for value-added PUFA biosynthesis	66
Thermostable aldolase	*Acinetobacter baumannii*	Structural and functional analysis	Biocatalyst for aldol condensation and cleavage with high specificity and stereoselectivity	67
GnnA protein	*Acidithiobacillus ferrooxidans*	Structural analysis	Biocatalyst for an oxidation of UDP-*N*-acetylglucosamine to UDP-3-oxo-*N*-acetylglucosamine, a precursor for lipopolysaccharide biosynthesis	68
Carboxylic acid reductase	*Mycobacterium marinum*	New function	Biocatalyst for esterification of carboxylic acids with a nucleophilic alcohol under aqueous conditions	69
Modified PET hydrolase	Human saliva metagenome	New function	New platform of polyethylene terephthalate detection with a split green fluorescent protein	70

°C and 45 °C compared to the wild-type enzyme, respectively. HadA catalyzes dehalogenation and denitration of toxicants derived from pesticides and pesticides; this enzyme is thus important for development of bioremediation applications and other important biocatalysis processes in the future (see more discussion in 4.2).[58–60]

To improve the efficiency and capability of substrate utilization, we have identified a bottleneck step in the reaction of tryptophan 6-halogenase (Thal) which is a flavin-dependent halogenase. The enzyme catalyzes important reactions in green chemistry because it can be applied in the synthesis of precursors for various drugs. Thal contains two active sites connected *via* a tunnel in which the flavin active site generates a reactive intermediate hypohalous acid (HOX) which diffuses through the tunnel to halogenate a tryptophan substrate in the second active site (tryptophan substrate binding site). In-depth mechanistic investigations have identified that the wild-type Thal has low catalytic efficiency due to leakage of the HOX intermediate. Using the program CAVER, the areas forming the tunnel connecting the two active sites were identified and enzyme engineering has been carried out to obtain the V82I variant which generates less leakage of HOX. The V82I variant also displayed various advantageous properties for biocatalysis including an increased T_m of 12 °C, expansion of substrate usage scope and increased pH tolerance of Thal. The engineered enzyme would provide a more efficient biocatalyst for future development of halogenase applications and also can be used in metabolically engineered cells for production of bioactive compounds in the future.[56]

Another successful example of increasing enzyme thermostability in Thailand is engineering of xylanase. Due to the high amount of biomass from agricultural wastes produced in Thailand, xylan is attractive as a feedstock of substrate for production of valuable compounds such as xylo-oligosaccharides, potential pre-biotics for human and animal health. Xylanase was engineered to improve hydrophobic interactions by adding disulfide bonds which stabilize the loops near the N-terminus and substrate binding sites, resulting in a 15.6-fold increase in catalytic efficiency compared to the wild-type enzyme.[61] Removal of the carbohydrate-binding domain also increased the catalytic efficiency of xylanase 6.5-fold.[62] These engineered xylanases should be useful as efficient biocatalysts in biorefinery and other applications.

Table 5
Optimizing cultivation in Thailand.

Organism	Production	Optimizing cultivation	Ref.
***Cordyceps militaris* iNR1329**	Cordycepin	Using *in silico* growth model prediction of optimal media formulation based on genome-scale metabolic models and optimal C:N ratios in synthetic media calculated from POPCORN modeling method to increase cordycepin production	77
Cyanobacterium *Arthrospira platensis*	PHB	Increase of PHB biosynthesis related to the increase of *phaB* and *phaC* expression when the cells were photoheterotrophically grown under nitrogen-deprived medium with organic carbon compounds	75
Mixed of microalgae consortia containing *Scenedesmus* sp., *Desmodesmus* sp., *Chlorella* sp., *Coelastella* sp. *and Phormidium* sp.	PHA	Mixed cultivation of microalgae consortia under nitrogen and phosphate limitation in batch cultivation	76
***Bacillus licheniformis* TISTR 1010**	Poly-γ-glutamic acid	Increase of poly-γ-glutamic acid production when using fed-batch fermentation supplemented with glucose, ammonium chloride and citric acid at each stage.	78
Engineered *E. coli* BP41Y3	1,3-Propanediol	Using the conventional method combined with statistical methods to attain high production levels in an aerobic-anaerobic fermentation process supplemented with fumarate, $(NH_4)_2HPO_4$ and peptone, under controlled pH	73

4.2. Construction of novel reaction cascades

Several research groups have also demonstrated *in vitro* cascade enzymatic reactions for production of valuable chemicals. Due to the high biomass production from agricultural industries, compounds derived from biomass are often targeted as substrates. One-pot cascade reactions using four enzymes (the engineered pyranose 2-oxidase (P2O), xylose reductase (XR), FDH and catalase) can be used to convert a common sugar, L-arabinose, to a rare and valuable sugar, L-ribulose, with 100% yield in aqueous solution at room temperature. L-Ribulose is a precursor for the synthesis of various L-nucleoside analogues which are anticancer and antiviral drugs. The key achievement of this work was the enzyme engineering of a substrate binding site in which the T169G variant was about 40-fold better than the wild-type enzyme in using L-arabinose as a substrate. Other enzymes in the reactions also work in tandem to complete the full catalytic cycle i.e. XR catalyzes reduction of 2-keto-arabinose intermediate to L-ribulose. FDH and catalase are auxiliary systems for regenerating NADPH for XR and removing the H_2O_2 by-product generated from P2O catalysis.[63]

As xylose is major component in hemicellulose found in agricultural waste, the effort has been made to use it to synthesize nicotinamide mononucleotide (NMN) as an intermediate for nicotinamide adenine dinucleotide (NAD^+) synthesis by NMN adenylyltransferase. There are two parts of this bioconversion process. The first part was D-ribose generation from xylose in the medium by the engineered *E. coli* MG1655. D-Ribose was used as a substrate for the second part of the cascade enzymatic reactions. The NMN biosynthesis is catalyzed by ribose kinase and phosphoribosyl pyrophosphate synthetase from *E. coli*, and nicotinamide phosphoribosyltransferase from *Chitinophaga pinensis*. Additionally, polyphosphate kinase II and pyrophosphatase were used to re-generate ATP from ADP and remove pyrophosphate, which are feedback inhibitors. This investigation can be further developed for metabolic engineering.[64]

A one-pot chemo-enzymatic cascade reaction of HadA-catalyzed biodetoxification of phenolic compounds such as nitrophenols and halogenated phenols, toxicants derived from pesticides and industrial chemicals, was developed. The reaction can convert phenolic toxicants into benzoquinone derivatives which can be condensed with D-cysteine to synthesize D-luciferin and its analogues. D-Luciferin is a high value compound as it is broadly used in biodetection applications in biomedical research. This reaction exemplifies the prototype reactions of toxic waste biorefinery and can also be applied as a detection tool for pesticides.[59] The reaction was further developed into an organophosphate pesticides (OPs) detection prototype called Luminescence Measurement of Organophosphate and Derivatives (LUMOS) technology. LUMOS can directly detect OPs from biological samples without any pretreatment at parts per trillion levels without sample pretreatment.[60]

4.3. Finding and creation of new enzymatic activities

Several groups have reported new enzymatic reactions of known enzymes upon new enzyme screening or testing reactions with unnatural substrates. For example, a new alkaline trehalose synthase from *Pseudomonas monteilii* was found to catalyze trehalose production from maltose in one step. Trehalose is used as a stabilizer and preservative for frozen food, and in cosmetic and pharmaceutical products.[65] A novel elongase isolated from fungal *Pythium* sp. BCC53698 was found to catalyze specific elongation of the Δ^6-18 carbon atom of desaturated fatty acids for polyunsaturated fatty acid biosynthesis.[66] Structural and mechanistic studies of a thermostable aldolase from *Acinetobacter baumannii* which catalyzes aldol condensation and cleavage found that the enzyme can catalyze aldol condensation of various short chain acids and aldehydes in which some reactions are stereospecific such as the synthesis of (4*R*)-2-keto-3-deoxy-D-galactonate, a chiral building block for synthesis of active pharmaceutical ingredients.[67] Additionally, the GnnA protein from *Acidithiobacillus ferrooxidans* was found to catalyze the oxidation of UDP-*N*-acetylglucosamine to form UDP-3-oxo-*N*-acetylglucosamine, a precursor for lipopolysaccharide biosynthesis, a crucial part of Gram-negative bacteria outer membrane used as a target of vaccine development.[68]

Through adapting the substrate usage based on an understanding of the reaction mechanisms, a new function of carboxylic acid reductase from *Mycobacterium marinum* (*Mm*CAR) has been discovered. CAR generally catalyzes conversion of carboxylic acids to aldehydes by forming an acyl-AMP as the reaction intermediate. By providing alcohols to act as nucleophiles instead of NADPH, CAR can catalyze esterification of carboxylic acids with nucleophilic alcohols to form various valuable esters such cinoxate, an active ingredient in sunblock. The reaction can be enhanced more than 10-fold in the presence of imidazole and can occur in aqueous environments. Unlike the reactions catalyzed by lipase or esterase, the advantage of CAR-catalyzed esterification is that the reaction is irreversible.[69] Recently, a new platform for polyethylene terephthalate (PET) detection by modified PET hydrolase (PETH) identified from the human saliva metagenome was demonstrated. The modified PETH was achieved by attaching a covalent linkage of 2,3-diaminopropionic acid at the modified catalytic residue of PETH with a split green fluorescent protein. Therefore, the fluorescence signal could be generated after the modified PETH bound to PET. This method can potentially be further developed to detect microplastic contamination in foods or beverages.[70]

4.4. Systems biology and modeling analysis of cells under different culture conditions

The medium composition and fermentation process are important factors which can govern levels of bioactive/metabolite production[71] because metabolic pathways for production of biomass and metabolites of interest can be altered by variation of carbon, nitrogen and phosphorus sources.[72] Various research groups in Thailand investigated the use of alternative carbon sources from agricultural by-products i.e. crude glycerol from biodiesel production in fermentation processes.[73–76] A growth rate and cordycepin overproduction by *Cordyceps militaris i*NR1329[77] were analyzed using an *in silico* growth model prediction to find an optimal medium formulation. The medium design is based on a genome-scale metabolic model approach that uses genome/transcriptomic data in combination with the amount of biomolecules produced by the cells to build metabolic networks of *C. militaris i*NR1329. The result demonstrated that by using glucose as a carbon source, *C. militaris i*NR1329 could produce the highest extracellular levels of cordycepin. As the biomass in the cells and cordycepin production was highly affected by the C:N ratio, further development based on a constraint-based modeling method called POPCORN (PrOPortion of CarbOn and nitRogeN) revealed optimum C:N ratios for growth and cordycepin production. This prediction was experimentally validated and was employed to increase cordycepin production by 3.5-fold. Another example is optimization of cultivation media to increase production of the bio-based polymer; polyhydroxybutyrate (PHB), polyhydroxyalkanoate (PHA) and poly-γ-glutamic acid production in natural bacteria by adjusting carbon and nitrogen sources.[75,76,78] For growth of Cyanobacterium *Arthrospira platensis*, a nitrogen-deprived condition with an appropriate organic carbon additive was employed. The addition of 0.5% (w/v) acetate resulted in a significant increase in the gene expression for production of PHB, which are the main enzymes involved in PHB biosynthesis, leading to the 2-fold increase in PHB accumulation. Poly-γ-glutamic acid production by *Bacillus licheniformis* TISTR 1010 was optimized through the types of nutrients and fermentation operation.[78] The fed-batch operation with substrate feeding could improve productivity of the poly-γ-glutamic acid production in a 5-Litre scale by about 3-fold. Likewise, 1,3-propanediol production by the engineered *E. coli* harboring genes encoding for enzymes which convert glycerol to 1,3-propanediol (glycerol dehydratase (DHAB), 1,3-propanediol oxidoreductase (YQHD) and YQHD activator protein) was also optimized by means of changing the nutritional conditions.[73]

5. Conclusions and perspectives

Although most of the current research in synthetic biology in Thailand is still in its infancy and at the proof-of-concept stage, the trends towards future development looks promising. Most of the current work still does not fully employ the Design-Build-Test-Learn cycle of synthetic biology, and genetic engineering work has mostly been done using plasmid systems. Genome integration of gene modifications would make the engineered cells more robust for bioproduction, especially for using feedstocks such as biomass of agricultural waste. However, a knowledge base in technical areas such as enzyme catalysis and engineering and systems biology is available in the country, which can strongly foster the development of synthetic biology in the future. Knowledge in these fields would strengthen future development of tool building and analysis of data obtained. In addition, awareness of the importance of synthetic biology by major players within the private and government sectors will hopefully further advance these technologies. Development of biocatalyst tools for bioremediation such as removal of heavy metals from industrial wastewater[79] or conversion of waste to valuable compounds[80,81] is also an important aspect of synthetic biology which allows it to make a contribution to sustainability efforts.

Perhaps, a key hallmark for raising awareness of synthetic biology in Thailand is the establishment of the SynBio consortium. Current members consist of 23 collaborators including the government, universities and private sectors. The recent SynBio Forum was conducted under the topic "SynBio for Sustainable Development Goals" on Nov 11, 2022. This effort is coaligned with the government policy to support the BCG model.[9] Because the amount of agricultural-derived materials in Thailand is massive, synthetic biology should be a suitable technology to plug-in and directly support the BCG policy.[82]

Based on analysis of the literature, we are optimistic about the future development of synthetic biology in Thailand. Leveraging the availability of domestic feedstocks and strong interests from public and private sectors, researchers in the synthetic biology community in Thailand should be able to find their niche areas of expertise, allowing them to contribute to the goal of making synthetic biology useable and scalable for bioeconomy applications. The awareness of the importance of the field by all sectors will support further development of sustainable bioindustries to increase the value of Thai natural resources by the BCG model which would ultimately support Sustainable Development Goals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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