# Biorefinery Research in Korea

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## Introduction

Industrial microbes have traditionally been developed through the repeated process of random mutation and screening for physiological characteristics that are beneficial in the production of various industrial chemicals (Park et al. 2008). However, due to hidden metabolic and regulatory characteristics from multiple random mutations, it is difficult to further improve the strain performance even with the various omics techniques and computational tools are available. Thus, a new strategy of rationally designing recombinant strains is needed to move beyond the classical methods of random mutations and create genetically well-defined strains with improved performance. Recent advances in systems biology, synthetic biology, and evolutionary engineering combined with metabolic engineering, or systems metabolic engineering, are now providing new insights for developing strategies to create the strains capable of efficiently producing targeted industrial chemicals (Lee et al. 2011).

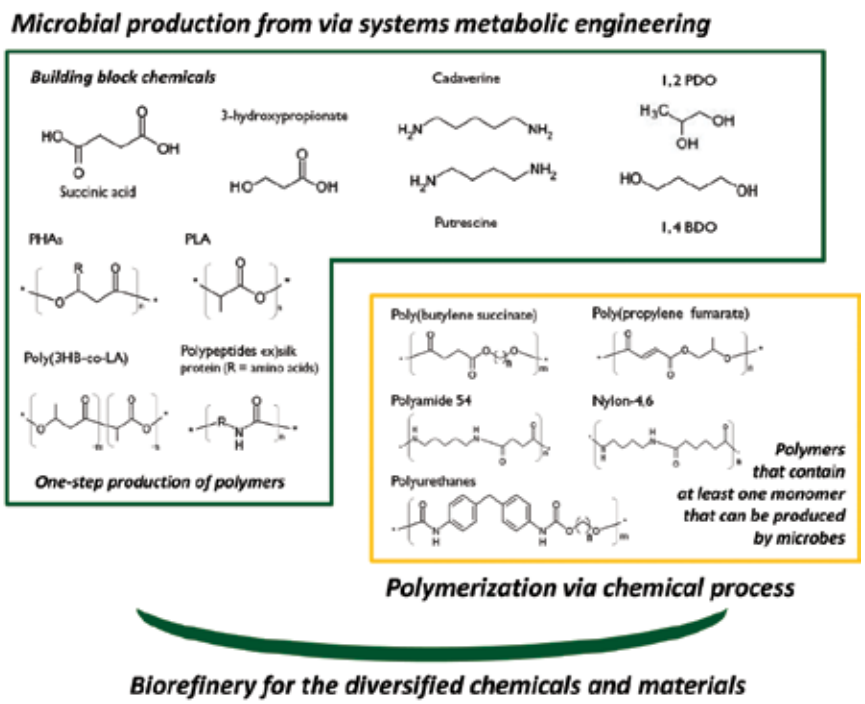


Figure 1. The advancement of systems metabolic engineering have allowed the improved production of various chemicals and polymers by microbes. Building block chemicals produced by microbes can be used to synthesize various polymers via chemical process. In some cases, biodegradable and biocompatible polymers can be produced directly from renewable resources using an engineered microbe, for example, polyhydroxyalkanoates (PHAs), polylactic acid (PLA), and polypeptides.

Biorefinery research has become an active field in Korea employing systems metabolic engineering to engineer recombinant strains for the production of a wide range of microbial chemical products. The diversity of microbial products being investigated in Korea have increased due to the contribution of synthetic biology, which allows for the creation of novel pathways by combining existing enzymes in nature and by generating novel enzymes, for the production of either natural or unnatural chemicals. This article introduces the status of biorefinery research activities in Korea with a focus on different microbial platform chemicals and biopolymers (Fig. 1) and will discuss how systems metabolic engineering will contribute synergistically for the successful development of biorefinery industry.

# Biorefinery research for platform chemical production

With advancement of systems metabolic engineering, the production of industrially relevant platform chemicals by microorganisms from renewable resources becomes economically feasible. Examples of industrial platform chemicals, such as succinic acid, putrescine, cadaverine, 1,4-butanediol (1,4-BDO), 1,2-propanediol (1,2-PDO) and 3-hydroxypropionic acid (3-HP), will be discussed. These platform chemicals have applications in a wide range of fields. For example, in the polymer industry, polyamide 54 is composed of cadaverine and succinic acid and nylon-4,4 is composed of putrescine and succinic acid. The diols 1,4-BDO and 1,3-PDO are constituents of various unsaturated polyesters where they form repetitive units with dicarboxylic acids. The polymer poly(propylene fumarate) is composed of 1,3-PDO and fumaric acid. Another biodegradable polymer, poly(3-HP), can also be obtained by polymerization of 3-HP. Traditionally, many of these chemical were synthesized using chemical processes from petroleum feedstocks. However, due to the volatile nature of the geopolitical environment surrounding petroleum reserves, high demand and consumption of energy and the increased consciousness of environmental consequences, an increased investment into exploring alternate renewable sources for the synthesis of these chemicals is observed. Recently, there has been a trend in employing microbial systems as cell factories for the production of these high valued chemicals. Systems level engineering of these microbial systems have resulted in the creation of strains which are capable of producing target chemicals from renewable feedstocks. This section introduces the researches in Korea that investigate the use of systems metabolic engineering on microorganisms to produce these platform chemicals.

## 1. Succinic acid

Succinic acid is used as an acidulants in the food and beverage industry, ingredients for lubricant and pesticide, and building blocks for polymers, such as poly(butylenesuccinicate). Succinic acid is also used as an important intermediate in deriving chemicals such as tetrahydrofuran, adipic acid, 1,4-butanediol, 2-pyrrolidone, and gamma-butyrolactone, all of which are commercially produced from petrochemical processes. Annual market size of succinic acid and its derivatives is approximately $15 billion. Various microbial succinic acid producers, including *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Corynebacterium glutamicum*, *Escherichia coli*, and *Mannheimia succiniciproducens*, were engineered to increase the production of succinic acid to levels which allow the microbial process of succinic acid production economically feasible. The bacterium *M. succiniciproducens*, a capnophilic rumen bacterium, was isolated from a Korean cow (Lee et al. 2002) and its genome was completely sequenced and annotated in 2004 (Hong et al. 2004). Genome-based engineering of *M. succiniciproducens* increased succinic acid production by blocking competitive pathways (Lee et al. 2006b). Inactivation of enzymes encoded by the genes *ldhA*, *pflB*, *pta*, and *ackA*, resulted in the production of 52.4 g/L succinic acid with yield and productivity of 1.16 mol/mol and 1.80 g/L/h, respectively. Several omics tools, including transcriptomics, proteomics, and fluxomics, were employed to better understand the metabolic and cellular status of *M. succiniciproducens* strains to further increase succinic acid production (Jang et al. 2007; Kim et al. 2008; Kim et al. 2007; Lee et al. 2006a). Studies on $CO_2$ concentrations in the media and the development of a defined medium for culturing *M. succinicproducens* were also investigated to optimize the fermentation environment for the succinic acid production (Song et al. 2008; Song et al. 2007). The combination of genetic engineering, omics tools, and the optimization of the fermentation environment for the recombinant *M. succinicproducens* strain resulted in the improved microbial production of succinic acid and provides a blueprint for further studies in other systems.

**With advancement of systems metabolic engineering, the production of industrially relevant platform chemicals by microorganisms from renewable resources has become economically feasible.**

## 2. Putrescine

Putrescine (1,4-diaminobutane), a four carbon diamine is an important platform chemical used to synthesize nylon-4,4 and nylon-4,6, by condensation polymerization with succinic and adipic acids, respectively. Recently, *E. coli* was metabolically engineered to produce putrescine in *E. coli* from renewable feedstock by our group (Qian et al. 2009). To obtain putrescine from *E. coli*, the genes involved in the putrescine degradation, utilization, and competition pathways were deleted to funnel the metabolic fluxes into the putrescine biosynthesis pathway. Then, genes (*argCDE* and *speC*) in ornithine biosynthetic pathways were amplified to convert the precursors to putrescine. Regulatory control was relieved through the deletion of *rpoS,* removing the RpoS-mediated stress responses which would negatively affect the production of putrescine. The combined genetic alterations resulted in a recombinant *E. coli* strain capable of producing 24.2 g/L of putrescine with a volumetric productivity of 0.75 g/L/h in fed-batch fermentation.

## 3. Cadaverine

Cadaverine (1,5-diaminopentane), a five carbon diamine, is also widely used as a building block chemical for polyamides and polyurethanes. Particularly, cadaverine is a constituent of polyamide 54 where 3.5 million tons are produced annually (Kind et al. 2010). Polyamide 54 is synthesized by condensation polymerization of cadaverine and succinic acid (Qian et al. 2011). The bacterium *E. coli* was also metabolically engineered to produce cadaverine (Qian et al. 2011). To achieve cadaverine production with *E. coli,* genes responsible for cadaverine degradation and utilization pathways were inactivated to allow the host to accumulate cadaverine. Competing pathways were also inactivated to direct the metabolic flux towards cadaverine biosynthesis. Then, the *dapA* gene, which encodes dihydrodipicolinate synthase

involved in the formation of L-lysine, an important intermediate for cadaverine, and the *cadA* gene, which encodes L-lysine decarboxylase that converts L-lysine to cadaverine, were overexpressed to amplify the cadaverine biosynthetic pathways in *E. coli*. The resulting recombinant *E. coli* was able to produce 9.61 g/L of cadaverine with a volumetric productivity of 0.32 g/L/h in a fed-batch fermentation.

## 4. 1,4-butanediol

1,4-BDO is one of four stable isomers of butanediol. This isomer has hydroxyl groups at both ends of the four carbon backbone. 1,4-BDO has traditionally been synthesized chemically from petroleum-based feedstocks, such as acetylene, formaldehyde, propylene and succinic acid. Industrial applications of 1,4-BDO include its use as a solvent in polymer synthesis and as a precursor to synthesize other important chemical compounds, such as tetrahydrofuran.

Using systems level metabolic engineering strategies, the production of 18 g/L 1,4-BDO in *E. coli* utilizing carbohydrate feedstocks was achieved (Yim et al. 2011). This report is the first reported use of microbial cell factory as a platform for 1,4-BDO production. Here the authors discuss the challenges in synthesizing the pathways for 1,4-BDO production, a non-native product for the host *E. coli*, and the hurdles in the synthesis of such a highly reduced chemical. Furthermore, strategies in the systems level utilizing the genome-scale metabolic model of the host organism and algorithms to optimize 1,4-BDO biosynthesis are discussed in this example.

## 5. 1,2-propanediol

1,2-PDO is a three carbon diol that is used in cosmetics, pharmaceuticals and as an antifreeze. However, 1,2-PDO is traditionally synthesized from petrochemical sources and an increasing trend toward employing renewable feedstocks has led to the investigation of microbial systems as a platform for 1,2-PDO production. In Korea, Lee and his colleagues at Sogang University, has reported the use of the yeast *Saccharomyces cerevisiae* as a host for production of 1,2-PDO (Jeon et al. 2009). To achieve 1,2-PDO production, the genes for the enzymes methylglyoxal synthase (*mgs*), from *E. coli,* and glycerol dehydrogenase (*gldA*), from *Citrobacter freundii*, was introduced into *S. cerevisiae.* This recombinant yeast achieved a production of 0.45 g/L 1,2-PDO (Jeon et al. 2009).

In another study from Oh and his colleagues at Korea University, the two genes were both taken from *E. coli* and expressed in *S. cerevisiae* that is deficient in triosephosphate isomerase 1 (*tpi1).* Glucose flask cultures of the recombinant strain yielded 1.11g/L of 1,2-PDO (Jung et al. 2008).

## 6. 3-Hydroxypropionic acid

The chemical 3-Hydroxypropionate (3-HP) has been employed to derive several important commodity and specialty chemicals. Some applications include, cross-linking agent for polymer coatings, metal lubricants, antistatic agents for textiles, and precursors for compounds such as 1,3-PDO, acrylic acid, methyl acrylate, acrylamide, ethyl 3-HP, malonic acid, propiolactone, and acrylonitrile. The use of *E. coli* to produce 3-HP has been reported in 2008 by Park and his colleagues at Pusan National University (Mohan Raj et al. 2008). Here, the *E. coli* BL21 strain was engineered to convert glycerol to 3-HP using enzymes encoded by genes from two other species: glycerol dehydratase (*dhaB*) from *K. pneumoniae* and aldehyde dehydrogenase (*aldH*) from *E. coli* K-12.

The resulting strain of *E. coli* (strain designation SH254) was then refined in later publications by the same group to improve the production and yield of 3-HP from glycerol (Mohan Raj et al. 2009). Furthermore, the enzymes that were introduced into the *E. coli* BL21 host were refined to improve their performance and the stability of the enzymes in the host (Rathnasingh et al. 2009). It was found that there was an imbalance between the two foreign enzymes introduced into *E. coli* BL21 and that the instability of *dhaB* was the limiting factor in 3-HP biosynthesis. This problem was dissolved by utilizing a different expression vector for *dhaB*, expressing a glycerol dehydratase reactivase (*gdrAB*), and utilizing an alternate enzyme for *aldH*, an α-ketoglutaric semialdehyde dehydrogenase (KGSADH). The resulting strain of *E. coli* (strain SH-BGK1) produced 38.7 g/L of 3-HP with a yield of 0.35 g/g glycerol.

# Biorefinery research for biopolymers production

Plastics are widely used and have been traditionally synthesized from fossil resources. However, due to the declining petroleum reserves and many environmental concerns, a search for alternatives to these petroleum derived plastics is in progress. Furthermore, the low biodegradability of these petroleum-based plastics has created an environmental problem. Biological polymers such as polyhydroxyalkanoates (PHAs), have presented themselves and suitable alternatives due to their similar properties and degradability in the environment.

## 1. Polyhydroxyalkanoates (PHAs)

PHAs are microbial polyesters which are accumulated by several bacteria, such as *E. coli*, *Ralstonia eutropha*, and *Alcaligenes latus*, under unbalanced growth conditions as intracellular carbon and energy reserves (Lee 1996). Since poly[3-hydroxybutyrate (3-HB)] (PHB) was first observed, more than 150 different monomers have been identified in PHAs produced by microbes depending on the different carbon sources utilized for growth (Keshavarz and Roy 2010; Yang et al. 2011). The diversity of monomers enabled the synthesis of various types of PHAs having different physicochemical properties according to their side groups and the possibility of co-polymerization with different monomers has been investigated (Fukui et al. 2002; Isemori et al. 2006; Kim et al. 2005; Lee 2006; Yang et al. 2011).

The production of PHB in *E. coli* was achieved by introducing the genes encoding the enzymes of the PHA biosynthetic pathways, including PHA synthase, β-ketothiolase, and acetoacetyl-CoA reductase, taken from native PHA-producing bacteria, such as *R. eutropha* or *A. latus* (Ahn et al. 2000; Choi et al. 1998; Schubert et al. 1988; Wang and Lee 1997). The host *E. coli* has been regarded as a good candidate of PHB production, compared to native producers, because its genetic manipulation is uncomplicated, it utilizes various carbon sources, it can accumulate PHB up to 90% of the dry cell weight, it

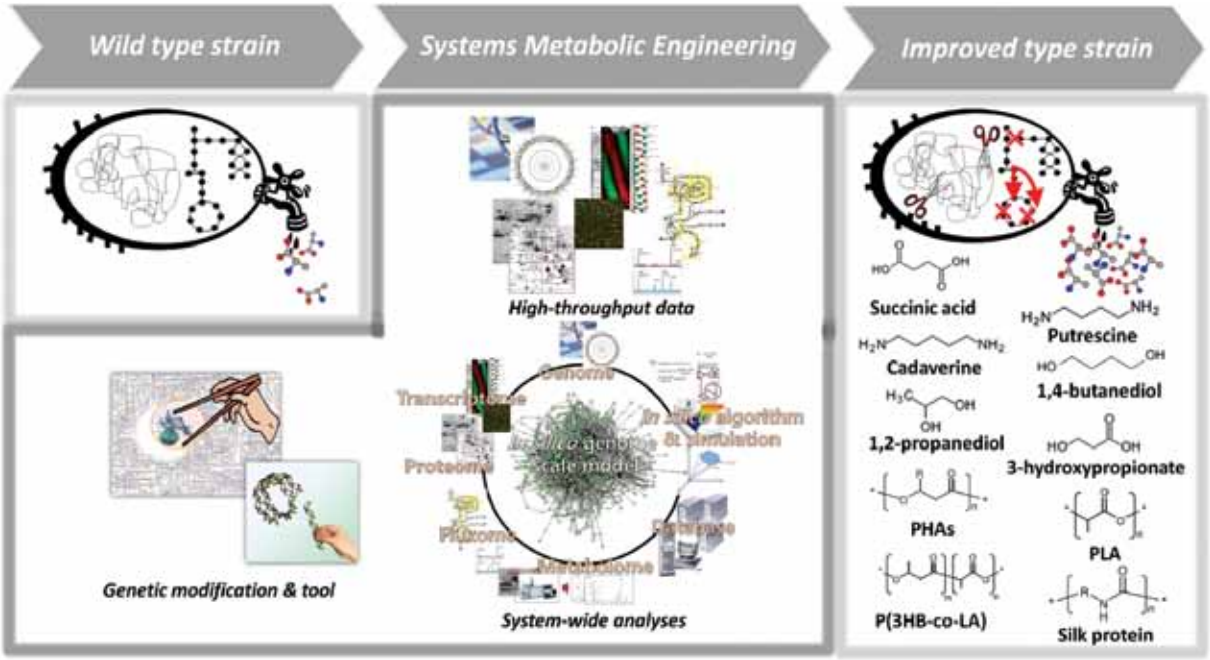


Figure 2. Systems metabolic engineering strategy for strain development on biorefinery by integrating systematically high-throughput data, computational methods and traditional experiments.

contains no intracellular depolymerase that can degrade PHB, and accumulated PHB in its intracellular space can be easily extracted due to its fragility (Choi et al. 1998; Fidler and Dennis 1992). However, it was found that the recombinant *E. coli* strains produced PHB with a lower productivity compared to the native PHB-producers. Therefore, to improve the PHB productivity of the recombinant *E. coli* strain, Choi et al. (1998) performed a pH-stat fed-batch culture and achieved a production of 141.6 g/L of PHB with 4.63 g of PHB/L/h of volumetric productivity (Choi et al. 1998).

## 2. Polylactic acid (PLA)

An unnatural polymer, PLA, was successfully synthesized by a metabolically engineered *E. coli* directly from fermentation, in distinction from existing two-step PLA production process where lactic acid was first produced via fermentation and was then chemically polymerized into PLA (Cho et al. 2006; Jung et al. 2010; Taguchi et al. 2008; Yang et al. 2011; Yang et al. 2010). PLA and its copolymers are promising alternatives to petroleum-derived plastics due to the biodegradability, biocompatibility, compostability, and low toxicity to humans (Jung and Lee 2011; Mehta et al. 2005; Vink et al. 2003). For the microbial production of PLA, lactyl-CoA is a key intermediate because it can be utilized as a substrate by PHA synthase and polymerized into PLA. Thus, two key enzymes, propionate CoA-transferase (*pct*) from *Clostridium propionicum* to synthesize lactyl-CoA and PHA synthase (*phaC1*) from *Pseudomonas sp. MBEL 6-19* to polymerize the lactyl-CoA into PLA, were engineered by directed evolution (Jung et al. 2010). Furthermore, *in silico* simulation suggested the inactivation of genes (*ackA*, *ppc*, and *adhE*) for competing pathways and the induction of genes (*ldhA* and *acs*) responsible for precursor-producing pathways *E. coli* strain containing the evolved propionate CoA-transferase and PHA synthase. As a result, the recombinant *E. coli* strain yielded 11 wt% PLA homopolymer from glucose and 56 wt% P(3-HB-co-LA) copolymer containing 55-86 mol% of lactate from glucose and 3-HB. This *E. coli* strain was further engineered to enable direct production of P(3-HB-co-LA) from glucose only. By introducing β-ketothiolase and acetoacetyl-CoA reductase from *Cupriavidus necator*, formerly *Ralstonia eutropha*, 46 wt% of P(3-HB-co-LA) copolymer containing 70 mol% of lactate was produced from glucose as a sole carbon source (Jung et al. 2010).

## 3. Silk protein

Fibers spun from spider dragline silk protein is exceptionally strong and elastic and is five times stronger by weight than steel and three times tougher than the highest quality Kevlar fiber. The spider dragline silk protein is primarily composed of two proteins: the major amullate spidroins 1 (MaSp1) and 2 (MaSp2). These spidroins are composed of a repetitive modular sequence and are flanked by nonrepetitive sequence of 100 amino acids. The repetitive regions are rich in glycine and alanine, where the alanine rich regions form the hydrophobic crystalline domains that give the silk the high tensile strength and the glycine rich regions are hydrophilic and give the elastic properties of the silk. Because of the attractive properties of high tensile strength and elasticity, utilization of spider silk in a variety of applications, including protective clothing and biomedical applications is desired. However, farming of spider silk is problematic in that the spiders are aggressive and highly territorial. Therefore, Xia et al. (2010) explored the use of microbial platforms for the production of spider silk (Xia et al. 2010).

Xia et al. reports the use of the bacterium *E. coli* to produce silk proteins of the organism *Nephila clavipes* rich in glycine with molecular weights of up to 290 kDa (Xia et al. 2010). The genes for the silk proteins were introduced into *E. coli* creating a recombinant strain and the expression of the genes were

optimized to the host. Furthermore, metabolic engineering was performed to increase the glycine pool in the host by up-regulating important glycine biosynthetic enzymes: serine hydroxymethyltransferase (GlyA) and the beta-subunit of glycyl-tRNA synthetase (GlyS). The combined effect of all the systems-level engineering of *E. coli* resulted in the increase spider silk production of up to 35-fold.

# Future perspectives

The world has been moving towards reducing the dependence of petroleum feedstock in recent years due to dwindling supply and increased prices and thus moving towards employing renewable resources for commodity and high valued chemicals. The use of microbial systems as biorefineries illustrates one approach towards this independence from petroleum. However, there is the problem that microbial systems are not often naturally optimized to achieve high production of the target chemicals and therefore economically infeasible.

Recently, systems metabolic engineering is actively employed to design new strains that can achieve high production rates and thereby making the biorefinery economically feasible. Systems metabolic engineering strategies have been enhanced by the development of high-throughput technologies, which include, but are not limited to, omics data for various environmental and genetic perturbations (Lee et al. 2011; Park et al. 2008). In parallel, advances in computational and systems biology have allowed us to investigate cellular metabolism and physiology at systems-level by integrating physiological omics data in a systematic manner. Systems metabolic engineering powered by the systems-level and genome-wide analyses and computational tools is providing a new paradigm for developing strains with advanced capabilities (Lee et al. 2005; Palsson and Zengler 2010; Park et al. 2008)

Thus, systems metabolic engineering for biorefinery will extend our scope of engineering, allow better understanding on cells, and generate novel knowledge on the biological systems (Fig. 2). Employing these systems metabolic engineering, biorefinery research in Korea has reached a critical point in developing the strategies and knowhow and must push forward to spearhead the research on biorefinery using microbial platforms worldwide.

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