Metabolic engineering is an integrating methodology of analysis and synthesis for the improvement of flux distribution of metabolic pathways in complicated bioprocesses, which are highly multi-hierarchical systems to extend from macroscopic to microscopic levels. Recent progress in metabolic engineering methodologies to improve metabolic pathways in microorganisms was reviewed with many studies in this paper. Metabolic flux distribution was analyzed under different environmental conditions, using a metabolic reaction model. The physiological states of microorganisms were understood by interpreting metabolic flux analysis (MFA). This analysis was also used for development of process operation and control strategy. Cell capability to form a targeted product was analyzed with a metabolic reaction model and linear programming (LP). The use of a $^{13}$C-enriched carbon source and nuclear magnetic resonance (NMR) and gas chromatography–mass spectrometry (GCMS) analyses of intracellular and extracellular metabolites enabled determination of a metabolic flux distribution more accurately than the flux distribution determined only by the metabolic reaction model, which involves not only metabolite balances but also energy and redox balances. The comparison of metabolic flux distributions between before and after genetic modification of cells yielded information on the mechanism of regulation of metabolic flux in microorganisms. Finally, integration of bioinformatics and metabolic engineering is discussed, and cyclic modification of the complex bionetwork and process development were emphasized.

**Key words:** metabolic flux distribution analysis (MFA), metabolic control analysis (MCA), on-line state recognition and process operation, $^{13}$C-NMR, bioinformatics

Bioprocess systems engineering was developed as a methodology for making engineering decisions in biochemical systems, when bioprocesses are applied to the production of target products and optimization of the process for the maximization of productivity and production yield. Since the bioprocess involves growing microorganisms as catalysis of biochemical reactions, the most significant issues are how the cell growth is enhanced and how cell capability of producing the target products is realized in the process. Reactor design, operation strategy including optimization, and process control are key technologies in this field (1, 2).

For making rational engineering decisions, mathematical models representing the behavior of microorganisms in bioprocesses are highly required. Due to the complex relationship among subsystems, and the nonlinearity and heterogeneity of the kinetic behavior of elements in the systems, which are characteristics of biological processes, it has been very important to construct good models for logical analysis of complex systems (3) and for realization of optimal decision-making (4).

Since the profile of metabolites is directly related to the behavior and phenotypes of microorganisms in bioprocesses, it is reasonable to analyze the microorganisms based on a metabolic network when bioprocess systems are optimized through molecular breeding and process development. Metabolic engineering is a methodology for optimizing bioprocesses through quantification of features of metabolic networks for purposes of conferring to the microorganisms’ desirable properties in order to develop optimal process operations. Metabolic engineering is expected to become a new framework of bioprocess systems engineering, in which not only macroscopic bioprocess optimization such as process development but also microscopic bioprocess optimization such as molecular breeding can be achieved systematically (5–8).

I. ORIGIN OF METABOLIC ENGINEERING

The first stage of studies exploiting techniques to quanti-
tatively analyze metabolic pathway information has been carried out with a technique using signal flow diagrams by researchers in the field of biochemical engineering in the 1970’s (9). Estimation of grades of metabolic reactions in citrate production was carried out based on material balances of intracellular metabolites (10). Sensitivity analysis of enzyme amount or activity against perturbation of biochemical networks has also been started in the field of theoretical biochemistry (11, 12). The technical term “metabolic engineering” emerged in the 1990’s and was defined as a targeted improvement methodology of product formation or cellular properties through the modification of specific biochemical reactions. The importance of analyzing the network rigidity in primary metabolic pathways (6) and of recruiting heterologous activities, such as heterologous enzymes and transport systems (5), has been emphasized.

II. PROGRESS IN METABOLIC ENGINEERING

Methodologies used in metabolic engineering and their applications are shown in Table 1.

**Metabolic flux analysis (MFA) and observability of metabolic pathway** One of the great contributions of metabolic engineering to biology and biotechnology is the integration of macroscopic analysis of entire bioprocesses such as fermentation and microscopic analysis of intracellular metabolic reactions such as networks of enzyme reactions. A set of reaction rates in a metabolic pathway is called metabolic flux distribution, and cell physiology can be interpreted and understood by observing the metabolic flux distribution under an investigated fermentation condition. The impact of a genetically engineered strategy on the flux distribution under an investigated fermentation condition can be also discussed by comparison of flux distributions between a wild-type strain of the microorganism and a recombinant microorganism, in which a specific gene was introduced, enhanced, or deleted genetically.

![Diagram of metabolic flux analysis](image)

Balance equations for the intracellular and extracellular metabolites included in the metabolic reactions given above can be represented (in Eq. 1) as (15)

\[ AR_c = r_m \]

where \( r_c \) and \( A \) are the calculated reaction rate vector (\( n \)-dimensional flux vector), measured reaction rate (\( m \)-dimensional reaction) vector, and \( (m \times n) \) matrix of stoichiometric coefficients, respectively. \( r_m \) involves intracellular and extracellular metabolite accumulation rates, and a pseudo-steady state (PSS) approximation is used for determining \( r_c \) of intracellular metabolites, that is, the accumulation rates of intracellular metabolites are zero. The concept is shown in Fig. 1.

The observability of the metabolic pathway is discussed in relation to the degree of freedom in the pathway, \( d \), defined as

\[ d = n - m \]

If \( d \) is equal to zero, that is, the number of unknown reaction rate is the same as the number of measured values and \( A \) is of full rank, metabolic fluxes are uniquely determined using measured values but consistency of the model cannot be checked. In this case, the system is called a “determined system”.

If \( d \) is less than zero, that is, the number of measured values is greater than the number of unknown reaction rates and \( A \) is of full rank, metabolic fluxes are determined using measured values with the least squares method as

\[ r_c = (A' A)^{-1} A' r_m \]
In this case, the consistency of the metabolic reaction model can be checked by comparison with the consistency index of the developed model, $h$, with $\chi^2$ statistics value of the degree of redundancy of the system (16, 17).

If $d$ is greater than zero, that is, the number of unknown reaction rates is greater than the number of measured values, metabolic fluxes cannot be uniquely determined with the measured values. The importance of developing analytical methods and equipment for determining the total number of metabolites ("metabolome") should be emphasized. Another possibility of computational study is the analysis of pathways from each of glucose and acetate for histidine production, cell growth, and ATP consumption for cell maintenance.

One example of metabolic flux distribution in the over-determined lysine synthetic pathway of *C. glutamicum* AJ3462 is shown in Fig. 2 (18). This strain is an S-(2-aminoethyl)-L-cysteine (AEC)resistant and leucine-, phenylalanine-, and tyrosine-auxotroph mutant (19). The flux distributions in the metabolic pathway in States 1 (cell growth) and 2 (lysine production) are compared in Fig. 2. The numerical values in this figure are the molar flux percentages in the metabolic pathway for lysine production, the upper and lower values being the molar fluxes in States 1 and 2, respectively. The flux of lysine production in State 2 was much higher than that in State 1, and the flux distribution evidently changed.

Using biochemical stoichiometric equations for the metabolic pathways, flux distributions were analyzed by solving a set of algebraic equations. The information on metabolic flux distribution was used in order to understand the phenomena in many fermentation processes (20–24). A metabolic reaction model was also used for optimization of fermentation processes (25).

**Capability analysis** Palsson and coworkers (26) developed an analytical method for investigating cell capability. In their method, Eq. 1 is considered to be a domain of the stoichiometrically allowable behavior of the investigated strain and this domain is defined as the "metabolic genotype" of the strain since it describes the metabolic flux distributions that can be achieved with the metabolic enzymes that the strain possesses. A particular solution for determining metabolic flux distribution is found by linear programming (LP) so that an objective function is maximized (for example, maximization of product formation). The determined flux distribution is the cell capability associated with the objective function set (26).

The capability of *Escherichia coli* to convert some sources, such as glucose, glycerol, acetate into amino acids and nucleotides was investigated using metabolic reaction models of *E. coli* by LP (27, 28). The change in metabolic flux distribution was also investigated when conditions such as supplies of oxygen (29) and carbon sources (30) were changed. The change in mole flux distribution was also predicted when some of the genes in the pathway were deleted. It is expected that bioinformatic data will be integrated for the construction of a map to be used for analysis (31, 32).

By comparison of the theoretical maximum flux in each pathway and the actual flux, performance of each pathway can be evaluated, namely, it can be evaluated how much percentage of cell capability is realized in the reactor. The metabolic flux pattern for histidine production by *Brevibacterium flavum* FERM1564 was analyzed when glucose and/or acetate was used as carbon source(s) (33). Total histidine production was enhanced when a mixed substrate (glucose and acetate) was used, compared with that when either glucose or acetate was used as the sole carbon source. Theoretical maximum carbon fluxes through the main pathways from each of glucose and acetate for histidine production, cell growth, and ATP consumption for cell maintenance were obtained by the LP method.

In order to investigate the roles of the main pathways for histidine synthesis, biomass, and m$_{\text{ATP}}$, the relative percentages among these three pathways were calculated and the results are shown in Fig. 3. In this analysis, the actual flux distribution was determined by superposition with the percentages of each original flux distribution for maximum histidine production, maximum cell growth, and maximum ATP consumption for cell maintenance. When glucose was used as the sole carbon source, the superposition of the flux distribution for the theoretical maximum histidine production was 8.2%, as shown in Fig. 3a, that for the theoretical maximum cell growth 34%, and that for the theoretical maximum ATP consumption 57%; these values gave the actual flux distributions. Thus, it is interpreted that a large amount of glucose (57%) was necessary for cell maintenance in glucose culture.

Much acetate (85%) was used for biomass formation in the acetate culture, as shown in Fig. 3b. The percentages of histidine production and ATP consumption were 4.7% and 9.7%, respectively. Furthermore, in the mixed-substrate cul-
ture, the percentages of both carbon sources were kept at the same level as shown in Fig. 3c. Flux distribution in the mixed culture was represented by a linear combination of glucose and acetate cultures.

**On-line estimation of flux distribution**  
Metabolic flux analysis was carried out through an on-line approach — that is, the fluxes of not only extracellular but also intracellular metabolites were calculated for lysine fermentation by *C. glutamicum* in order to recognize the physiological state (18). In lysine production by *C. glutamicum* AJ-3462, a high productivity level was attained at a low leucine concentration in leucine-limited and glucose-limited fed-batch cultures. Thus, in order to produce lysine at a high yield, the leucine concentration should be kept low. However, the concentration of leucine cannot be measured on-line, moreover, sometimes the leucine concentration needs to be controlled at a level lower than the detection limit of an amino acid analyzer.

The extended Kalman filter method was applied to estimation of the specific growth and glucose consumption rates on-line, after which lysine production rate and metabolic flux distribution were estimated only from on-line measurable rates, including the filtered specific growth and glucose consumption rates calculated using the metabolic reaction model. Figure 4 illustrates sample results of physiological state recognition — the transition from cell growth (State 1) to lysine production (State 2). The top and middle sections of the figure show the leucine and lysine concentrations measured off-line using an amino acid analyzer, respectively. The lysine concentration increased immediately after the depletion of leucine. The on-line calculated lysine production rate, $r_L$, obtained using the metabolic reaction model described above, increased to more than 0.0075 mol/h after the depletion of leucine, and a high lysine production was attained. In this example, glucose was fed to the fermentor at 10.5 h, after the transition of the physiological state from State 1 to State 2 was recognized.

Furthermore, a small change in metabolic flux of lysine synthesis due to the addition of leucine was detected in order to maintain not only the high lysine production rate but also the high molar production yield of lysine from glucose aided by the metabolic engineering approach (34). Another study on the on-line flux analysis was reported in acetone–butanol fermentation (35).

**Improvement of metabolic pathway by recombinant DNA technology and molecular biology**  
One of the most significant aims in metabolic engineering is to systematically confer a desired characteristic to the microorganism. Recent progress in recombinant DNA and other molecular biological techniques enables us to carry out well-defined genetic perturbation, that is, amplification, disruption, and/or modification of the targeted genes that encode the enzymes in the metabolic pathways in many kinds of industrial microorganisms (8). Concrete strategies to this end are summarized as follows (6, 7): enhancement of bottleneck reaction (36) in biosynthesis, repression of by-product synthesis (37), enhancement of redox and energy generation (38, 39), and recruiting heterologous genes to give new characteristics (40, 41) were all investigated. Hereafter, genetic improvement studies for amino acid synthesis only are briefly reviewed due to limitation of the space.

Amino acids have been produced at the current rate of more than $10^6$ t/year in the world and glutamate and lysine are produced during fermentation by *C. glutamicum*. The genome size of this microorganism is about 3.3 Mbp and its entire genome has been completely sequenced (Nakagawa et al., Eur. patent 1,108,790, 2001) (42); thus its genome data are available in the database. There have been many studies concerning the mechanism of overproduction of these amino acids, which are summarized in review papers.
Since the key branch points of glutamate and lysine syntheses are 2-oxoglutarate and oxaloacetate in the TCA cycle, respectively, supply of these compounds is very important for production of these amino acids through anaplerotic pathways. The roles of pyruvate carboxylase, phosphoenolpyruvate carboxylase, and phosphoenolpyruvate carboxikinase were studied (45–48). Deletion of phosphoenolpyruvate carboxikinase activity resulted in increase in lysine biosynthesis (49). Enhancement of dapA encoded dihydriodipicolinate synthase resulted in improvement of lysine synthesis (50) and determination of the transporter gene of lysine llysE enabled determination of the mechanism of lysine overproduction (51).

The mechanism of glutamate overproduction is not yet fully understood. Thus far, a combination of three significant points regarding the above mechanism is discussed: (i) decrease in 2-oxoglutarate dehydrogenase activity, (ii) possible presence of a specific exporter, (iii) change in states of the membrane barrier. It has been well known that there are some triggering phenomena for glutamate production by C. glutamicum: depletion of biotin which is required for cell growth, addition of a detergent such as polyoxyethylene sorbitan monopalmitate (Tween 40), and addition of β-lactam antibiotics such as penicillin. Enhancement of glutamate dehydrogenase (GDH) (52) and isocitrate dehydrogenase (ICDH) activities does not have significant impact on glutamate overproduction (53). On the other hand, a decrease in the activity of the 2-oxoglutarate dehydrogenase complex (ODHC) and enhancement of glutamate production were observed after these triggering operations (54). Molecular cloning of 2-oxoglutarate dehydrogenase (odhA) was carried out (55). Other important factors related to dtsR gene (56) and alterations of the states of cell wall barrier (57–59) were reported. Improvement of the histidine and tryptophan synthetic pathways was also studied in detail (60, 61).

**Metabolic control analysis (MCA)** To improve metabolic pathways systematically, their quantitative regulation is highly desired. An important issue to this aim is to elucidate the parameters responsible for the control of flux in metabolic pathways. The theoretical basis for the metabolic control analysis (MCA) was developed in the 1970’s (11, 12) and generalized in the 1990’s (62, 63). The theory was applied to investigation of the control mechanism of many complicated metabolic networks. The concept of metabolic control analysis allows the establishment of the quantitative regulation of metabolic flow through sensitivity parameters called control coefficients.

Flux control coefficients, FCCs, $C^i_k$, are defined as the relative effects of infinitesimal changes in the activities of enzymes on changes in the steady-state fluxes.

\[
C^i_k = \frac{\partial J_i/\partial e_j}{e_i/e_j}
\]

where $e_i$ and $J_i$ are the enzyme activity of reaction $i$ and the flux of reaction $k$, respectively.

In MCA, two approaches to determine FCCs were proposed and studied, that is, bottom-up approach and top-down approach (7, 15). The bottom-up approach is based on the kinetic model of a metabolic pathway, which is very robust if the kinetic model represents the real metabolic pathway (64, 65). However, there exist difficulties in constructing the real kinetic model because it is necessary to identify many kinetic parameters in the model. The top-down approach is based on a large perturbation of enzyme activities and involves target gene modification and comparison of flux distribution before and after the introduction of perturbation. The large-perturbation theory is easy to extend to the analysis of a branched point, using small number of perturbation experiments on enzyme activities and metabolic flux distribution data (66–69), and the effectiveness of this method was confirmed in lysine synthesis (70).

In glutamate production, the quantitative degree of impact of changes in the enzyme activities around a key branch point, 2-oxoglutarate, on a target flux of glutamate was investigated by the top-down approach (71). A metabolic reaction (MR) model was constructed for the central carbon metabolism and glutamate synthetic pathways, and consistency of the model was statistically checked. Metabolic flux distribution, particularly, flux distribution at the 2-oxoglutarate branch point was investigated in detail. The enzyme activities of isocitrate dehydrogenase (ICDH), glutamate dehydrogenase (GDH), and the 2-oxoglutarate dehydrogenase complex (ODHC) at the branch point were changed using two genetically engineered strains and controlled environmental conditions. GDH and ICDH activities were enhanced by transformation using each plasmid, which involved homologous gdh and icd genes, respectively. The ODHC activity was attenuated under the biotin-deficient condition. The FCCs were experimentally determined by the method based on the large-perturbation theory without precise kinetic information.

Figure 5 shows the redistribution of fluxes around the 2-oxoglutarate branch point. It was found that the enhancement of ICDH and GDH activities did not significantly affect the flux distribution at the 2-oxoglutarate branch point. Even though ICDH and GDH activities were enhanced to 2.96 and 3.21, respectively, more than 70% of carbon flux still flows into the TCA cycle. On the other hand, when the ODHC activity decreased to around 50% after the depletion of biotin, marked changes in fluxes of GDH and ODHC were observed. More than 75% carbon was directed to glutamate production, which is the effect of three enzymes on glutamate production.

FCCs were determined together with flux and enzyme activity data, and calculation results are summarized in Table 2 (71). The FCCs of ODHC attenuation for glutamate production became negative because glutamate production was enhanced in the decrease of ODHC activity, namely, a

<table>
<thead>
<tr>
<th>Flux</th>
<th>Enhanced ICDH activity</th>
<th>Attenuated ODHC activity</th>
<th>Enhanced GDH activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$J_i$ (ICDH)</td>
<td>0.32</td>
<td>0.33</td>
<td>0.19</td>
</tr>
<tr>
<td>$J_i$ (ODHC)</td>
<td>0.08</td>
<td>3.43</td>
<td>0.22</td>
</tr>
<tr>
<td>$J_i$ (GDH)</td>
<td>1.67</td>
<td>−16.9</td>
<td>0.025</td>
</tr>
</tbody>
</table>

The mechanism of glutamate overproduction is not yet fully understood.
competitive pathway showed negative sensitivity. The most important issue shown in Table 2 is that the greatest impact on glutamate production indicates the highest absolute value of the FCC. The highest value of FCC is that of the ODHC attenuation. The second impact on glutamate production was observed during enhancement of ICDH activity, and enhancement of GDH activity was not effective for glutamate production. The flux distribution analysis and FCCs data quantitatively supported the notion that the enhancement of the GDH and ICDH activities does not affect glutamate production (52, 53) but ODHC attenuation after biotin depletion is a trigger of glutamate production (54). Summation of all FCCs in each raw for the enzyme activity changes for each flux was not unity. One obvious reason is that there are no constraints related with the summation theorem in the FCC determination method used here. This suggests that there are enzymes other than those that did not exist around the 2-oxoglutarate branch point that possibly control the metabolic network flux. It was reported that deletion of phosphoenolpyruvate carboxikinase and block the backward flux of anaplerotic pathway from phosphoenolpyruvate to oxaloacetate was effective for production lysine (49). The modification of the pathway might be also effective for further glutamate production.

**Experimental determination of flux distribution by isotope labeling** Although metabolic flux analysis is a powerful technique for investigation of metabolic pathways, the use of material balances and measurements of reaction rates of extracellular metabolites has limitations in the elucidation of metabolic pathways, such as (i) flux distribution at split points that converge at another point of the network, (ii) metabolic cycles, (iii) an unknown network structure and (iv) unknown consumption and regeneration of cofactors. Through the use of a $^{13}$C-labelled carbon source and measurement of the labelling pattern of intracellular metabolites by nuclear magnetic resonance (NMR) and gas chromatography–mass spectrometry (GCMS), it becomes possible to apply balances for individual carbon atoms in addition to metabolite balances (7, 15, 72, 73).

Experimental studies in the field of microbiology on isotope labelling and measurement of enrichment with labelled carbons are not new (74, 75); the systematic determination methods used in these studies were developed in the 1990’s (76). In the lysine synthetic pathway, the succinylase reaction and dehydrogenase reaction with diaminopimeric acid, which converged at lysine, were determined experimentally (77). Bidirectional reaction steps in the central metabolism of *C. glutamicum* were determined with exchange fluxes that were defined as the minimum absolute value of the forward and backward reaction rates. $^{13}$C-NMR and GCMS were applied to the investigation of metabolic flux distribution of hybridoma cells (78), and cells of *Corynebacterium melassecola* (79), *Penicillium chrysogenum* (80). Recently, a two-dimensional NMR (81, 82) technique and *in vivo* NMR (83) were also applied.

The split ratio of a key branch point, 3-ketovaleryl CoA, in the biodegradable co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) was analyzed for mole fraction control of the monomer unit, 3-hydroxyvalerate (3HV) in *Pseudomonas aeruginosa*. 3HV mole fraction was measured by

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**FIG. 5.** Comparison of flux distribution at 2-oxoglutarate (αKG) branch point in glutamate production. Flux distribution of wild type strain (a) was perturbed by enhancement of the *icd* gene (b), enhancement of the *gdh* gene (c), and attenuation of ODHC by biotin depletion.
\(^{1}\text{H}-\text{NMR} \) and \(^{13}\text{C}-\text{NMR} \) methods and the split ratio of the branch point was constant, even though the specific consumption rate of carbon sources (ethanol and \(n\)-pentanol, in this case) changed. From this information, a feeding strategy for mole fraction control was developed (84).

**Kinetic study of metabolic pathways** Since metabolic flux analysis based on stoichiometric equations does not provide any kinetic information, it is impossible to simulate the kinetic behavior of a metabolic pathway in the microorganisms. Thus, it is necessary to build a kinetic model by the bottom-up approach to metabolic control analysis. A kinetic model of the metabolic pathway in *Saccharomyces cerevisiae* was exploited and the dynamics of intracellular metabolites at short-range intervals was discussed (85).

### III. FUTURE PERSPECTIVES IN METABOLIC ENGINEERING

Many microbial genomes have been fully sequenced and the assignment of functions to open reading frame (ORFs) is ongoing. Thus, much information on genomic and biochemical functions will be available in the future. Transcriptome and proteome data are now available. This gives rise to one question: “How will bioinformatics influence metabolic engineering? (31, 86)”. Figure 6 shows bioprocess systems and their elements, in which we can see highly multi-hierarchical systems and the extent of bioprocesses to macroscopic systems and microscopic systems.

As mentioned above, one objective in the field of engineering is to maximize productivity and production yield. To achieve this objective, bioprocess analysis should be performed. In classical biochemical engineering, the concept of specific reaction rates is exploited not only as an important parameter that indicates microbial activities but also as a kinetic parameter in the field of reaction engineering that is used for optimal design of bioreactors and the development of operation strategies. Since the specific reaction rates are defined as reaction fluxes of extracellular metabolites such as substrate and main products, they are useful indicator parameters. Metabolic engineering is a field in which metabolic reactions are focused on and changes in metabolic flux distribution in the cell and extracellular reaction rates are analyzed from a microscopic viewpoint.

Recently, transcriptome analysis by DNA microarray techniques was performed. In a batch culture of *S. cerevisiae*, the gene expression profiles of the microorganisms were studied when its diauxic (glucose and ethanol) growth was observed (87). The gene expressions in *Escherichia coli* (88) and *Corynebacterium glutamicum* (89) were also investigated in fermentation processes.

One contribution of metabolic engineering to bioinformatics field is metabolic flux distribution analysis. Essential gene products of *E. coli* were identified by metabolic flux analysis and optimization techniques under aerobic and anaerobic culture conditions (90). The maximum number of tolerable gene deletions was also predicted (91). Not only gene deletion but also gene addition or introduction were formulated in the network and implemented using a computer (92).

Systematic experiments on perturbation of the metabolic network, namely, systematic deletion of single gene (93) in the specific bio-network, were performed experimentally. Gene and protein expression levels were measured. From the results of the systematic deletion studies of genes involved in the metabolic pathway, many of genes were found to be “silent”, that is, they did not show distinct phenotype, in terms of growth rate or other metabolite fluxes when they were deleted from the genome (94). Even though the specific single gene involved in a particular metabolic pathway was deleted, an alternative pathway should complement the lack of the path.

From this discussion, a strategy for improving metabolic
pathways and for making superior cells, namely, development of a systematic algorithm of modification cycles is recommended in metabolic engineering with many tools of bioinformatics, such as genome database, DNA array, 2-D electrophoresis, mass-spectrometry, and so on (7, 95). The first step in this strategy should be the introduction or disruption of single gene. However, we often encounter situations where genes interact in intricate, nonlinear and unpredictable ways due to alternative and unknown metabolic pathways enrolled in the cells. Therefore, multiple genetic modifications should be carried out to reach the final goal. After several cycles of genetic modifications and process development are performed, if the performance of the cells and metabolic pathway are found to be suitable for the given engineering objective, the cycle can be finalized. By comparative genomic analysis and reconstruction of classically derived production strain with only relevant mutations were also proposed (96).

During cyclic modification of the cells, profiles of gene and protein expressions, metabolic flux distributions should be carefully analyzed. These bioinformatic data of parent cells and modified cells should be compared. Since such bioinformatic data are significantly large and multivariate, data mining technology for selection of significant variables that can represent the difference in physiology of the cells becomes very important (97–100). Multivariate analysis and several types of neural networks used for clustering gene expression patterns (101, 102) and other bioinformatic data treatment are also necessary (103).

According to the careful analysis of a large volume of bioinformatic data, the next modification cycle of the gene network and process development should be designed and developed. Finally, the importance of integrating bioinformatic data of multi-hierarchical biological systems, i.e., gene, protein, and metabolic network systems should be emphasized for attaining the objective. Metabolic engineering methodologies discussed in this paper are powerful tools in cyclic modifications.

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