Microbial Strain Engineering

Chapter · July 2020

DOI: 10.1007/978-981-15-2604-6_2

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Microbial Strain Engineering

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Gaurav Sanghavi, Prabuddha Gupta, Mahendrapalsingh Rajput, Tejas Oza, Ujwal Trivedi, and Nitin Kumar Singh

Abstract

The utilization of microorganisms for the production of chemicals at industrial scale requires improvements/changes at physiological, metabolic, and genetic levels. Natural or wild-type isolates produce minimal quantity of metabolites/compounds required as a matter of survival. Hence, to use these microorganisms at industrial level, different tools are required for strain improvement. These tools will improve the metabolite production of industrial importance. The strain improvement program traditionally employs classical mutagenesis approach followed by screening and selection of mutant strain. Today, in-depth understanding of genetics and recombinant DNA technology helps in strain improvement via metabolic and genetic engineering. These strain improvement approaches has increased the product yield with subsequent cost reduction. These approaches have also served other goals like reduction of undesirable products and elucidating the complex biosynthetic pathways. Further combination of different omics approaches like transcriptomics and proteomics with recombinant DNA technology has increased the prediction of accurate genes responsible for overproduction of metabolites/compounds.

Keywords

Mutagenesis · Physiology · Metabolic engineering · Genetic engineering · Omics

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V. Singh et al. (eds.), *Engineering of Microbial Biosynthetic Pathways*,
https://doi.org/10.1007/978-981-15-2604-6_2

2.1 Introduction

Microbes, the microscopically tiny miniatures, are the most abundant species present in the environment. Microbes are found in all the ecological niches and working as pillars of life on earth. Diverse microbes like bacteria, archaea, fungi, algae, protozoa, and viruses are in existence from at least 3500 million years ago and are supposed to be only present life forms on earth during that time. These life forms encompass the most phylogenetically diverse life on earth with many lineages. They dwell in every habitat including the terrestrial, aquatic, atmospheric environments (Sean and Jack 2015). Their presence reshaped many ecological, aquatic, and terrestrial niches including the extreme environments. Large diversity makes them suitable to live, adapt, and tolerant in many conditions like extreme salty environment, anaerobic conditions, limited water availability, extreme pH. Although, the microorganism grows in different niches, most important thing which makes them special in the way the metabolic pathways change for their existence in a particular environment. For example, although the microorganisms are microscopic with the addition of simple nutrients, they can grow on nutritive media and can be easily visualized by the naked eyes. This makes microbes to study them better. Additionally, microbes have a surface area where it can easily absorb the nutrients and release the end products. They also possess high metabolic activity making the system highly reproductive.

The presence of novel enzymes, high- and low-molecular-weight compounds, and metabolites makes microbes the best suitable source for industries to replace the chemically synthetic procedures with bio-based processes. In a real scenario, microorganisms can act as chemical factories for the production of commercially important compounds. Microbes, isolated from the natural environment and maintained in in vitro conditions, are mostly used for the industrial processes. Nowadays with the indispensable role of microbes in biotechnology, the use of microbes is increasing nearly in all the industries. The industrial sector like health care, pharmaceutical, food and beverages, agriculture, and chemical all are making efforts for generating bio-based/microbe-based process. The microbes, especially from diverse niches, can work as an important source for the discovering novel industrially important entities.

In any industrial process, it is important to consider that substitution to the chemical source needs as effective as any organic/synthesized molecule and also stable under different conditions. Microorganisms offer the best alternative as they are easy to handle and its maintenance is very much economically viable. The most challenging thing in the production of any compound using microorganism is to maintain the efficiency of the microbial line throughout the generations. However, in comparison to the mammalian and plant cells, microorganisms have a greater potential to produce/grow at high density within a short time frame. This makes the microbes feasible for large-scale industrial usage. The major disadvantage might be there is a frequent genotypic change in microorganism due to which microbes are highly variable and prone to the frequent mutations. Microorganisms could provide

everlasting solutions for environmental and societal issues. It can be used for the production of large volume commercially important drug, but the genetic stability with low fermentation economics needs to be prioritized.

For industries, the production of compounds requires to be at relatively lower cost with higher yields. Microorganisms that are used usually go through the strain improvement program for obtaining/screening the best suitable strain for commercial production of the industrially important compounds (Parekh et al. 2000).

2.1.1 Need and Significance for Strain Improvement

Microbes that live freely in the diverse ecological state are less likely to be suitable for the production of novel commercially important compounds. The metabolism of the wild-type isolate is inadequately adapted to the environment which is producing the enzymes and intermediates needed for surviving in a particular physicochemical condition. The regulation of metabolic and genetic machinery is controlled by the sequence of genes in genome. To improve these microbial strains, there is a prerequisite to alter the genes of metabolic pathways for overproduction of desired metabolites. In some cases, these changes lead to structural alteration in specific enzymes which increases the ability of enzyme to enhance its catalytic activity. Also, there are chances that due to alteration in a specific region of the gene (promoter), it can cause the deregulation of gene expression and metabolite overproduction. With the preset data set enzymes function, rate-limiting steps in metabolic pathways, environmental factors controlling growth help in designing screening strategies for the generation of industrially important mutant. However, the outcome of any strain development/selection depends on the kind and type of improvement we expect from the microbes (Elander and Vournakis 1986).

Fermentation economics is majorly dependent on product's manufacturing cost and the raw material prerequisite for production. Although lower fermentation cost can be availed by process designing like fermenter design and constructing material of fermenter, but the improvement in microbial strains offers the best opportunity for the cost reduction. Production enhancement through strain improvement for fermentation process is the prime factor which makes a major impact in fermentation economics.

Microbial engineering technique changes the genetic makeup of the microorganisms. It has played an exemplary role in biotechnology due to its unique features and ease of manipulation using recombinant DNA technology (Kou et al. 2016). For any strain to be used in industries, improvement is concerned with the development or modification for exploiting its properties for the production of compounds with less production cost and cheap raw material. The change in fermentation dynamics works well for optimizing the process for maximum production, but the strain improvement will give the desirable results at long time. The different methods for the strain improvement are given in Fig. 2.1.

Strain improvement/engineering encompasses creation of strains with the following properties.

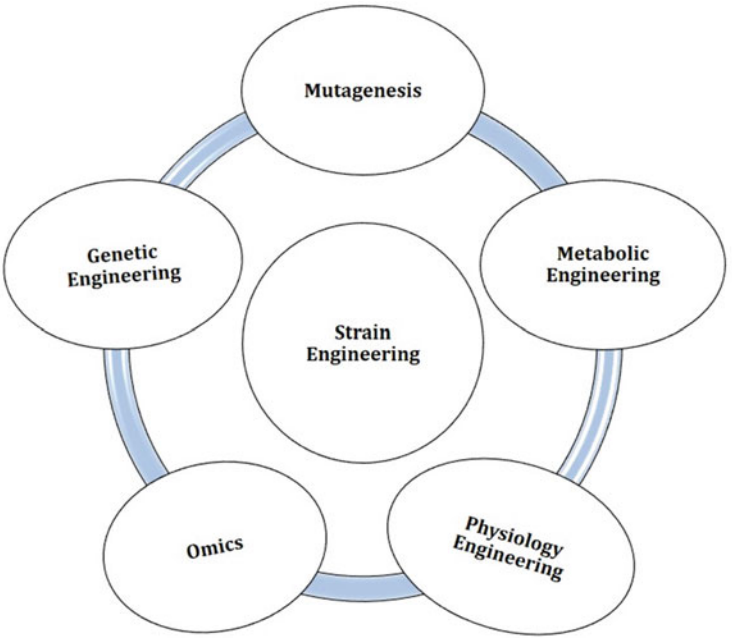

Fig. 2.1 Approaches of strain engineering

(a) Proficient assimilation of low-cost and complex raw materials.
(b) Removal of byproducts and change in the product ratios.
(c) Overproduction and excretion of native and foreign products.
(d) Short duration of fermentation time and easy scale-up.
(e) Tolerance of various metabolites produced (Parekh et al. 2000).
(f) Provide morphological changes in cell which is suitable for product separation.

The traditional empirical approach for the strain improvement is mutagenesis of the isolated/screened strain. The mutagenesis in this approach is random and is followed by the direct titer of large number of isolates. This approach has been successful in most of strain improvement program in pharmaceutical industries (Vinci and Byng 1999; Parekh et al. 2000). Although, the mutation is used to shift the proportion of metabolites produced in the fermentation broth to a more favorable distribution, elucidate the pathways of secondary metabolites, the yield of new compounds, etc. The major disadvantage of these techniques is a laborious procedure as a large number of isolates are processed in order to detect/screen the improved strain. The possibility to reduce the amount of work can be better understood by elucidating the biochemical and genetic mechanisms controlling the different metabolic pathways. Recently, most of the research and academic centers are developing the model organisms which can work effectively for system designing at industrial scale (Rowlands 1984). In context to the above lines, the present

chapter portrays different methods for strain engineering for improved production at industrial scale.

2.2 Mutagenesis

Current research trend focuses on influencing the regulation of specific biochemical pathways and strive for strain improvement to achieve the desirable conditions, metabolite, or phenotype. The strain improvement can either be achieved via a coherent metabolic engineering approach or by randomized mutagenesis strategy (Zhang et al. 2015). Whatever the case may be, a comprehensive understanding of genome and underlying metabolic mechanism of the microbial strain is of prime importance. In order to improve strain by using mutagenesis approach, the information of metabolic regulation mechanism, a well-defined rational plan and a robust method is required (Hu et al. 2017). One of the key challenges in strain improvement programs is involvement of enormous amount of time, cost, and need of skill labor arising due to the complications in understanding multifarious interactions of metabolic pathways. Furthermore, due to the poor understanding of underlying mechanism and pinpointing of precise sequence for manipulation, the radical metabolic engineering approach is always a time-consuming process (Lee and Kim 2015). On the other hand, random mutagenesis involving physical mutagen such as UV is rapid, relatively cost-effective, requires limited training and skill set, and is safer to handle compared to the chemical mutagen. However, physical mutagen generates mutations arbitrarily, and has a possibility to generate nonspecific or multiple mutations in genome.

2.2.1 Physical and Chemical Mutagenesis

The most common physical mutagen uses ionizing radiation such as gamma rays ($\lambda < 0.01$ nm), X-rays ($\lambda = 0.01$–10 nm), alpha particles, and non-ionizing radiation such as UV rays ($\lambda = 10$–400 nm) (Kodym and Afza 2003). The role of physical mutagen operates by two mechanisms, direct and indirect. The direct effect involves the direct ionizing of DNA strands owing to ejection or excitation of electrons to a higher energy level (Ravanat and Douki 2016) (Table 2.1). The indirect effects are produced by shifting of electrons to induce activate molecules, also known as free radicals ($OH^{\bullet}$ and $H^{\bullet}$) that arise from OH^{-} and H^{+}, resulting in base modification and/or single−/double-stranded breaks in the DNA (Morita et al. 2009). These free radical or reactive oxygen species (ROS) can result in the double strand breakages (DSBs) causing deletions and translocations. In some cases point mutation can arise owing to single-strand breaks (SSBs), or due to nitrogen base switchover for example, the conversion of pyrimidine bases to 5-(hydroxymethyl) uracil, 5-formyluracil, 5-hydroxycytosine, and 5-hydroxyuracil (Min et al. 2003).

DNA damage due to UV light exposure can be explained by two mechanisms. UVC ($\lambda = 200$–280 nm) and UVB ($\lambda = 280$–320 nm) are absorbed readily by

Table 2.1 Physical mutagenesis of some key microbial organism

Strain	Species	Mutagen type	Target metabolite	References
Bacteria	*Sporolactobacillus inulinus ATCC 15538*	UV mutagenesis	Lactic acid	Zheng et al. (2010)
	Streptomyces xanthochromogenes RIA 1098	UV mutagenesis	Compactin-resistant	Dzhavakhiya et al. (2015)
	Streptomyces griseoaurantiacus	UV mutagenesis	Production of endoglucanase and β-glucosidase	Kumar (2015)
	Streptomyces flavoviridis G-4F12	UV mutagenesis	6′-Deoxy-bleomycin Z	Zhu et al. (2018)
	Sporosarcina pasteurii (MTCC 1761)	UV mutagenesis	Urease and calcite production	Achal et al. (2009)
	Lactobacillus delbrueckii NCIM 2365	UV mutagenesis	Lactic acid	Kadam et al. (2006)
	Acidianus brierleyi	UV mutagenesis	Tolerance to $Cu2^{+}$ and a high bioleaching rate of chalcopyrite	Meng et al. (2007)
	Clostridium tyrobutyricum ATCC 25755	Heavy-ion irradiation ($^{12}C^{6+}$)	Ameliorate butyrate by 68%	Zhou et al. (2014)
	Dietzia natronolimnaea	Heavy-ion irradiation ($^{12}C^{6+}$)	Boost canthaxanthin	Zhou et al. (2013)
Fungi	*Penicillium janthinellum NCIM 1171*	UV mutagenesis + ethyl methyl sulfonate (EMS)	Boost cellulase	Adsul et al. (2007)
	Rhodosporidium toruloides 8766 2-31 M	UV mutagenesis	Lipid productivity	Yamada et al. (2017)
	Fusarium maire K178	UV mutagenesis + diethyl sulfate (DES)	Paclitaxel (taxol)	Xu et al. (2006)
	Aspergillus terreus CA99	Heavy-ion irradiation $^{12}C^{6}$	Increase lovastatin by four times	Li et al. (2011)
	Trichoderma viride	Heavy-ion irradiation $^{12}C^{6}$	Increase cellulase	Li et al. (2016)
	Aspergillus niger	Heavy-ion irradiation $^{12}C^{6}$	Increase cellulose	Wang et al. (2015)

Algae	*Scenedesmus* sp.	UV mutagenesis	Lipid productivity	Sivaramakrishnan and Incharoensakdi (2017)
	Nannochloropsis oceanica IMET1	Heavy-ion irradiation $^{12}C^{6}$	Boost biomass productivity and growth	Ma et al. (2013)
	Desmodesmus sp.	Heavy-ion irradiation $^{12}C^{6}$	Lipid productivity	Hu et al. (2013)
Cyanobacteria	*Spirulina* sp.	γ-Rays (^{60}Co)	CO_2 fixation	Cheng et al. (2017)

nitrogenous bases, resulting in the trigger of excited states and formation of pyrimidine dimers (Brash 2015). Whereas UVA (λ = 320–400 nm) and to some extent visible light (λ = 400–740 nm) interact via photosensitizers (produces chemical modification in another molecule by means of photochemical reaction) to initiate the DNA damage (Epe 2012).

Often, ionizing radiation results in the biological injuries to the cells and tissues. Therefore, before starting the mutation studies, the exposure and dose should be selectively controlled and maintained. Moreover, bacterial tissues are soft and hence needs lower dose of radiation and exposure. In most of the scenario, the dosing is often limited to 2–3 doses along with control; however, if the primary goal is to obtain a decent amount of mutants in surviving population, the optimum dose should be standardized to obtain the highest proportion of desirable mutants (Sauer 2001).

Unfortunately, either of induced or spontaneous mutations are of little use as the exact mechanism of mutation needs to be thoroughly understood for its repetitive and specific usage. From past many years, specific chemical mutagens like base analogs, deaminating agents, alkylating agents, and intercalating agents are used for the site-specific chemical mutagenesis to obtain desired mutant having industrial application. Certain chemical compounds may result in changes in DNA structure or its sequence, resulting in mutation. Such chemicals, which induce mutations, are known as chemical mutagens. Chemical mutagens or genotoxic compounds are natural as well as man-made. A strategy of using chemical agents to generate mutations in desired strains is known as chemical mutagenesis. Base analogs and DNA intercalating agents are two biggest class of chemical mutagens. A base analog can replace a DNA base during replication and can result in transition mutations. Whereas, intercalating agents are molecules that may get inserted in between DNA bases, resulting in frameshift mutation during replication. Other chemical mutagens may act by the generation of reactive oxygen species (ROS), deamination, alkylation, etc. (Table 2.2).

Chemical mutagens cannot perform a site-directed mutation and generally their effect is hence random that to at multiple sites of genome. Mutagenesis has been used since long in several microorganisms to enhance the performance and productivity arising from single or multiple gene traits (Giudici et al. 2005). A very good example of such strain improvement is production of penicillin antibiotic from *Penicillium chrysogenum* with huge increase of more than three orders of magnitude, attained after 65 years of research and development using multiple mutagenesis techniques (Demain 2010).

2.2.2 Mutation Signature

A mutation signature is specific mutation owing to the unique mutagenesis process (Brash 2015). The mutation signature helps to provide insight to screen out potential mutant colonies and can further be used for targeted therapies, in case of oncology (Forbes et al. 2017). However, the mutation signature currently is limited only in oncology studies but can also be extended for the microbial cells. One of the key

Table 2.2 Mode of action of physical and chemical mutagenic agents used for strain improvement

Agents	Mutagens	Mode of action	Type of mutation
Physical	Ionizing radiations (γ-rays, X-rays, α-particle)	Single- and double-strand DNA breaks, deamination, and dehydroxylation of nitrogenous bases	Point mutation
	Non-ionizing radiations (UV-A, UV-B & UV-C)	Pyrimidine dimers, mitotic crossing over; hydroxylation of nitrogenous bases and cross-linking DNA strands	Frameshift mutations, base pair substitutions, transversions, and deletions
Chemical	Base analogs (5-FU)	Thaimine analog tauntomerizes and pairs with guanidine	Transition
	DNA intercalating (EtBr, Proflavine)	Inserting an extra base opposite an intercalated molecule	Transition and frameshift mutation
	Alkylating agents (EMS, ENU, MNNG)	Ethyl or methyl group transfer to nitrogenous bases, cross-linking of DNA strands	
	Deamining agents (HNO_2)	Interstrand cross-linking of DNA, deamination of the amino group of adenine, and cytosine to an ether group	Transition

example of mutation signature is UV signature, where in the UV light is used to target the two pyrimidines (C or T) adjacent to induce CC → TT substitutions (Brash 2015). Another example involving mutation signature is the bacterial cells which undergoes stress during the radiation dose, owing to cellular and DNA damage. In such case, the cellular repair mechanism becomes active, for example, the *recA* is expressed due to DNA damage and thus expression of *recA* promoter is a suitable choice for screening out the potential mutant colonies (Min et al. 1999).

2.3 Engineering Physiology of Microbes

For industrial application of any microbe, microbial physiology plays a major role for the identification of production hosts and in designing strategies for strain improvement. The metabolic activity gives reflections of the physiological responses/adaptation to the external environments in which microorganisms are growing. The physiological performance of the microorganism is a type of selection criteria for its industrial usability. It is also influenced by the change and combination in components present inside cells with respect to the external environments, viz. the conditions prevailing during the fermentation process. Important physiological characteristics like fitness, tolerance, and robust nature ensure the industrial value of microorganisms. In this concern, the engineering of the microbial physiology is important to make the process more industrial viable (Zhang et al. 2009).

Fig. 2.2 Steps in physiological engineering

For physiological engineering, the most important thing to understand is the mechanism of cells sensing and adapting to the environmental changes. Also, it is important to critically analyze the specific adaptation of few organisms in extreme conditions like high pH, temperature, and solvents. For successful engineering of microbial physiological functions, below-mentioned steps can become helpful in designing an industrial strategy for the strain improvement (Fig. 2.2).

1. Define the desired physiological characteristics.
2. Candidate screening.
3. Selection of host strain.
4. Engineering within host strain.

2.3.1 Desired Physiological Characteristics

The selection of the target strain possessing desired physiological characteristics is based on the bioprocess used for compound/metabolite production. The factors involved in the selection of process are mainly the market demand and cost of process parameters like methods for strain improvement (upstream) and for purification (downstream). For example, sulfuric acid is used for ethanol production from the corn straw. During the process, to avoid contamination, acidic fermentation at high temperature is prerequisite (Shaw et al. 2008). Also, with acidification at high temperature, the microbial cells should also perform simultaneously hydrolysis of the sugar to produce ethanol (Wisselink et al. 2009).

2.3.2 Candidate Screening

Strains with more desired characteristics are screened using tools like directed evolution, non-specific mutagenesis, and stress-induced adaptations (Foster 2007; Galhardo et al. 2007). The screened strain will be ideal/model candidate for generation of library for candidate strains for further screening. With much collection of screened strains, the library contains strains which are having mutation at different sites or have engineered genes important for the industrial applications. In practical scenario, the targeted strain will possess the desired physiological characteristics by using the different genetic tools and mutation strategies. However, once the

collections of screened microbial strains are available, the efficiency of the process to extract the desired screen heavily relies on high-throughput screening process.

2.3.3 Selection of Host Strain

Once the strain having desired characteristics is identified, further it is important to understand the metabolic regulatory network/pathways along with the genes by genome-wide annotations. However, the other microbial physiological characteristics like fitness, tolerance, and robust nature depend on complex cell components. Therefore, it is utmost important that during designing an physiological engineering strategy, it is better to understand first the underlying physiological characteristics of screened microorganism (Lee et al. 2006; Jeffries et al. 2007). This gives a clear idea for selection of suitable host. The host which shows/exhibit the most complex physiology and which is fully genetically characterized can be the best suitable host for physiological engineering.

2.3.4 Engineering into the Host Strain

After successful screening of strain and identifying the host strain, the remaining part is expression or infusion of the desired characteristics by various engineering approaches. If the host microbe is strong and fit, the first target to be engineered should be microbial regulatory metabolic capabilities. The host should also be adaptable to the engineered changes so that the expression of the desired product can be easily screened. Another approach can be the reverse engineering. In this type, the host is not fit and robust, but it is highly metabolically active. The promising route to proceed with such strain is to elucidate/decode the evolutionary mechanism of stress-tolerant microorganisms. The rationale for selection of stress-tolerant microorganisms is that the physiological characteristics can be transferred to host strain by the DNA altering genes, transposons, or by genome shuffling (Foster 2007).

2.4 Metabolic Engineering

Metabolic engineering is intended for direct improvement in formation of product or its cellular properties via alteration/modification in particular biochemical pathways or by intruding the new set of specific regulatory genes with aid of recombinant DNA technology (Stephanopoulos 1999; Nielsen 2001). Metabolic engineering has emerged as new designing/engineering in which microorganisms are capable of novel compound production. Technique is amalgamation of control of fluxes with molecular tools and at the same time quantifies the fluxes with analytical methods for getting the desired genetic alteration. The metabolic engineering has revealed that the flux associate with a metabolic pathway is not a single rate-limiting step; instead

it is dependent on many steps involved in the biochemical pathways (Kacser and Acerenza 1993).

2.4.1 Methodologies and Tools for Metabolic Engineering

Before designing the different strategy/methodologies for metabolic engineering, it is important to identify the key fundamental essential requirements:

1. Detailed information of biosynthetic pathway of compound to be produced.
2. Set of genes encoding the enzymes and its regulatory pathways.
3. Methods to transfer and express the desired gene in the host organism.
4. Different tools for in vivo and in vitro gene mutation.

Metabolic engineering normally starts with the genetic alteration which is followed by characterization of gene expression and further analysis of change in the metabolic pathway of the mutant. Many strategies are used for designing a pathway for the metabolic engineering. Few major strategies are as follows:

2.4.2 Engineering of Biosynthetic Pathways

For engineering the biosynthetic pathway, the first step is to understand the key components of pathways and its regulatory points.

(a) Increase the number of genes coding for rate-limiting steps in biosynthetic pathway (Cremer et al. 1991).
(b) Increase/amplify the genes responsible for the branch or end point enzyme which we can give direction to the intermediate compound to move the process.
(c) Infuse heterologous enzymes with unusual structures which can allow them to bypass regulatory step.
(d) Infuse heterologous enzymes having diverse mechanisms which are functionally more advantageous (Ikeda et al. 1994).
(e) Inclusion of the enzyme which is divergence point for the central metabolic pathway. This will lead to increase the flow of carbons in biosynthetic pathway.

2.4.3 Central Metabolism Engineering

Central metabolic pathways are the main suppliers of energy and precursors for biosynthesis of many essential compounds. Engineering the central metabolic pathways is very complicated as it is regulated globally, and the identification of regulatory network pattern is yet to be resolved completely. For example, the omission of phosphenolpyruvate carboxylase from the biosynthetic pathway has led to increase the production of threonine by 40% in *E. coli* (Hermann 2004).

Another example is of increase in lysine production in host strain *C. glutamicum*. 250% increase in lysine is reported by overexpression of pyruvate carboxylase and aspartate kinase (Koffas et al. 2003). The pentose phosphate pathway which aids in the synthesis of aromatic amino acids is responsible for major supply of NADPH, ribose-5-phosphate, and erythrose-4-phosphate. By amplification using error-prone PCR for transketolase gene, it increases the production of erythrose-4-phosphate which leads to mutant having higher tryptophan production capacity (Ikeda and Katsumata 1999).

2.4.4 Transport Engineering

Mutants that have modified transport systems can continue to thrive at low intracellular level of the product. Such mutants are not subject to feedback control. For example, there is significant increase in tryptophan and threonine yields using a *C. glutamicum* and *E. coli* mutant generated by transport engineering (Ikeda and Katsumata 1995). Mutants having dynamic efflux system and weak uptake system can overexpress the amino acid without deregulation of biosynthetic pathways (Ikeda 2003).

2.4.5 Engineering the Whole Cell

Majority of metabolic pathways and flux associated with the central metabolism is extensively studied and elucidated in few microorganisms. However, the regulation of flux and physiology is not reported in the microorganisms which are of industrial interest. With much development in genetic tools, currently it is difficult to predict possible product outcome of metabolic pathways when it is redirected. For example, the intersection between the glycolysis and TCA cycle is most important for the regulation of amino acid synthesis. Moreover, the control of pathway flux is not controlled but shared. In this context, the inverse metabolic engineering, physiology engineering, and systems biology have become important tools for designing an industrially important mutant (Koffas and Stephanopoulos 2005). Novel approaches like functional genomics and genomic breeding are trending as these tools can identify and remove the unwanted mutants from the process (Petri and Schmidt-Dannert 2004).

2.5 Genetic Engineering

The production level of desired compound from the natural wild type isolate is always too low for the industrial applications. Henceforth, the strains are improved using different engineering methods. Among all, the most important is genetic engineering. In normal approach, by single step improvement, there is no significant improvement in the product yield. In comparison, the genetic approach is specific for

microbial engineering of industrial importance (Verdoes et al. 1995). The following points needs to be considered for constructing the genetically engineered strain.

(a) Screening of the desired microbial strain.
(b) Cloning of desired gene required for the synthesis of desired protein/metabolite.
(c) Amplification for creating multiple copies of genes.
(d) Investigation of the expressed product.

2.5.1 Screening

The molecular basis of selection of specific strain depends on the specific enzyme activity of the microbial strain (Van Gorcom et al. 1990). Once the specific strain is selected, the desired enzyme/protein of interest is cloned from this strain. Many methods are reported for gene cloning using different fungal species as host organisms (Timberlake 1991). Routinely, the "reverse genetics" is used for insertion/cloning of desired genes. This method works with very simple principle of elucidation of genomic or cDNA from the isolated proteins from the cell extracts. Briefly, the desired protein from the culture filtrate is purified and amino acid sequence of purified protein is resolved. From the resolved amino acid sequence, oligonucleotides are designed. These nucleotides are used for screening of cDNA library. Further, DNA is amplified by PCR for further experimentation (Choi et al. 1993; Gomi et al. 1993).

2.5.2 Gene Expression

Host strains which are used for overproduction are mostly constructed by insertion of multiple copies of gene of interest. However, the direct selection is not possible of strains having multiple copies, henceforth the indirect selection of the strain having multiple copies is done using a selectable marker. This selectable marker is inserted in same vector as gene of interest. The transformed mutants have desired sequences which are stable by integration with genome by homologous or nonhomologous recombination. After insertion of the gene sequence, it is most important that the gene is expressed at desired level. For example, in certain cases it was found that even though strains possess multiple copies of gene but the expression level is not significant. Therefore, for improvement of gene expression level, it is necessary to design a system in which the expression levels of gene of interest can be controlled or modified for higher yields (Verdoes et al. 1995).

2.5.3 Enzyme (Over)Production and Posttranscriptional Control

It is important to study a correlation between copies of gene of interests and protein production. A previous finding suggests that with high copy numbers protein

production levels are lower than expected. The amount of protein expressed in the culture medium not only influences levels of expression but also the protein degradation pattern was observed in a study conducted for overproduction of pectin lyase enzyme (Kusters-van Someren et al. 1992).

Another approach can be possibly to associate posttranscriptional mechanisms for tuning the expression of multiple genes in the operons. Pfleger et al. (2006) has successfully demonstrated the library of tunable intergenic regions (TIGRs). TIGRs consists of many control elements like mRNA, RNase cleavage sites, and couple of sequestering sequences. TIGRs was able to modify the processes of transcription, mRNA stability, and initiation of translation. Combination of RNAse site and TIGRs in particular cleavage sites has helped to fragment (decouple) the coding region stability helping the self-regulating expression variations. This method was successfully utilized for the optimization of flux for mevalonate pathway using host strain *E. coli*. Using this method, significant increase of sevenfold increase in mevalonate production was reported. This overexpression was observed due to counteractive mechanism of HMGS and tHMGR reduction activity. Therefore, it is utmost important to understand that combinatorial strategies for strain improvement will be able to rescue the phenotypic variant for the beneficial changes in strain improvement. Also, the combination will make the system much more understandable and clearer for designing of novel tools for strain engineering.

2.6 Omics for Strain Engineering

With the advancement in DNA sequencing technologies, the data obtained from DNA sequencing is much more rapid, reliable, and specific. Nowadays, for most of the model organisms, complete genome sequences are there which increase the postgenomic research in the strain development field. With help of complete data set, it is easy to predict the probable outcome of the genotypic changes to be incorporated for the strain improvement. Transcriptomics and proteomics allow parallel analysis of mRNA and protein expression levels using the DNA microarrays and two-dimensional gel electrophoresis system or mass spectrometry methods. The other approach is metabolomics which quantifies the metabolites and intermediates using the analytical technique mass spectrometry or nuclear magnetic resonance spectrometry. The study of flux in the metabolic pathways; fluxomics allows the quantification of metabolic fluxes based on balancing of metabolites or isotope analysis.

2.6.1 Genome Analysis

Comparative genome analysis is a simple but very useful technique for the identification of desired genes that can be inserted or deleted for achieving the desired phenotypic change. Using the genome analysis, easy comparison is possible for the wild type and mutant/engineered strains. In one approach, the only essential or active

genes helpful for the metabolic functions are retained while the unnecessary genes are deleted without any genomic or metabolic burdens (Kolisnychenko et al. 2002). This technique used is called minimum strain development. The minimum strain development suggests that only the metabolically active gene sets are present in the host microorganisms. Ohnishi et al. (2002) has compared the genome sequence of the wild-type *corynebacterium* strain to locate the genes having point mutation and are also responsible for the overproduction of L-lysine. Even though, we have succeeded in engineering of few industrially important strain at genome scale, but the initiation in this field has led us to understand the engineering of local reactions in the biosynthetic pathways and possibly can lead to significant improvement in the performance of screened microorganism (Lee et al. 2005). The other major advantage of genome analysis is that using the data of whole genome in silico metabolic models can be developed which can give the prediction of expected gene expression levels.

2.6.2 Transcriptome Analysis

With development of throughput DNA microarrays, the accuracy has increased for quantification of the changes at the gene transcription levels by monitoring the relative changes in mRNA level in multiple samples. Comparing the transcriptome profiles of wild-type and mutant-type strains at different time points/culture conditions helps to identify/locate the regulatory networks and probable target genes to be altered/manipulated. The new information and understanding in this way can be further used to improve the performance of microorganism. For example, transcriptome profiles of *E. coli* used for the production of human insulin-like growth factor was analyzed by high cell density culture method. From the data sets of nearly about 200 genes, only those genes involved in the amino acid or nucleotide biosynthetic pathways were targeted. Among these genes, the amplification of prsA and glpF genes has shown increased production of IFG-I_f production from 1.8 to 4.3 g/L. These two genes were encoding for phophoribosyl pyrophosphate and glycerol transporter. The cited example suggests that the strategy for targeted engineering-based approach using the global information will allow the identification of gene which would be helpful for the construction of superior strain giving high yield at industrial scale.

2.6.3 Proteome Analysis

Proteome analysis, a prevailing tool for the comparative analysis of two or more protein spots, needs to be done showing varying intensity under different genetical and environmental conditions. Also, proteome analysis is much more useful as the central metabolic activities are correlated or mediated via proteins. Henceforth, proteome analysis will give us edge to understand more regulatory networks of metabolic pathways. The major disadvantage of proteome analysis is that every

protein spot is not identified yet, and therefore the information gathered after proteome profiling may be less compared to the transcriptome profiling. Best example for the proteome analysis is proteome comparison of two *E. coli* strains. The first strain was gathering the biodegradable polymer 3-hydroxybutyrate and the other is wild-type/native *E. coli* strain. This comparison has helped in the identification and understanding of the importance of protein Eda (2-keto-3-deoxy-6-phosphogluconate aldolase) in poly(3-hydroxybutyrate) production by mutated/engineered *E. coli* strain (Han et al. 2001). Another classic example is of comparison of *E. coli* strains overproducing the human leptin. Interesting observation was that the expression of few enzymes was decreased significantly, indicating the possible limitation in serine biosynthetic pathways. At quantification levels, the content of serine in leptin is about 11.6% which is significantly higher than the serine content 5.6% found in proteins expressed in *E. coli* (Han et al. 2003). The above-cited examples indicate even though there is limited information in proteome profiling/analysis, it can result into successful designing of a new strategy for strain improvement.

2.6.4 Fluxome and Metabolome Analysis

2.6.4.1 Metabolome Analysis

The varied and dissimilar chemistry of different metabolites and availability of only a limited number of detectable chemicals/standards turns the whole cell metabolome profiling realistic. These analyses were possible due to the development of the high-throughput quantification methods like NMR, gas chromatography (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and MALDI-TOF. Usually, the number of metabolites in cells are always fewer than the number of genes expressed in specific conditions which give edge to metabolome analysis compare to the transcriptomic and proteome analysis. For example, the low-molecular-weight metabolites in *S. cerevisiae* is estimated to be only 560 which is very less compared to genes or proteins expressed. Also few researchers have tried to integrate the metabolome and transcriptome analysis for construction of mutant strain giving the high yield. Furthermore, Wittmann and Heinzle (2001) has used the metabolome profiling to understanding of flux distribution in *Corynebacterium glutamicum*.

2.6.4.2 Fluxome Analysis

For better understanding of cellular metabolic status, it is important to go for metabolic flux analysis. As intracellular flux cannot be easily quantified, they are usually measured using the bioinformatics approach. For calculation of fluxes, practical data like substrate uptake and secreted product are constraints for proper analysis. The isotope analysis/experiments can provide important information on the intracellular fluxes. Usually, for the isotope analysis, the 13c carbon is labeled uniformly on substrate in the process. Whenever, the substrate is metabolized in the cell, isotope distribution can be located, and intracellular flux ratios can be calculated (Wittmann and Heinzle 2001).

2.6.5 Combined Omics Approach

The real integration of all omics approaches is still not a realistic. There are several reported approaches where different combination of omics approach was tried for improvement of strain. For example, the combination of transcriptome and proteome was carried out in *E. coli* strain for overproduction of L-threonine. The results show that genes involved in the TCA cycles, amino acid biosynthesis, and glyoxylate shunt were upregulated compared to the downregulated ribosomal proteins. Due to this combination, significant overproduction of L-threonine was reported (Lee et al. 2003).

Another classic example is of improvement of *Aspergillus* strain for production of lovastatin. In this, a combination of transcriptome and metabolome analysis was carried out. First, the libraries were constructed of the desired strains having desired gene expressions. These screened strains were further characterized by the metabolome and transcriptome profiling. By using these combination approach, the resultant mutant strain was able to secret 50% more lovastatin compared to the wild/native strain (Askenazi et al. 2003).

2.7 Conclusion

Microorganisms have natural tendency to produce compounds of industrial importance. The power of microbial culture needs to be appreciated due to fact that even simple molecules which are similar to synthetic compounds are produced by microorganisms using different fermentation process. Strain improvement programs are completely required for commercial production of compounds at industrial scale. To obtain these desired properties, it is utmost important to define the right host strain having specific physiological, biochemical, and genetical functionalities. Furthermore, advances in high-throughput screening and omics approaches have enabled rapid isolation of mutant strains having desired expression profiles. We believe that this review will provide the valuable insights for identifying and designing strain optimization strategies to improve product yield and reduce the fermentation economics.

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