Improving Microbial Robustness Using Systems Biology

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42.1. MICROBIAL ROBUSTNESS IN INDUSTRIAL PROCESSES

What is robustness regarding microorganisms and microbial processes? We define robustness to be a collection of properties a microorganism has that allow it to grow, conduct biochemical processes, and survive in a changing environment better than less robust microorganisms. Robustness requires the microbe to be able to rapidly adjust to different stresses derived from temperature and pH changes, nutritional limitations, presence of chemical toxins, and pathogen attacks such as by viruses and phages, while both surviving and continuing to function (Table 1). Indeed, these adaptations theoretically could result in a microbial strain that is less competitive under nonstressed conditions. Still, these characteristics are sought for industrial and environmental processes such as large-scale fermentations or bioremediation applications. Robustness is not necessarily a requirement for highly productive microorganisms that are developed for well-controlled environments, although robustness is nevertheless quite beneficial there too. Therefore, robust microbial processes are sought for all microbial processes used in the medical, food, feed nutrition, cleaning, chemical, bioenergy, and environmental industries. In this chapter, we aim to outline aspects of microbial robustness desirable for specific industries and processes and examine the current status and potential benefits of application of systems biology analysis tools to further improve these processes.

42.1.1. Biological Robustness

While robustness is a key recognized property of biological systems, the term "robustness" is frequently applied across a broad range of biological attributes, ranging in scale from genes to biological populations. For example, an enzyme can be described as robust if it performs its function across a wide range of conditions, such as pH and temperature, although optimal (peak) activity is defined by a narrow range of pH and temperature, and stability is temperature dependent (Mosier and Ladish, 2009). Likewise, the genetic makeup of an organism can be regarded as robust if it contains redundant genes and pathways for key metabolic activities or incorporates sophisticated metabolic control schemes. Additionally, "robustness" is used to describe both the ability of biological systems to respond rapidly to perturbations and their ability to adapt to evolutionary pressures

on a much longer timescale. Such broad use of the term underscores the significance of understanding biological robustness and has motivated several attempts to more precisely define the term (117).

In the context of industrial microbiology, the value of robustness can be seen across processes, organisms, and biological scales. It is a topic of both fundamental and applied value, the centrality of which has been essentially rediscovered with the development of systems biology tools. For example, robustness of even very simple biochemical networks has been shown to be important to bacterial chemotaxis, circadian rhythms, and the yeast cell cycle (10, 13, 116). Such analyses draw heavily from the network theories and computational tools applied previously to physical networks and incorporate consideration of dynamic stability, control theory, and biochemical "noise" (66, 69, 80).

This application of systems biology and network theory to biological robustness has generated valuable insights that may potentially be useful in identifying or developing industrial organisms with improved robustness. At the genetic level, latent metabolic pathways have been shown to be as important as excess metabolic capacity when organisms adapt to the loss of key metabolic enzymes (31). Perhaps not surprisingly, such metabolic flexibility often exists around core metabolic pathways that synthesize or utilize metabolites essential for biomass formation under all environmental conditions (5, 63). For example, high-throughput, integrated "omics" tools were used to evaluate *Escherichia coli* strains with disrupted glycolytic and pentose phosphate pathways. Despite the alterations to these central metabolic networks, the organism was able to reroute fluxes and exhibited only minor changes in gene transcription and protein expression (57).

In many organisms, genotypic variation far exceeds phenotypic variation, and a great many genes and gene variants can be considered inconsequential in terms of normal function. However, such variants may impart phenotypic or evolutionary capacitance, which enables the organism to behave in a normal phenotypic manner even when environmental conditions or mutations would otherwise make the system less robust (11, 84). While such phenotypic capacitance is not yet well understood, it may play a significant role in determining the robustness of industrial organisms designed around so-called "minimal" genomes and metabolic functionality. In a practical example of

TABLE 1   Preferred characteristics of a robust microorganism

Able to use inexpensive organic and inorganic sources of nitrogen and other nutrients
Able to utilize multiple complex carbohydrates as substrates for carbon and energy
Able to withstand a temperature or pH shock with minimal loss in viability
Multiple restriction modification systems to minimize virus and other external genetic attacks
Presence of metabolic and genetic redundancy for core biological processes
Able to enter dormant state to survive inhospitable environments or nutrient limitations
Ability to withstand brief oxygen deprivation or exposure for aerobes or anaerobes, respectively
Able to multiply rapidly and respond appropriately to changing environments
Reaches and maintains very high viable cell density
Able to withstand high levels of metabolic products and input inhibitors

robustness of enzymes, substitution of several amino acid residues was sufficient to enhance the stability of an alkaline protease 10-fold (43). Hence, robustness comes from a range of potential biochemical, metabolic, and genetic characteristics.

Robustness gained through genetic or metabolic redundancy is not without trade-offs, as such redundancy requires that an organism have additional genetic complexity, that it utilize more sophisticated regulatory networks, and that it carry a greater metabolic burden. These extra burdens exert pressure on biological systems to find an efficient and effective balance between redundancy, adaptability, and robustness. One possible means for accomplishing this balance is reliance upon a few simple yet complementary strategies for responding to predictable environmental stresses, such as nutrient limitation. Elementary node analysis of E. coli central metabolism, when applied in such a case, indicates that while only a very small fraction of permissible metabolic pathways are ecologically competitive, they represent enough diversity to account for the observed range of metabolic responses (16). In this way, a robust response results from the combined application of only a few metabolic strategies.

42.1.2.   Genetic Adaptability

A key aspect of attaining robustness is a population's ability to adapt genetically to perturbations to the environment to not only survive but also possibly thrive due to improved fitness. Evolutionary geneticists distinguish adaptation as different from natural selection primarily in that adaptation includes mutations that in and of themselves do not result in large genetic changes but individually move an organism to a more fit optimum (101). Such a process may have been involved in evolution of the very successful variety of beverage yeast, which is discussed below, that developed over time through genetic paths that will remain unknown. An ongoing difficulty is that beneficial, adaptive mutations are difficult to study due to low frequencies (on the order of 4×10^{-9} per cell and generation), and they are difficult to identify compared to deleterious mutations (auxotrophy and loss of other critical functions) (52). Using phage evolution in the laboratory, Wichman et al. determined that fitness can be the result of multiple mutations, many defined as adaptive yet resulting in a similar improved organism or, in this case, a phage genome (130). In their study, they examined the adaptation of φX174 to high temperature and an altered host. They found that two different lines had 15 mutations and documented the specific amino acid changed within five proteins. Most interestingly, they determined

that seven of these mutations were precisely identical as to amino acid affected and the nature of substitution, but critically, the order of their appearance was very different, suggesting that no single change or order of adaptation was decisive regarding ability to survive in a stressful environment. In the case of bacterial evolution, a laboratory 3-liter fermentor of E. coli approaching stationary phase ($\sim 2 \times 10^9$ cells/ml) should contain at least one mutation in every one of the $\sim 4,600$ genes in the genome. Using this microorganism, two different studies analyzed adaptation of E. coli across hundreds of generations under specific adaptive pressures. The authors concluded that many of the genomic changes are highly reproducible although not identical as with the aforementioned study, improving E. coli with many mutations that had small impacts and few "silver bullets" resulting in sudden fitness (52, 141). So such a population will likely contain many silent or nearly silent mutations from an evolutionary standpoint, many of which have the potential to improve the fitness when responding to metabolic challenges or perturbations.

42.2.   SYSTEMS BIOLOGY TOOLS

Systems biology tools include measurement of the level of transcripts, proteins, and metabolites in a biological system at any moment in time. With rapid sampling, these tools can be used to paint a picture of the basal operation of cellular functions in a temporal context, including gene and protein expression plus carbon and metabolic flux. Similarly, the cellular response to an external change such as environmental alterations, metabolic limitations, or exterior assaults by pathogens or infective agents can be measured and compared with respect to the basal metabolism to detect genetic and metabolic responses to the new situation. Consequently, these tools are ideal to begin to further define the nature of "robustness" in microorganisms, and cellular and genetic characteristics that comprise this beneficial survivability. Certainly, a metabolic model of such robust microorganisms should have components not found in many "lesser" microorganisms. Systems biology tools have been the subject of a number of excellent reviews (1, 61, 64, 66) and so are reviewed here only briefly.

42.2.1.   Genomic Analysis and Resequencing

The genetic sequence of the organism under analysis is a fundamental requirement for systems biology tools. Fortunately, the technology for sequencing is continuing to evolve rapidly from the "ancient" gel-based sequencing to fluorescent-tag-based sequencing to the evolving Roche

454 Genome Sequencer FLX™ and FLX Titanium™ and the Applied Biosystems SOLiD™ instruments. Interestingly, large numbers of repeated sequences can compromise assembly of contiguous sequences (contigs), especially for newer short-contig sequencing technologies. Even with the acquisition of genomic data, fully assembled, the function of many genes will remain unknown or hypothetical within all genomes, including that of very-well-studied organisms such as *E. coli*, due to the lack of homologous genes found in other organisms. Therefore, gene annotation remains one of the most limiting factors for genome analysis. At this writing, there are approximately 761 bacterial genome sequences, 55 archaeal microorganism genomes complete, and 100 animal and plant genomes complete. Table 2 highlights a sampling of the numerous microorganisms with fully sequenced and annotated genomes, along with areas of research that motivated their genomic analysis. Each of these microorganism's genomic data fulfill a fundamental requirement for application of systems biology tools to investigate these many important research topics.

Genome resequencing refers to determination of changes to a previously known genome sequence after a designed modification and/or evolution of the genome. An obvious example is a mutagenesis and selection procedure, but also interesting is detection of changes after multiple generations under mild selection pressure, including continuous culture. With proper genome sequence knowledge completed at the outset of a designed selection, as opposed to a genome sequence available from public data banks, there is a good likelihood a relatively limited number of genetic changes, whether they are single nucleotide polymorphisms or deletions or genome rearrangements, will be identified, providing valuable information regarding genetic changes relative to phenotypic changes. For example, the power of genome resequencing is described in the amino acid production section. In short, the genome sequence permitted specific rebuilding of a production strain, avoiding existing unintended potentially deleterious genetic changes found in the conventionally developed amino acid producer.

42.2.2. Transcriptomics for Gene Expression

Given availability of a genomic sequence, microarray gene expression analysis has made it possible to determine differences in gene expression under two or more conditions. Briefly, a microarray contains presynthesized oligonucleotide segments of selected regions of the genome, usually all the open reading frames/genes in the genome. The mRNA is isolated from an organism and converted into labeled DNA segments by enzymatic synthesis, producing genetic probes that represent the genes active under the selected condition. These labeled DNA segments derived from the mRNA are used to challenge the microarray by hybridization. If a gene is active, the mRNA is present as its labeled DNA equivalent, which will hybridize to the microarray oligonucleotide segment representing that gene. Since the locations of all selected genes are known on the microarray, hybridization and therefore expression of each of the genes can be determined. As an example, using a bacterial culture, genes expressed by a wild-type strain can be compared to a mutant to detect those genes not expressed or those whose expression has changed due to the mutation. Additionally, microarrays are very valuable to determine temporal expression of genes, for example, during the course of fermentation. Since thousands of genes are active during normal metabolism of an organism, a very large number of signals will be detected by the microarray.

The best microarray data come from differential expression of clearly definable differences, as with the example mentioned above, and completed with multiple biological and technical replicates to provide statistical validation of the results. As the technology matures, the number of arrays and replicates of arrays that can be placed on one analysis slide has grown considerably, providing tens of thousands of gene spots per microarray, yielding multiple gene coverage for simpler genomes with one microarray.

An additional tool, serial analysis of gene expression (SAGE), is used to evaluate gene expression patterns in a comprehensive manner (125). The technology relies upon the uniqueness of an ~14-bp segment (called a tag) from a gene obtained from expressed mRNA. The multiple tags are amplified and linked together for sequencing. The key advantage is that genes can be identified by these small sequences that can be linked together, allowing a sequencing run to identify dozens of genes expressed at a particular time. Using homology searching, the cells that are being examined do not require a genomic sequence because sequences of related (micro)organisms or homologous genes from anywhere could be used to identify the tagged gene. This technology has been useful for analysis of *Saccharomyces cerevisiae* and other yeasts (125, 126), as well as other cellular systems, including cancerous cells. Like all new techniques, improvements have been made with longer tags (25 to 27 bp) called super-SAGE, allowing better identification of gene location (90), and other variants aimed at microRNA expression quantitation, especially for cancer research (140).

42.2.3. Proteomics for Protein Synthesis

Proteomic analysis involves the examination of the proteins present in an organism. While early work was based on two-dimensional gels, technological improvements in genomics and mass spectrometry (MS) have permitted identification of the majority of proteins present in simple microorganisms, far more than before. For example, gel-based techniques required sufficient protein to form a detectable spot and required its structural characteristics to permit it to be within the pH and molecular weight range separable in the gel. While shotgun proteomics requires the partial purification of proteins, the technology permits many more proteins to be identified than by gel-based methods. The key to the technique is the ability to cleave the proteins specifically by site-specific proteases that generate peptides of predictable molecular weight. The peptides are separated by high-performance liquid chromatography (HPLC) and detected by either matrix-assisted laser desorption/ionization or electrospray ionization possibly coupled with time-of-flight technology (2). Computer analysis is required to connect the hundreds of peptides of specific molecular weight to the protein encoded by the genome of the targeted organism. MS protein identification can be challenging when there is extensive posttranslational modification of proteins by phosphorylation, glycosylation, transferases, or other modifications that increase the peptide molecular weight, making the computational identification of those peptides impossible without additional treatment of the protein sample. Interestingly, coupled with genomic data, experience has shown that proteins expressed from microorganisms resident in a complex matrix such as soil or biomass can still be identified in the presence of these environmental "contaminating" proteins, making proteomic technology very powerful for dissection of complex biological materials (108, 132).

TABLE 2   Examples of industrial organisms with completed genome sequence and potential areas of applied use[a]

Organism	Use(s)
Acidithiobacillus ferrooxidans	Bioleaching, bioremediation
Acidothermus cellulolyticus	Bioremediation, environmental
Actinobacillus succinogenes	Succinic acid production
Anaerocellum thermophilum	Energy production
Anaeromyxobacter sp.	Bioremediation, environmental
Arabidopsis thaliana[b]	Model plant species
Arthrobacter chlorophenolicus	Bioremediation, environmental
Aspergillus niger[b]	Citric acid production, enzyme production
Aspergillus oryzae[b]	Beverage alcohol, food industry
Bacillus amyloliquefaciens	Antibiotic production, suppresses plant pathogens
Bacillus licheniformis	Biotechnological, enzyme production
Bacillus subtilis	Biotechnological, enzyme production
Bacillus thuringiensis	Insect pathogen
Bradyrhizobium japonicum	Soybean nitrogen-fixing symbiote
Caldicellulosiruptor saccharolyticus	Cellulases, biofuels
Cellvibrio japonicus	Cellulases, biofuels
Chlamydomonas reinhardtii	Model plant species
Clostridium acetobutylicum	Acetone, butanol, ethanol production
Clostridium beijerinckii	Acetone, butanol, ethanol production
Clostridium cellulolyticum	Cellulases, biofuels
Clostridium phytofermentans	Cellulases, biofuels
Clostridium thermocellum	Cellulases, biofuels
Corynebacterium efficiens	Amino acid production
Corynebacterium glutamicum	Amino acid production, food industry
Desulfovibrio vulgaris	Bioremediation, environmental
Escherichia coli	Biomaterials, biofuels, biochemicals
Geobacter sp.	Bioremediation, microbial fuel cells
Gluconobacter oxydans	Vitamin C production
Hydrogenobaculum sp.	Hydrogen production
Klebsiella oxytoca	Biofuels
Lactobacillus casei	Lactic acid production
Lactobacillus delbrueckii subsp. *bulgaricus*	Lactic acid production
Lactobacillus plantarum	Lactic acid production, food industry
Lactococcus lactis	Cheese production
Mannheimia succiniciproducens	Succinic acid production
Methanocaldococcus jannaschii	Methane production
Methanosarcina barkeri	Methane production
Methanospirillum hungatei	Methane production
Nostoc (Anabaena)[b]	Alga model organism
Pichia stipitis[b]	Energy production, biofuels
Populus balsamifera subsp. *trichocarpa*[b]	Biomass source
Ralstonia eutropha	Bioplastic producer
Rhizobium leguminosarum	Bean nitrogen-fixing symbiote
Rhodobacter sphaeroides	Bioenergy, biohydrogen
Rhodopseudomonas palustris	Hydrogen production
Saccharomyces cerevisiae[b]	Biofuel production, food industry
Sinorhizobium meliloti	Alfalfa nitrogen-fixing symbiote
Streptococcus thermophilus	Yogurt production
Sphingomonas (Pseudomonas) elodea	Biogum production

TABLE 2 *(continued)*

Organism	Use(s)
Streptomyces avermitilis	Antibiotic production
Streptomyces coelicolor	Antibiotic production
Thermoanaerobacter ethanolicus	Biofuels
Thermoanaerobacter pseudethanolicus	Biofuels
Thermobifida fusca	Cellulases, biofuels
Trichoderma reesei[b]	Enzyme production
Vitis vinifera L.[b]	Grapes and wine
Yarrowia lipolytica[b]	Citric acid production
Xanthomonas campestris	Biogum production
Zymomonas mobilis	Biofuels

[a]Data gathered from www.genomesonline.org; www.genolevures.org.
[b]Eukaryote.

42.2.4. Metabolomics

Biological systems are constructed of chemical molecules either obtained from the environment (i.e., food) or synthesized from portions of these input materials by cellular metabolism. While much can be learned from examination of gene expression and the resulting protein expression, identification of the chemical molecules that comprise internal cellular metabolism can be critical to understanding regulation of cellular gene and protein/enzyme expression. Metabolomics aims to accomplish this by use of advanced separation and detection tools to identify small metabolic molecules. Typically, a sample is taken and quickly extracted to remove the small-molecule metabolites by use of very cold solvents, such as methanol, to halt metabolic activity as quickly as possible. Special approaches are needed for charged molecules or those not readily solubilized by cold solvents. As metabolism is dynamic, temporal sampling of a biological system in rapid succession can detect changes in metabolic processes which can confer robustness after the organism is challenged by inhibitors or other stressful or selective conditions. These small molecules are typically separated by chromatography (gas chromatography, HPLC, or capillary) and detected by either nuclear magnetic resonance spectroscopy or MS, which are particularly suited to small molecules, with modifications to separate charged molecules such as phosphorylated molecules common in cellular processes. Unlike with the previous "omics," the genome sequence is not required to delve deeply into the metabolism of biological systems using metabolomics (89).

42.2.5. Fluxomics for Metabolite Flow Analysis

Analysis of the flow of metabolites within an organism is termed fluxomics and is a natural extension of metabolomic analysis. Indeed, temporal analysis of the metabolome comprises the fluxome, with flow of cellular small molecules delineated in a linked metabolic pathway, permitting analysis of the flow of materials, as shown in *Saccharomyces cerevisiae* (14). Pathway engineering in combination with fluxomic analysis provides a powerful approach by which to understand the changes engineered into a metabolic chain of reactions. Such understanding can be critical to understanding an organism's ability to survive stresses and is therefore closely aligned to understanding differences between robust (micro)organisms and more sensitive relatives. Fluxomics has been helpful for production of small molecules such as amino acids and is being used to analyze production of ethanol production from both corn starch and cellulosic feedstocks.

42.2.6. Metabolic Model Development

Metabolic models are valuable tools to begin to structure and test understanding of cellular processes. Models can have different degrees of complexity and can attempt to model the whole cell or, more often, specific aspects of cellular processes such as carbon flow, DNA replication, or bacterial sporulation. As computational power has grown, the possibility to model hundreds of genes and enzymes and link them to the fluxome has become realistic. Models provide platforms to test interactions of different cellular processes using multiple approaches including all the "omics" tools described briefly above. Metabolic modeling is particularly useful for fermentation chemicals such as organic acids, alcohols, and amino acids, as outlined below.

42.3. INDUSTRIAL ENZYME PROCESSING AIDS AND PRODUCTION STRAINS

Industrial enzyme production is a mature industry that contributes to numerous products and processes, including food production, improved cleaning products, pulp and paper processes, textiles, animal feed, and niche markets such as chemical detectors, research enzymes, and chiral chemical processes. Due to their complex structure, enzymes are synthesized exclusively by biological processes. Microorganisms are most commonly used for enzyme production, although there are examples of enzymes isolated from plant and animal sources. Since enzyme production is largely a microbial process, highly productive fermentation processes have been developed yielding enzymes in excess of 50 g of protein per liter, which is now required in many instances to be commercially competitive.

Bringing an enzyme to the commercial market requires a number of steps. Initially an enzyme must have catalytic and stability characteristics that meet the requirements of the application for which they are destined. Such functional criteria are typically very stringent, especially now with the many enzymes that have been developed over the last three decades (106). For example, early commercial enzyme producers quickly realized that process knowledge was essential, consistent with the formation of the strategic partnership between Procter & Gamble and Genencor International developed in the 1980s for development of detergent enzymes. This partnership permitted Genencor to understand the very stringent requirements needed for a successful and superior detergent and processing enzymes needed in Procter & Gamble product lines. As is discussed in some detail below, Henkel KGaA (Düsseldorf, Germany), Staley/Tate & Lyle, and CPC

International are three of a small number of companies that developed in-house capability for enzymes used in detergent and sweetener applications. Proprietary details for "doing the laundry" or production of high-fructose corn syrup were readily available to in-house microbiologists/geneticists seeking improved enzymes for their processes. This proprietary process knowledge has been one of the significant hurdles facing nascent enzyme companies, such as Diversa/Verenium, Dyadic, Direvo, and Maxygen, whose products must compete with and hopefully displace carefully crafted existing enzyme products that are commercially successful using existing processes with attendant depreciated capital.

Selection of an enzyme that provides the best match to existing or anticipated process needs is critical, as it provides the springboard for both enzyme improvement and high-level production. Enzyme production has been accomplished through the use of convenient, reasonably productive original host microorganisms such as *Bacillus* or *Streptomyces*, as well as filamentous fungi such as *Aspergillus niger*. However, it was understood early on that improved production can be accomplished better by transferring the enzyme structural gene to a new, more suitable host, such as genetically modifiable fungi or bacteria, initially with associated production improvement by simple gene dosage. Such gene cloning opened the doors to genetic improvement by numerous approaches, including random gene mutagenesis, site-directed modification, gene and nucleotide shuffling, and gene recruiting. Productivity improvements were gained by modification or recruitment of regulatory sequences, most prominently a paired promoter/Shine-Dalgarno sequence with a strong terminator, but more sophisticated improvements continue to be developed (113).

Selection of the best microbial production host organism is extremely critical, since follow-on genetic and fermentation process development will be built around this organism. Approaches have taken and continue to take two general forms. The first is the development of a process around a microorganism that can easily be genetically modified, with the expectation that deficient characteristics can be genetically improved. The alternate approach is the selection of a robust host microorganism that has a known track record of functioning in an industrial process, and struggling with the usually difficult task of genetically modifying a microorganism for which the requisite tools are not developed. Both approaches have merit, and their selection often hinges upon the value of the product as well as the complexity of the protein regarding required posttranslational modification. For example, *E. coli* K-12 was chosen by Eli Lilly as the host for production of human insulin even with its lack of protein transport and potential for endotoxins because the high value of the product supports more expensive, highly regimented regulation-driven reproducible production and downstream processing. By contrast, industrial carbohydrases, such as glucoamylases, and cellulases continue to be produced by filamentous fungi, even with the associated complexity of production due to required extensive posttranslational modification and since their applications do not require highly purified enzyme products.

42.3.1.   Detergent Enzymes and Production Strains

As mentioned previously, robust production strains are highly beneficial economically because their performance in industrial fermentors, 50 m^3 and larger, is predictable and dependable. Henkel KGaA, as the largest producer of laundry detergent in Europe, was faced with the need to produce an improved detergent protease to replace their in-house-produced *Bacillus. licheniformis* alkaline protease

P300 or to purchase a protease from an enzyme manufacturer such as Novo Nordisk at a higher price. The current production host had been subjected to multiple rounds of random chemical mutagenesis, which yielded a strain able to produce active protease at economically competitive levels. In-house research identified a more effective protease from *Bacillus lentus* which passed required stability tests in company applications laboratories, which included the required perborate bleach and temperature stability needed for European laundry practices. The structural gene for the protease was cloned, and production in *Bacillus subtilis* rose modestly but did not match the productivity of the older enzyme, P300 (133), being produced on a large scale from *B. licheniformis*, a well-characterized high-yielding enzyme producer. To take advantage of this production strain, the *B. lentus* protease gene was cloned behind the P300 protease promoter and Shine-Dalgarno ribosome binding sequence (133). However, it was not known whether the signal sequence for the *B. lentus* enzyme would function as efficiently in the new industrial host, so the new protease gene was either left linked to the resident homologous gene or attached to the *B. licheniformis* P300 secretion sequence prior to cloning into the robust, dependable production host, *B. licheniformis*. Results showed that the secretion sequence homologous to the host and the new structural gene was significantly more productive, permitting the well-characterized production process to now produce a superior alkaline protease at protein concentrations obtained previously with the inferior enzyme. Subsequently, the genetic construction was inserted in the chromosome after multiple rounds of site-directed mutagenesis, yielding one of the best alkaline proteases for detergent applications, all being produced by the original superior production host strain (43, 44).

While production of alkaline proteases, such as described above for Henkel KGaA, involves quite mature technology where possibly only small incremental improvements remain, much can be learned from analysis of the production process with common heterologous host-enzyme combinations using systems biology tools. For example, sequencing of the genome of the production strain will yield valuable hints as to why this host organism is so effective in protein production under industrial conditions. Furthermore, with the genome sequence available, it could be determined if the protein production proceeds consistently throughout the fermentation run using both transcriptomic and proteomic approaches, although the latter will be a challenge in the presence of very high levels of protease. Certainly protein synthesis has a high energy demand, and potential limitations in synthetic capability may become evident with gene expression analysis. For example, a detergent protease expression was modeled for mass flux looking at enzyme synthesis (15). Furthermore, Henkel, as an oleochemical company, could benefit from this information were they to want to produce lipases or other enzymes for detergent, chemical, or personal-care businesses with a well-understood host organism.

42.4.   FERMENTED BEVERAGE INDUSTRY

One of the oldest uses of microorganisms is production of fermented beverages by yeasts and other microorganisms, which has evolved from a spontaneous process to the current well-developed art and science of production of wine, beer, and processed fermentation beverages such as various liquors. Indeed, alcoholic fermentation was used by early societies to preserve beverages from fresh fruits and other sources of fermentable sugars. For example, *Zymomonas* has

long been used for production of pulque from fermented agave sap and has appeared as a troublesome contaminant in wine making. S. cerevisiae and other yeasts have been used for wine and beer production since antiquity (9, 92). Currently the technology is very advanced, with dramatic improvements in quality and quantity due to improved processing equipment and selection and development of improved fermentation yeasts. Indeed, studies using systems biology tools are beginning to delineate differences between laboratory yeast strains and those developed through the ages as robust fermentative strains that permit the production of high-quality alcoholic beverages.

Laboratory strains of S. cerevisiae have been the subject of intense analysis since the genome sequence was determined in 1996 (45, 134); such analysis includes generation of a metabolic network model for a portion of the intracellular metabolism, including over 700 genes/open reading frames, or about 16% of the genome (32). For example, the linkage of transcriptomic analysis with the metabolome during a sudden increase in carbon source (glucose) in S. cerevisiae showed that within 5 min, the organism can respond to addition of glucose both with increased intracellular metabolites and with activation of genes associated with purine synthesis due to the sudden demand for ATP (71). Interestingly, the demand for the energy carrier's metabolites was severe enough that the mRNA degradation rate increased to provide purine building blocks, giving a clue with respect to the extent of cellular metabolism reprogramming expected due to a sudden change in the external environment. Finally, a comprehensive multiomic study correlating the gene expression (transcriptome), protein expression (proteome) and the external and internal metabolome with a chemostat-grown S. cerevisiae strain permitted the delineation of some of the details of growth control of this laboratory yeast (19). Genes associated with growth under nutrient-limited and -sufficient conditions were identified, as well as stress- and stimulus-related genes, and provided a guide for integrated systems biology analysis of a complex biological system and possible approaches to understand how robustness can be detected and quantified in a cellular system.

Regarding robustness, laboratory yeasts cannot successfully compete with commercial wine and fermentation yeasts, which have been selected to be very hardy and survive a very stressful environment while rapidly fermenting metabolizable sugars to ethanol. When a yeast is pitched into a wine must, it is suddenly exposed to very high sugar levels (i.e., >22% sugar by weight) with associated high osmotic pressure, plus low pH (pH 3 to 4) due to high organic acid levels, especially for white wines. Relatively low nitrogen levels and high levels of sulfur dioxide used to kill indigenous yeasts and bacteria present with incoming grapes plus the onset of anaerobiosis (105) provide other stresses. Beer production exposes the beer yeast to a similar environment, with the exception of minimal sulfur dioxide levels, but can include limitations in expression of genes associated with membrane synthesis that require small levels of oxygen (106). As a result of this stressful and inhospitable environment, yeasts have presumably evolved that are quite different from laboratory S. cerevisiae.

Transcriptome and proteome tools require a genome sequence for gene identification. Wine and beer yeasts, including Saccharomyces bayanus, Saccharomyces kudriavzevii, Saccharomyces mikatae, Saccharomyces paradoxus, and Saccharomyces pastorianus (Saccharomyces carlsbergensis), are among the approximately 117 Saccharomyces genomes being sequenced which will greatly facilitate systems biological analysis (www.genomesonline.org; www.genolevures.org).

Wine production often uses S. cerevisiae or S. bayanus, while beer yeasts comprise a wider group of Saccharomyces species. Initial analysis has shown considerable genome and chromosome rearrangement, and there is considerable divergence between beer yeasts for top-fermenting ales and bottom-fermenting lagers (62, 70, 115), providing for different characteristics among fermentation strains likely to affect the resulting product. The availability of a large and growing number of genome sequences, with multiple strains being sequenced from the aforementioned species, will greatly broaden the potential for understanding the value of the genetic differences found in industrial yeasts.

During wine production there is no need for starter cultures, as dry wine yeasts are typically pitched into the must, where they have to quickly adapt to very different environments upon rehydration. Having been produced in a rich and rapidly fermenting aerobic fermentor, harvested, and spray dried, the starter yeasts find themselves now in a high-sugar, acidic, anaerobic, rather toxic milieu comprising the wine must. Surprisingly, a few studies have failed to detect activation of classical stress response genes, which might be expected under these conditions. The rehydrated yeasts undertake activation of anaerobiosis genes, glycolytic genes, and catabolism repression due to the high sugar levels (98, 111), suggesting that these yeasts have been selected to avoid an energy-consuming stress response in favor of quickly adapting to the environment of an industrial wine yeast, i.e., are able to undertake fermentation in the environment and not become the classical "stuck" fermentation common to laboratory yeasts when facing high sugar levels. As the fermentation proceeds, an additional stress is self-introduced as the ethanol levels rise due to fermentation. Indeed, levels above 25% (by weight) sugar are common with wine production yielding over 12% ethanol, a level that is highly toxic to laboratory yeasts and nearly all bacteria. One common response is accumulation of the sugar trehalose, as well as expression of genes related to membrane fitness and electron transport and, finally, generalized stress response genes (110, 124). Cellular metabolism slows down in late stationary phase due to minimal sugar and nitrogen and high levels of ethanol in readiness for harvesting of the product by the enologist.

By comparison, a true wild-type yeast isolated from a spontaneous grape fermentation caused by a yeast resident on the grape exterior was analyzed for protein changes during fermentation (121). During the fermentation, when the glucose level dropped significantly, there was a dramatic decrease in protein synthesis for about 50 proteins and evidence of proteolytic activity. Also noted was upregulation of classical stress response genes, a response not seen in selected wine yeast strains, indicating that the natural yeast indigenous to vineyards was not as robust or resilient as selected commercial wine yeasts.

Beer yeasts are more diverse and, as mentioned above, have multiple fermentation modes divided at least between top- and bottom-fermenting yeasts plus other proprietary fermentation approaches. Analysis of brewing yeasts has shown chromosomal diversity regarding structure and number, as first noted by Casey (17). Using comparative competitive genomic hybridization techniques, various laboratories have found that wine and lager yeast chromosomal structures have distinct site-specific changes in a number of genes at or near the mating type (MAT) loci (12, 53, 70). These hybridization studies have identified regions of S. cerevisiae-like and non-S. cerevisiae-like genes, suggesting interspecies mating and hybridization, which lead generally to two situations. First, an addition of large regions of chromosomes after

mating clearly accelerates evolution of traits that can be advantageous if selection pressures are imposed. Additionally, analysis of industrial yeast strains has shown them to have poor mating and sporulation ability, providing genetic isolation eventually. This provides for stability and maintenance of traits such as robustness in the face of the challenges of alcoholic beverage manufacture, for example, but makes conventional strain improvement via natural gene transfer difficult. The use of genetic engineering to further improve yeast strains for beverage fermentation can, therefore, be limited to modern genetic engineering approaches, raising concerns regarding genetically modified organism (GMO) application for products destined for human consumption. The application of systems biology tools such as those underway with laboratory strains (19, 32) can be applied to wine and especially lager beer production to build an integrated model of gene response and metabolite flows as the fermentation process proceeds. With such a model, non-GMO approaches can be used after identification of genes that should be either removed (knockout) or overexpressed to improve further the robust industrial nature that the brewmaster has selected through time.

42.5. AMINO ACID PRODUCTION

Amino acid production is a mature industry, with very large volumes of selected amino acids being produced primarily for food and feed applications. For example, lysine is deficient in corn-based rations for animals, so a number of companies such as Ajinomoto, ADM, Cargill, Kyowa Hakko Kogyo, and DSM produce lysine as well as glutamic acid by microbial fermentation with additional amino acids, including threonine and phenylalanine. Corynebacterium glutamicum has been developed for very-large-scale fermentations (>50 m^3) using low-cost substrates with current production of glutamic acid reaching 1.5 tonnes annually, primarily for food use, and lysine production of more than 600,000 tonnes as a feed additive.

Historically C. glutamicum was identified as a glutamic acid producer, and this discovery started the continued development of this microorganism for industrial processes, with many years of conventional mutagenesis and selection. Early work included the use of amino acid analogues to select for overproduction (115) and improved fermentation yields were improved with time. Fermentation conditions were developed that took advantage of the flexible nature of Corynebacterium. For example, many corynebacteria do not demonstrate diauxic lag for use of multiple carbohydrates beside glucose and as gram-positive microorganisms are associated with a very broad and diverse substrate utilization potential. Due to these advantages, such microorganisms, which include Brevibacterium, have been developed as producers of multiple amino acids, with the advantage of employing similar fermentation regimens for different amino acid products.

C. glutamicum was first used for glutamic acid production in the late 1950s, and work to increase yields was begun using fermentation process development and strain improvement, the latter with conventional mutagenesis and screening with amino acid analogues. Strain improvement continued as gene cloning and expression contributed to productivity, which included early flux analysis and substrate pathway modification for higher yields (51, 65). While the productivity of these conventional and genetically improved C. glutamicum and related Brevibacterium organisms provided sufficient economic productivity, the advent of genomic sequencing opened additional investigative avenues of strain improvement using systems biology tools (60). For example, initial comparative analysis of related genomes revealed that the ability of Corynebacterium efficiens to produce amino acids at higher temperatures was due to both higher G+C content and altered codon usage, providing a beneficial change to production at higher temperatures, and likely improved stability to temperature spikes, an important aspect of robustness.

Industry and academia have used microarray technology that followed the emergence of genome sequences. Microarrays generate hundreds to thousands of signals, often leading to data overload and potentially reducing the perceived value of this tool. However, with well-designed experiments, a specific metabolic quandary was solved by microarray analysis involving valine production. The sequenced C. glutamicum strain ATCC 13032 was genetically modified to overproduce valine, and unexpectedly it was discovered to be inhibited by exogenously added valine (as was a related lysine producer). Microarray analysis comparison of the overproducing valine strain with the wild type detected an isoleucine limitation overcome by adding isoleucine, thus identifying an actionable outcome for further genetic engineering (74). As another example, microbial genome sequencing permitted an additional unique approach to strain building. It is well known that conventional mutagenesis followed by screening permitted significant progress in developing industrial fermentation strains, but it often resulted in unrelated deleterious mutations causing poor growth and stability. Whole comparative genomic analysis of a C. glutamicum production strain was used to examine the lysine pathway, along with transport and supportive genes. Initial work was limited to specific genes, but further research used the full-genome analysis, which detected many mutations in the conventionally derived strain, along with the identification of key genetic changes that caused overproduction of amino acids. These specific mutations predicted to be critical to amino acid production were recruited for "genome-based strain reconstruction" in which the critical mutations were added to an unmutated C. glutamicum strain, which resulted in a much more robust rapidly fermenting production strain with no known detrimental mutations (88, 99).

In addition to genetic analysis, analysis of the intermediate metabolites is progressing with advanced analytical tools with increasing sensitivity. For example, an automated method for gas chromatography-MS analysis of C. glutamicum metabolites able to detect over 1,000 compounds with conclusive identification of a selected 164 of these has led to improved understanding of internal metabolism (118).

These examples highlight the growing potential of the "omics" toolbox that can be beneficially applied to industrial microbes that were developed before the advent of genetic engineering. Competing with these efficient industrial coryneform bacteria for amino acid production is E. coli, which was investigated as a preferred host for phenylalanine production by GD Searle in the 1980s for NutraSweet production. Apparently it was decided that E. coli was advantageous due to its ease of genetic modification and that strain development research could overcome economic advantages of the competitor's Corynebacterium and Brevibacterium strains. This approach discounted any apparent benefits from robustness of the gram-positive microorganism, likely due to patent concern, with the conclusion that E. coli could be developed for high levels of phenylalanine production quickly. Interestingly, GD Searle successfully developed E. coli as a phenylalanine producer from two different directions. The first included conventional mutagenesis and selection with amino acid

analogues at their Harbor Beach, MI, facility. Subsequently the unpublished cloning of the entire phenylalanine pathway was completed by a research team led by S. Primrose in the Searle High Wycombe, England, research facility (33), leading to another E. coli production strain for phenylalanine. The initial nonrecombinant E. coli strain went into production, providing important knowledge regarding large-scale E. coli phenylalanine production. Among the knowledge gained from this work is the lack of robustness of E. coli regarding phage infections, as early fermentations were plagued by viral attacks due to a nearby waste treatment facility, until additional engineering modifications were added to ensure sterility of incoming air. Indeed, this is a real concern for any bacterial fermentation process that is potentially worse for E. coli hosts due to ubiquitous coliform-containing waste management facilities. This new information was undoubtedly included in facility designs as the fully recombinant strain went into production by Searle in their Georgia facility. This example demonstrates that nonindustrial microorganisms can be harnessed successfully and compete with robust counterparts if proper facility and process practices are included.

42.6. XANTHAN PRODUCTION

The origins of the industrial production of bacterially derived biogums, such as xanthan, can be traced back to work done in the 1950s at the U.S. Department of Agriculture Laboratory in Peoria, IL. Current production of xanthan gum for use in foods, pharmaceuticals, cosmetics, oil drilling, and industrial processing amounts to more than 50,000 tonnes per annum globally. Xanthan production leaders include companies such as CP Kelco, ADM, Cargill, Danisco, Jungbunzlauer, and Deosen, the majority of whom have been shifting production capacity to China to remain competitive in this growing but cost-sensitive market (25).

The production of xanthan gum by Xanthomonas campestris has been extensively studied and is the subject of several good reviews (30, 41). Produced aerobically via submerged fermentation, xanthan is a large, anionic, water-soluble heteropolysaccharide with a primary structure containing repeated pentasaccharide units of glucose, mannose, and glucuronic acid. Even early mucoid isolates of X. campestris were capable of producing >50 g/liter xanthan, so much of the development of the xanthan process has focused on industrial scale-up and application development, especially as a food ingredient. The development of techniques for large-scale xanthan production have focused on overcoming the challenges inherent in the highly viscous fermentation, including controlling dissolved oxygen, temperature, and pH as the acidic polysaccharide is produced (87). Xanthan gum fermentations exhibit a strong dependence on oxygen transfer rate, as it affects cell growth and xanthan production rates, as well as the mean molar mass of the xanthan produced. As the fermentations are viscous, typically >100 cP, various carbon and nitrogen feeding strategies are used to maintain productivity during the course of fermentation (30, 41). As the commercially relevant properties of the xanthan gum differ among strains, cultivation techniques, and purification schemes, these have also been investigated in detail (30, 37, 112).

The genomes of three strains of Xanthomonas campestris pv. campestris have been published since 2002 (24, 107, 128). While the genomic information has been primarily interrogated to better understand the pathogenicity of the microbe, which is the causal agent for black rot disease in crucifers, the genome sequences have also been utilized to reconstruct the metabolic pathways for synthesis of xanthan and its precursors, as well as to perform comparative genomic analyses (128). The reconstruction of the xanthan pathway was nontrivial, for while the genes responsible for xanthan gum production were first reported in the 1980s, the number of cyclic metabolic pathways involved in xanthan synthesis complicates stoichiometry. In addition to providing insight into xanthan synthesis, the genome provides evidence for a large number of carbohydrate import systems. Following publication of the genome, the links between the import systems and specific carbohydrates have been further elucidated using transcriptomic data (114).

While structured kinetic models of X. campestris growth and xanthan production have been developed and shown to be useful in predicting bioprocess performance, the development of a genome-scale metabolic model has not yet been reported (39, 40, 83). Metabolic flux analyses have been developed and utilized to evaluate an advanced reactor configuration, to evaluate the energetics of xanthan synthesis, and to increase understanding of the relationship between nutrient addition and product attributes (30, 50, 81). Proteomic analysis has also been utilized with X. campestris, although the focus has been largely restricted to its role as a phytopathogen (20, 129). The presence of copious amounts of exopolysaccharide creates challenges in the "omic" investigation of X. campestris, as it can interfere with standard techniques for proteomic, transcriptomic, and metabolomic sampling and analysis. To overcome this issue, researchers have utilized modified methods or nonmucoid strains (20, 82). To make real progress with "omics" tools, method development aimed at extraction of genetic transcripts and microbial proteins for transcriptomic and proteomic analysis, respectively, must be accomplished. Once these methods are available, a full "omic" analysis of gum synthesis will be possible and potentially yield a metabolic model with hopefully actionable outcomes to further improve a very unique microbial fermentation process.

42.7. CELLULOSIC ETHANOL PRODUCTION

The conversion of cheap, abundant cellulosic biomass into alcohol fuels has been pursued for more than a century. Like ethanol derived from cane and corn, ethanol derived from cellulose will require low-cost operations possible only in very large, simply designed fermentation equipment. However, while relatively inexpensive, cellulosic feedstocks are much more difficult and costly to process than feedstocks providing starch or sucrose. Taken as a whole, the robustness challenges of cellulosic ethanol production are numerous (Table 3).

Cellulosic biomass is composed mainly of cellulose, hemicellulose, and lignin. While precise compositions vary significantly across types of cellulosic biomass, such as grasses and trees, typical dry biomass is >50% carbohydrate by weight, with lignin comprising between 20 and 35%. While the major portion of biomass is typically cellulose, composed of $\beta(1\rightarrow4)$-linked D-glucose units, a significant portion is composed of pentose-rich polysaccharides such as xylan and arabinan, or hexose-rich polysaccharides such as galactan and mannan. In part due to the presence of lignin, which sheathes cellulose fibrils and is cross-linked within the hemicellulose, the biological breakdown of lignocellulosic biomass is challenging. To attain the conversion rates and yields required of industrial fermentation processes, this recalcitrance to biological decomposition must be overcome.

TABLE 3 Robustness challenges in biomass ethanol production

Type of challenge	Description
Substrate	
Concentration	Processes employing prehydrolysis require rapid growth at high initial sugar concentrations.
	Processes featuring simultaneous saccharification release sugars slowly, typically over several days.
	Process economics favor very high solid concentrations, often >20% (by wt), resulting in limited free water, difficult mixing, and potential for local accumulation of sugars and/or ethanol.
Heterogeneity	Biomass feedstocks exhibit considerable compositional variability.
	Processes require the use of both pentose and hexose sugars.
Environment	
pH, temp, nutrients	Low-cost, large-scale fermentation systems have minimal mechanical mixing, resulting in potentially large spatial variations in pH, temperature, and nutrient availability.
Product accumulation	Process economics favor ethanol titers in excess of 6% (vol/vol).
	High concentrations of dissolved carbon dioxide can be anticipated due to equipment scale and mixing.
Infection	Scale, feedstock, and equipment limitations increase the likelihood of bacterial and phage infections.
Biomass-derived inhibitors	
Cellulose	Many pretreatments convert a portion of the hexose sugars into HMF, levulinic acid, and formic acid.
Hemicellulose	Many pretreatments convert a portion of the pentose sugars into furfural; acetyl groups are converted to acetic acid.
Lignin	Many pretreatments convert a portion of the lignin polyphenolics into vanillin, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and syringaldehyde.

As early as 1900, concentrated acid was employed to hydrolyze cellulose into glucose. However, low yields and high cost led researchers to look for other means of liberating sugars from cellulosic biomass. Most current research is focused on processes that employ pretreatments which disrupt the lignocellulosic structure prior to biological conversion to ethanol. A wide variety of pretreatment approaches have been proposed, including processes that operate at low pH (dilute acid and sulfur dioxide), high pH (ammonia and lime), and near neutral pH (liquid hot water and steam explosion). In other processes, such as wet oxidation, pH is a matter of choice (103). In addition to disrupting the cellulosic structure, the low-pH pretreatments hydrolyze the hemicellulose fraction and yield monomer sugars such as xylose. Pretreatments at high pH tend to solubilize lignin, without hydrolyzing the hemicellulose to a significant degree. The various approaches to pretreatment and their performance trade-offs are the subject of several good reviews (36, 49, 95). Pretreatments vary in terms of their ability to enhance enzymatic digestibility of different cellulosic feedstocks, but for most cellulosic feedstocks of interest, one or more pretreatments have been shown to be effective at rendering the cellulosic biomass digestible (135, 136).

While the various pretreatments improve biomass digestibility, many of them also make the biomass hydrolysates inhibitory to the growth of the fermentative microorganisms. Growth inhibition by biomass-derived inhibitors has been a recognized challenge since the earliest days of concentrated acid hydrolysis (79). Various means of detoxification have been tried, including overliming and activated carbon treatment of liquid hydrolysates and neutralization and washing of the insoluble materials. While several of the methods have been shown to be effective at reducing inhibition, their cost has motivated efforts to both minimize the production of inhibitors during pretreatment and identify or develop robust organisms capable of tolerating biomass-derived inhibitors (76, 94).

The exact identity and mechanism of the biomass-derived inhibitors are not well understood. Degradation products of glucose and xylose, such as 5-hydroxymethylfurfural (HMF) and furfural, have been a focus of attention for many years. The same is true of lignin degradation products such as vanillin and syringaldehyde. However, recently developed analytical methods capable of quantifying a wide range of potentially inhibitory water-soluble compounds have made it clear that dilute-acid-pretreated biomass hydrolysates have many more potential inhibitors (21, 22). The inhibitor profile following alkaline pretreatments differs considerably from that following pretreatments at lower pH and can result in pretreated biomass with low toxicity. For example, both wheat straw pretreated by alkaline wet oxidation and corn stover pretreated using ammonia fiber expansion have been shown to exert little or no inhibition on S. cerevisiae (67, 77). For ammonia fiber expansion and other mild types of pretreatments, such as liquid hot water (93), carboxylic acids such as acetic and formic acids are the primary biomass-derived inhibitors.

Among the potential inhibitors, furfural and HMF have received the most attention. Examination of furfural and HMF toxicity in yeasts led to the recognition that both compounds are converted to their corresponding alcohols during the course of the fermentation (86, 97, 127). While the sensitivities and responses to furfural and HMF vary widely among yeast strains, under anaerobic conditions, cell growth is typically inhibited until the conversions to the less toxic alcohol derivatives are complete (38, 93, 102, 119). In S. cerevisiae the reduction of the aldehyde group is catalyzed by one or more alcohol dehydrogenases, using both NADH and NADPH as cofactors (85). Researchers at Lund University utilized mRNA expression profiles to identify adh6 as the gene responsible for conveying NADPH-dependent HMF conversion. Later, the same group identified a second, NADH-dependent alcohol dehydrogenase using electrospray ionization-MS and demonstrated that overexpression of the two alcohol dehydrogenases in a laboratory strain of

S. cerevisiae markedly improved anaerobic fermentation in the presence of HMF and furfural (6, 73, 104). The bacterial ethanologens *Zymomonas mobilis*, *E. coli* KO11, and *Klebsiella oxytoca* P2 have also been evaluated with respect to furfural and HMF toxicity and conversion. As with the yeast, the bacteria appear to detoxify the aldehydes by converting them to their respective alcohols (47, 109, 139).

While the appearance of HMF and furfural in pretreated biomass is associated with the degradation of otherwise fermentable carbohydrates, the presence of acetic acid is an unavoidable outcome of the depolymerization and hydrolysis of hemicellulose. Microbial sensitivity to acetic acid is strongly pH dependent, as toxicity is well correlated to the protonated form of acetic acid. Acetic acid toxicity has been shown to be significant for *E. coli* and *Z. mobilis*, as well as *S. cerevisiae* (38, 78, 102, 109). Interestingly, a gene that provides sodium acetate resistance in *Z. mobilis* has been identified by resequencing the genome of a mutant strain, in conjunction with transcriptomic profiling and genetic studies (S. Brown, Oak Ridge National Laboratory, personal communication). Microarray analysis of *S. cerevisiae* grown in glucose-limited chemostats showed hundreds of up- and downregulated transcripts when exposed to acetic ethanol at sublethal levels (1). Analysis of *S. cerevisiae* strains adapted to acetic acid, utilizing either whole-genome microarrays or lipidomics, highlights the adaptive importance of membrane composition and ion pumps (42, 137).

Microbial inhibition due to lignin-derived inhibitors such as vanillin, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and syringaldehyde has also been shown to be significant (26, 68, 75, 139). While genetic elements responsible for tolerance to these compounds have not been reported to date, this can be expected to change. For example, recent analysis of an *S. cerevisiae* deletion library identified 76 mutants with increased sensitivity to vanillin. The analysis indicated a key role for ergosterol biosynthesis, as well as for genes in the functional categories for chromatin remodeling and vesicle transport (29).

In addition to the robustness challenges posed by inhibitors deriving from pretreated biomass, microorganisms to be used in cellulosic ethanol production also need to be able to withstand high levels of ethanol and dissolved carbon dioxide. In cellulosic ethanol production, like starch- and sugar-based processes, attaining high ethanol concentrations has a significant impact on production costs. Existing commercial ethanol processes often achieve ethanol yields in excess of 10% (vol/vol), with much of the ethanol production occurring after cell growth ceases. High partial pressures of CO_2 accompany high ethanol titers, especially in large-scale ethanol production processes where economic considerations include very large (>500,000-gal) fermentation vessels with very limited agitation.

The effects of dissolved carbon dioxide on microorganisms have been studied at length, including its impact on aerobic and anaerobic yeast cultivations (28, 58). High levels of dissolved CO_2 have been shown to influence cell growth and protein production in aerobic systems as well as cell growth and product formation in anaerobic ethanol production processes (72, 96, 120). Analysis of these systems using microarrays has led to new insight into the underlying genetic mechanisms of CO_2 inhibition at the regulatory and transcriptional levels (3, 96). The physiological effect of dissolved carbon dioxide has also been examined in continuous cultures of the bacterial ethanol producers, including *Z. mobilis* and *Clostridium thermocellum*, although without the benefit of systems biology tools.

Ethanol tolerance has been of applied and fundamental interest since before the modern era of biotechnology, especially in wine making. Several good reviews have been written on ethanol tolerance in yeasts and other industrial organisms, which highlight the impact of ethanol on cell membranes, glycolysis, end production distribution, and cell growth (18, 23, 54, 56). Despite years of study, the biological mechanisms involved in ethanol tolerance, and how they are affected by temperature, medium composition, and genetics, remain the subject of some debate.

Genetic-level understanding of alcohol tolerance in yeasts has been significantly advanced through the use of systems biology. In 2006, separate groups published studies in which *S. cerevisiae* deletion libraries were screened for ethanol tolerance. While dozens of genes were correlated with ethanol sensitivity, genes encoding proteins involved in vacuolar function were identified by both groups as being critical in ethanol tolerance (35, 123). Since the initial reports, screening of more comprehensive libraries has expanded the list of genes which potentially influence ethanol tolerance (138).

Microarrays have also been employed in examining the response of yeast to ethanol. Alexandre et al. showed that more than 3% of the genes in the yeast genome are upregulated within 30 min of an ethanol challenge (4). Similar challenge experiments with a variety of alcohols showed significant upregulation in genes associated with stress response, glycolysis, metabolism of energy reserves, and synthesis of amino acids (34). Transcriptional profiling has also been applied to evaluate the difference between ethanol-adapted and parental yeast strains during growth in the presence of elevated ethanol. Genes related to ribosomal proteins were highly upregulated in the ethanol-tolerant strain. In addition, when cultivated under ethanol stress, both parental and adapted strains showed upregulation of genes associated with mitochondrial ATP generation and oxidative stress response (27). The number of transcriptional differences seen in response to short- and long-term ethanol exposure underscores the complexity inherent in yeast fermentations. Further evidence of this can be seen in research showing that modifications in global transcriptional machinery can impart significant improvements in overall ethanol and stress resistance (7, 8).

Systems biology tools have also been applied to evaluation of ethanol-adapted and parental of bacteria, such as *C. thermocellum* and *E. coli*, engineered for ethanol production. When performing transcriptional profiling to compare ethanol-adapted and parental strains of *E. coli*, researchers found that gene expression levels for more than 5% of the genome differed significantly. Among the affected genes were those involved in aromatic amino acid transport and synthesis, cell structure, energy metabolism, and stress response (46). In proteomic analysis of a *C. thermocellum* strain adapted to grow in 5% ethanol, the majority of membrane proteins were shown to be differentially expressed. Most of the expression difference was the result of downregulation of membrane proteins, especially those related to carbohydrate transport and metabolism (131). Subsequent resequencing, using 454 pyrosequencing and microarray-based comparative genome sequencing, revealed hundreds of mutations between the ethanol-adapted strain and the ATCC type strain from which it was derived (Brown, personal communication). Undoubtedly, additional systems biology investigations will reveal additional clues regarding the response of ethanologens to this important industrial chemical.

The development of processes for biomass ethanol production will require strains that tolerate the combined effect of ethanol, acetic acid, and degradation products arising from biomass pretreatment. While the impact of individual inhibitors on growth and product formation may not severely impair performance, the effect of the combined stresses may be more than additive (59, 100, 102). So the quintessential multiple-inhibitor-resistant super-robust ethanologen has yet to be obtained.

In biomass ethanol production, economic considerations necessitate the use of organisms capable of fermenting both cellulose- and hemicellulose-derived carbohydrates, including cellobiose, glucose, xylose, arabinose, galactose, and mannose. In practice, this requires broadening the range of sugars fermented by proven ethanol-producing organisms, enhancing ethanol production in organisms natively capable of using numerous biomass-derived sugars, or both. While metabolic engineering to obtain these improvements falls beyond the scope of this chapter, it is the subject of several good reviews and an area of active, ongoing research that includes multiple examples of successful application of systems biology tools (48, 55, 91, 122).

42.8. SUMMARY

Development of a vigorous, rugged microorganism by adaptation or as the result of directed selection is of selective value to microbes and economic value to industrial microbiologists. This robustness will have many characteristics derived from a combination of selection/adaptation and genetic changes, resulting in better survivability under stressful conditions. One of the best examples of robustness is yeast strains derived for beer and wine manufacture through the centuries which resulted in many phenotypic and genetic changes in the yeasts, leading to very beneficial survival responses during alcoholic fermentation. Similarly, engineered microorganisms for enzyme or other products are being developed to be very rugged and potent, which is part of the definition of robustness, but this microbial soundness requires a protected, engineered production environment to maintain and demonstrate their hardiness. Still, in either situation, the microorganism's response to the environment, however controlled, can be dissected and defined in ever-increasing detail by employing systems biology tools. This process starts with identification of gene and genome changes, and resultant data are used for analysis of both expression of genes and attendant proteins and their impact on the flux of metabolite in the cell and environment. As these differences become known, new or expanded modification strategies can be planned to further improve the microbe's robustness as needed for each industry.

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