

Fermentation

G O E S L A R G E - S C A L E

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THE TREND TOWARD ENVIRONMENTAL sustainability and development of renewable resources has significantly increased interest in the recovery of fermentation products, such as organic acids, feed or food additives, and industrial chemicals. Consequently, the range of products produced by fermentation is expanding beyond the traditional high-value low-volume compounds, such as pharmaceuticals, and is beginning to compete with traditional synthetic production of commodity chemicals. As fermentation moves into lower-value higher-volume chemicals, it becomes necessary to maximize efficiency, and minimize costs and waste byproducts to compete effectively against traditional options. Achieving these goals means approaching the design of fermentation and downstream separations as a single, integrated process. As stated by Williams (1): "The bioreactor should be regarded as an integrated unit operation with both upstream and downstream unit operations."

However, all too often, the design of fermentation and downstream separations are regarded separately in process development. Typically, a separation specialist takes on the challenge of designing steps to separate the various components of a complex fermentation broth that the fermentation-process designers included to maximize fermentation performance. Hence, if the required separation becomes complex and costly, the most efficient fermentation may not necessarily yield the optimum overall process.

Typically, 50–70% of the total production cost in classical processes is due to downstream processing, whereas in fermentation that employs recombinant DNA, the fraction can reach up to 80–90% (2). This large percentage is often due to separation and purification of the fermentation product.

ECONOMY OF SCALE

Fermentation broths are complex aqueous mixtures of cells, soluble extracellular products, intracellular products, and converted substrate or unconvertible components. The particular separation techniques useful for any given bioprocess depend not only on the location of the product

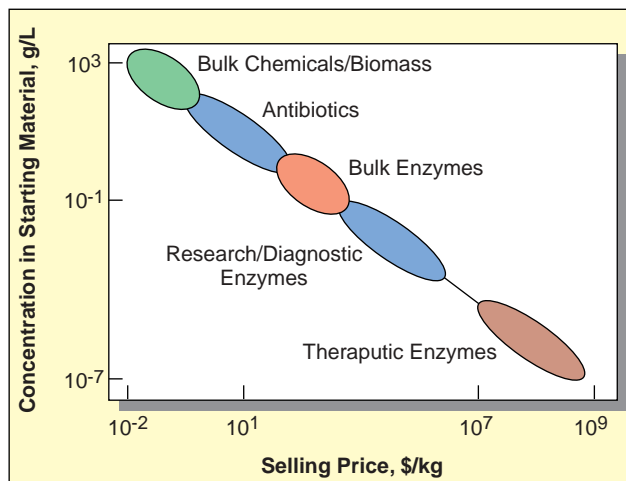
Designing an economically competitive and environmentally sustainable fermentation route means considering the downstream separation needed to capture the final product during the initial process design.



Photo courtesy of Cargill Dow.

(intracellular vs. extracellular) and its size, charge and solubility, but also on the scale of the process itself and the product value. For example, chromatography is generally useful for high-value pharmaceuticals or biologicals, such as hormones, antibodies and enzymes, but is expensive and difficult to scale up.

As with other chemical processes, fermentation for producing commodity chemicals and products is also aimed at minimizing production costs. Due to the significant impact of raw substrate materials and down-



■ Figure 1. Economy of scale: correlation between achievable product concentration and selling price for common fermentation products.

stream processing on the total production cost, process optimization is primarily focused on finding new, competitive and, above all, sustainable production technologies.

In addition, many fermentation processes are hampered by the accumulation of products in the fermenter. The recovery cost as a percent of the total production cost can vary from as low as 5–10% in, for example, the production of single-cell protein (SCP) and extracellular enzymes such as proteases, amylases, etc., to as much as 90% for the bacterial production of poly(3-hydroxyalkanoates) used to produce biodegradable thermoplastics (Table). The high cost of recovery is due to the generally low product concentrations in aqueous fermentation broths; the complexity of the broth mixture, especially when liberating intracellular products by cell disruption; and the multiple, discrete separation and purification steps in purifying the product. The latter steps lead to high capital and operating costs, as well as multiple operations that each can result in significant product yield losses and generation of waste streams that must be disposed of or recycled.

Therefore, fermentation development is bound by the overall recovery strategy chosen for a certain product. This depends on the required product purity, which can be as high as 99.999% for diagnostic and therapeutic proteins. In addition, economic consideration must be given to the feasibility of minimizing unit-operation complexity and waste production, while maximizing yield and productivity.

The impact of fermentation on the overall production economics strongly depends on the type of product made, *i.e.*, industrial vs. therapeutic proteins; and specialty vs. bulk chemicals. This depends on the desired vs. achievable product concentration in the final broth. According to the economy of scale, the selling price of a product is inversely correlated with its achievable concentration at the end of fermentation, and can range by 9–10 orders of magnitude (Figure 1).

Regardless of the product, the highest purity in the final broth is the most desirable. Since, for bulk chemical and biomass routes, fermentation already represents a large fraction of the total production costs, it is here that so-called “clean” fermentation development (*i.e.*, integration of fermentation and separation to reduce the environmental ‘footprint’) can have a major impact on the overall economics. There is continual pressure for technical improvement on the upstream bioreactor section to yield a final broth of higher-product concentration to lower the cost of separation and recovery.

DEVELOPMENT OF LARGE-SCALE FERMENTATION TECHNOLOGY

The most obvious benefits that can be achieved from integrating fermentation and downstream processing are

Table. Recovery or purification cost as a percentage of total production cost for typical fermentation products.

Source	Product	Recovery cost as of percentage of total production cost
Whole-cell yeast biomass	Single-cell-protein, yeast extract	5%
Bulk chemicals	Lactic, citric and malic acids	10–50%
Extracellular enzymes	Amylases, proteases	10%
Antibiotics	Penicillin	20–50%
Intracellular enzymes/proteins	Human insulin, interferon	90%

Box. Roadmap for Integrated Process Development

- Analyze of economic and process constraints based on preliminary process design
- Identify opportunities for improvement, *e.g.*, reduced waste streams, energy use, impurity levels and raw material use
- Put together a wish list of physiological characteristics and downstream separation performance
- Evaluate feasibility of achieving the wish list based on technical difficulty and economics
- Define the best strategy for addressing each opportunity by taking into account both downstream and fermentation capabilities, such as high cell density, extractive fermentation, simplify broth, etc.
- Integrated fermentation and downstream process development

minimizing waste, raw materials, capital and energy. An often-forgotten and more-challenging-to-achieve benefit is environmental sustainability. As questioned in a recent article in *Time*, if there could ever be a system that’s perfectly efficient, the author answered, “Yes, it already exists, and we call it nature. The same materials have been recycled for billions of years. The new industrial revolution is all about absorbing the lessons we should have learned from nature long ago. ... Efficient use of energy and materials and a reduction in waste can help the bottom line.” (“New War on Waste,” *Time*, pp. A28-A31 (Aug. 26, 2002)).

As fermentation is becoming an increasingly integral part of the development of many high-value products and is replacing conventional routes for commodity products, these processes will need to be integrated similarly to the way the petroleum industry has worked on this over the last few decades (Box).

In general, large-scale fermentation development comprises of the following steps:

1. Organism selection, with regard to:
 - substrate versatility
 - byproduct formation characteristics
 - robustness of the organism, *e.g.*, to process upsets
 - viability with regard to cell recycling
 - physiological characteristics (maximum growth rate, aeration requirements, etc.)
 - genetic accessibility.

2. Metabolic and cellular engineering:

- improve existing properties of the organism
- introduce novel functions, for example, by simplify-

ing product recovery, expanding substrate and product ranges, and enabling fermentation to occur under non-standard conditions

3. Fermentation process development:

- culture and media optimization (from complex to defined minimal media)
- optimization of cultivation parameters that take into account product recovery and purification (minimize byproduct formation, minimize chemical inputs, and develop high-cell-density cultivation)
- incorporation of cell retention/recycling

4. Introduction of downstream unit operations within a fermentation process:

- examples are extractive fermentation, electro dialysis and in-line membrane separation technologies.

INTEGRATION TIPS

Integration can be approached from different angles. The following examples by no means comprise a comprehensive list, but relate to the steps involved in large-scale fermentation development.

Simplify the fermentation broth — In principle, any ingredient added to the broth that does not end up as product will have to be removed. It therefore behooves the fermentation-process designer to eliminate any unnecessary ingredients from the broth. For example, many fermentation processes employ complex media, such as yeast extract or corn steep liquor (initial waste stream of corn wet milling operation; generally used as a rich source for nitrogen in fermentation media). These media are inexpensive and ample in nutrients. Designing defined fermentation media from salts and vitamins requires a considerable development effort to provide a recipe capable of supporting microbial production at the desired levels. However, it is sometimes worth considering the tradeoff between slightly reduced fermentation performance and a greatly simplified downstream process. In addition, if a complex medium component must be used, the

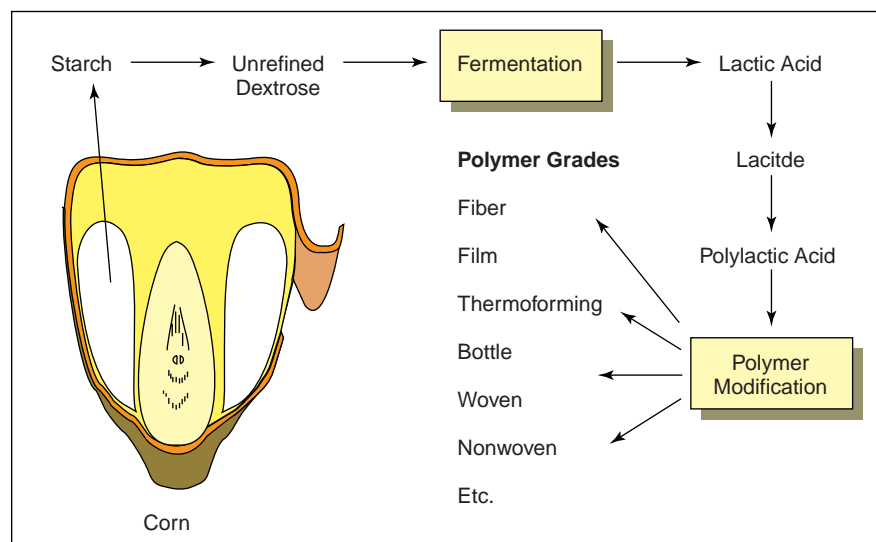
component may contain elements that do not directly benefit the fermentation, yet provide a separation challenge downstream. In some cases, it may be feasible to separate out these non-beneficial nutrients before they reach the broth.

Ease separation by altering the product form — In some cases, different forms of the product are easier to separate downstream than others. A common example of this is organic acids, where the free-acid form of the product may be easily extracted from a fermentation broth, while the salt is not easily removed. If fermentation is designed separately from downstream processes as is usually the case, an acidification step will be required downstream. However, this requires that a neutralizing base be added to the broth, which must later be removed by adding an acid, with both base and acid becoming a waste salt that must then be disposed of. This inefficiency can be avoided if the fermentation can be carried out at a low enough pH to provide the product predominantly as the acid. The resulting savings in acid, base and waste disposal costs may offset a considerable amount of decline in fermentation performance resulting from the more unfavorable acidic fermentation conditions.

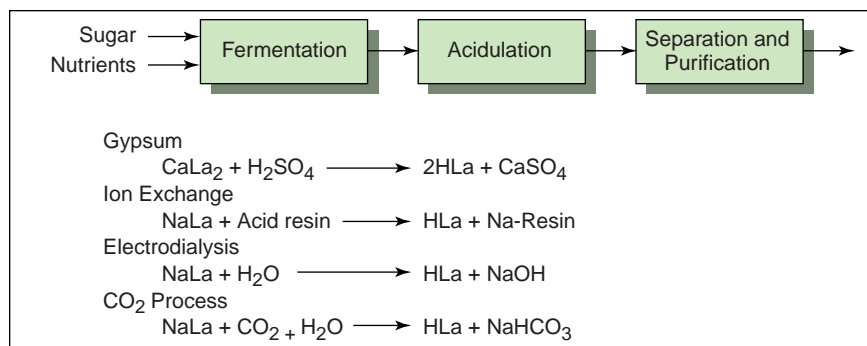
Reuse the fermentation broth components — Significant improvements in fermentation raw-material yield and production rate can be effected by reusing components of the broth. For example, recycling cells, although a technical challenge, holds promise for improving fermentation efficiency. Separation of the biomass from the broth requires that the cell fraction be treated cleanly to prevent contamination of subsequent fermentations, and that the cells not be subjected to unnecessary stresses to maintain their viability. However, any live biomass that can be reused reduces the amount of new substrate that is required for biomass growth, as well as reducing the challenges of biomass disposal. Also, an increased concentration of cells can increase the production rate in the fermenter, which will reduce fermentation capital costs. Even if the biomass cannot be recycled viably, if it can at least be recycled cleanly, it can become a nutrient source for later biomass production.

Another strategy is reusing some or all of the broth after product separation. Often, optimum product synthesis and biomass growth take place when medium nutrients are present in excess. However, this results in nutrients being left over at the end of fermentation. Reuse of the broth allows a reduction of nutrient addition to the next batch, as well as avoiding the cost of treating a high biochemical-oxygen-demand (BOD) waste stream.

Remove the product during fermentation — In many fermentations, the product acts as an inhibitor to the production reactions. This can limit the



■ Figure 2. Simplified pathway for the production of polylactic acid (PLA) and derived consumer products.



■ Figure 3. Various lactic acid acidification technologies are aimed at eliminating salt byproduct formation.

concentration that can be achieved in the fermenter. However, as said above, the product concentration in the finished fermentation broth is a clear inverse indicator of cost. Removing the product during fermentation increases the yield by allowing more to be produced from a given amount of biomass, plus increases the production rate by reducing the accumulation of an inhibitory product. Using continuous extraction, a side-stream can be pumped out of the unit and the extracted broth returned to it. Further, two-phase fermentations have been developed to extract the product from a biomass-containing aqueous phase into an organic phase, which can then be removed on-line.

Reduce the water content of the broth — Typically, as much as 90% or more of the broth is water, which must be removed. This is not only costly to separate, but also produces a large aqueous stream that must then be disposed of or recycled. Integrative approaches to water reduction include increasing the biomass concentration (*i.e.*, high-cell-density (HCD) fermentation), engineering the organism to tolerate higher product concentrations, and removing inhibitory elements from the fermentation recipe.

Several examples will now illustrate how costs can be kept down. The large-scale fermentation for the production of lactic acid (3), baker's yeast (4, 5) and recombinant human albumin (6) embody an integrated approach to process design that integrates the steps listed above to reduce waste-management and production costs.

LACTIC ACID

Lactic acid is a commodity chemical that is used in the production of medicines, foods and beverages, and polymers. In the U.S., a large portion of the lactic acid is used as a feedstock to make polylactic acid-based (PLA) polymers for the production of fibers, films, nonwovens, etc. (Figure 2). Lactic acid is generally produced by fermenting dextrose found in biomass via bacteria, yeast or fungi. PLA production is environmentally sustainable, with 20–50% more PLA being produced using the same amount of fossil fuel vs. petrochemical-based plastics (7). PLA-based plastics derive their carbon from plant carbohydrates which, in turn, are produced from carbon dioxide via photosynthesis. This ultimately results in lower net carbon dioxide emis-

sions. In the near future, PLA is expected to be produced on multi-billion dollar/year scale. However, there are still challenges to be resolved to produce inexpensive pure lactic acid monomers in large quantities from renewable resources.

High production yields of lactic acid and a robust, simple production system with minimum byproduct formation are of key importance, since, generally, the sugar feedstock ac-

counts for over 50% of the fermentation cost. Lactic acid is usually produced as a salt, such as NaLa or CaLa (where La = lactate), since the pH of the fermentation process has to be maintained far above the pKa of the acid species (= ~3.8; *i.e.*, at pH = pKa, the salt-form:free acid form is 50:50). A subsequent acidification obtains the undissociated form, HLa. Various acidification methods can retrieve undissociated lactic acid. These include using sulfuric acid, acid resins in ion-exchange chromatography or CO₂/triethylamine treatment.

This step should avoid producing large amounts of salts (calcium, ammonium or sodium sulfate), which have little added economic value, and are mainly discarded or sold as fertilizer. As an example, acidifying a lactic-acid fermentation-broth with sulfuric acid yields equal amounts of gypsum (CaSO₄) (Figure 3). Several separation and purification techniques can concentrate the acid, such as esterification with ethanol, and subsequent distillation and hydrolysis; direct distillation of HLa; or liquid/liquid extraction. Any optimization should have a high yield and meet the proper product quality.

With certain bacteria as biocatalysts, lactic acid can be made in high yields (> 90% of the sugar carbon is converted straight into lactic acid carbon) and high volumetric productivities (0.1–10 g/L/h). The biocatalysts used for lactic acid production are efficient, thus, small amounts are needed (< 5 g/L). This is favorable in terms of economics, since in bulk-chemical production, where the product is entirely made from sugar carbon, any loss of carbon (*e.g.*, to biomass production) is a loss of product yield.

Integration of the fermentation-process development for lactic acid focuses on reducing salt byproduct formation by developing new salt-regeneration strategies and lactic acid technologies at low pHs. Further reductions in capital and operating costs have been effected by: simplifying separation operations; developing clean, defined media; improving biocatalyst efficiency by metabolic engineering; using alternative feedstocks; and applying technologies that reduce lactic acid accumulation in the broth.

HIGH-CELL-DENSITY (HCD) BAKER'S YEAST PRODUCTION

HCD fermentations are defined by the presence of cell dry-weight biomass concentrations that exceed 100 g/L (vs.

5–20 g/L for other fermentations). The amount of protein derived per amount of feedstock, as well as the specific productivity, are generally two to three orders of magnitude lower compared to bulk chemical production. Therefore, high biomass concentrations are required to achieve high product concentrations in the final broth. HCD fermentations are being developed for the production of baker's yeast and for recombinant human serum albumin (rHSA), a major blood-plasma protein with the yeast *S. cerevisiae*.

In HCD fermentation, a substantial part (> 25%) of the culture volume is occupied by cell biomass. This has implications for downstream processing in protein production, since additional water and extracellular proteins tend to adhere to the cells at high concentrations, requiring washing of the biomass to liberate the proteins.

To make HCD fermentation for protein production successful, developments focus on media optimization with respect to use of carbon sources, inorganic nutrient packages, complex vs. defined media formulations, vitamins and foaming. Due to the large amounts of biomass generated, attention is being put on oxygen transfer and heat transfer. For HCD, large excess additions of nutrients should be avoided to eliminate osmotic and toxicity effects, reduce the cost of media, facilitate downstream processing of extracellular products, and manage waste-stream production. Therefore, development of balanced, "clean" media has high priority; requirements are species- and even strain-dependent.

The production of baker's yeast is about 2,500 kton/yr (5). Process optimization, focused on improving dough-leavening capacity, has been on strain selection and empirical optimization of environmental conditions during the fed-batch production process. The large demand for high-

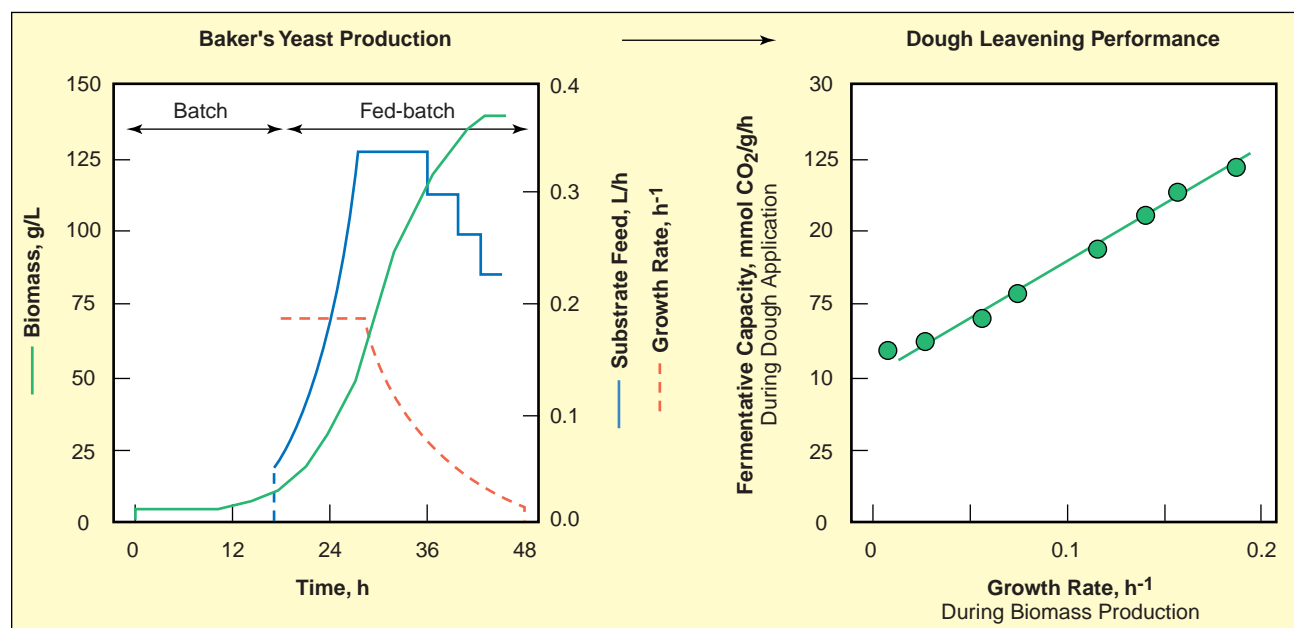
quality (drinking) water in downstream processing of commercial baker's yeast (> 15 m³ washwater used per ton yeast produced) provides an environmental and economic incentive for novel fermentation processes on food-grade media. The final yeast produced can be used in food applications without the need for downstream processing.

A further incentive for sustainable production technology is that, due to improvements in beet and cane sugar refining and the quality of molasses, the conventional feedstock for baker's yeast production is declining. The major challenge is to reconcile the conditions necessary for HCD cultivation with the quality requirements for industrial baker's yeast (high dough-leavening power and high storage stability). Dough-leavening power can strongly depend on the growth-rate history of the biomass (Figure 4). Developing cleaner HCD production technology necessitates understanding how to improve the dough leavening capacity at low growth rates in baker's yeast cultures (gassing power is the selling trait in baker's yeast, not the biomass itself).

HCD HETEROLOGOUS PROTEIN PRODUCTION: RECOMBINANT HUMAN ALBUMIN (rHA)

Similar developments have been happening in the production of rHA. This protein is abundant in human blood (at about 40 g/L) and it serves various physiological functions. Therapeutically, it is used in multi-gram doses for the treatment of shock or burns, and also to compensate for blood loss (6). With an annual market of 400,000 kg, an estimated product concentration of 5 kg of rHA/m³ (~ 5 g/L), and 80% recovery in downstream processing, roughly 1,000 HCD fermentations of 100 m³ are run each year.

One of the major challenges is to find expression sys-



■ Figure 4. The fermentative capacity of baker's yeast, as manifested during the dough leavening application, is strongly dependent on the final specific growth rate during biomass production in high-cell-density fed-batch cultures.

tems for production of the protein under HCD cultivation conditions (i.e., under low growth rates) and to minimize downstream purification requirements.

CRITICAL ISSUES IN FERMENTATION DEVELOPMENT

Irrespective of producing proteins or biomass, HCD yeast biomass production requires aerobic conditions. Due to the low oxygen solubility in aqueous solutions and limited oxygen transfer/cooling capacity in large-scale fermenters, it is necessary to control the specific growth rate of biomass by controlling the carbohydrate feedstock addition-rate to maintain fully aerobic conditions in the fermenter. Concomitant with extremely low specific growth rates, a large amount of consumed sugar carbon is simply burned by the yeast biomass to carbon dioxide to provide energy for cell maintenance. This is a general observed phenomenon with many organisms that reduces the overall product yield in biomass and protein-production processes. In baker's yeast production, low, specific growth rates result in low dough-leavening capacity of the final product. Therefore, the focus in the baker's yeast production industry is to improve this parameter at low, specific growth rates.

In heterologous protein production, the specific rate of protein production, q_p , is a key parameter that is rarely reported in the scientific literature. Values of q_p generally range from 0.5–2.5 mg/g-h at a growth rate of 0.1/h and represents generally less than 10% of the overall cellular production rate (β). Efforts are on understanding the relationship between the specific growth rate of the culture and the specific rate of protein production. This relationship is needed to further optimize the protein expression system with respect to factors such as the stability of expression constructs (over a large number of cell generations), selection markers (cost, stability), constitutive vs. inducible promoters, and the dependency on expression conditions, among other factors. As with baker's yeast production, work on HCD protein-production systems also focuses on improving expression levels in slowly growing cultures.

Over the past few years, research in this field has been directed towards innovative fermentation process design that can reduce downstream processing costs significantly or even eliminate them completely (as in baker's yeast production). Further, designing new-generation bioprocesses increasingly depend on engineering process-compatible microorganisms. The latter, whether through genetic or physiological manipulations, can be greatly assisted by metabolic engineering. To achieve these goals, more fundamental knowledge is needed about metabolic pathways, control mechanisms and process dynamics to optimally design integrated systems. This knowledge will enable industry to select the right biocatalyst in clean fermentation processes, as well as introduce and express new or improved properties of the biocatalyst via genetic engineering to facilitate and/or improve downstream processing. Chemical engineers,

metabolic/genetic engineers and microbial physiologists will have to work together on this journey.

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