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# *Bacillus* and the Story of Protein Secretion and Production

*Giulia Barbieri*[1], *Anthony Calabria*[2], *Gopal Chotani*[2], *and Eugenio Ferrari*[1]

[1] *Dipartimento de Genetica Molecolare Batterica, Universidad di Pavia, Pavia PV, Italy*
[2] *DuPont Industrial Biosciences, Palo Alto, CA, USA*



## 1.1 *Bacillus* as a Production Host: Introduction and Historical Account

Contrary to logical thinking, the use of enzymes in daily activities may actually predate the development of modern agricultural societies. Nomad populations of hunters and gatherers exploited rennin produced by the stomach of ruminants for the cheese-making process; while the development of fermentation processes for alcohol can be traced back to more than 7000 years (McGovern et al. 2004; Alba-Lois and Segal-Kischinevzky 2010). However, it is only in the nineteenth-century that enzymes were identified as responsible factors for century old processes such as leather tanning and conversion of starch to sugar (Payen and Persoz 1833).

At the beginning of the twentieth-century, thanks to the work of Otto Rohm, enzymes started playing a wider role in industrial processes as well as in household applications (Wallerstein 1939; Maurer 2010). Two US patents were granted on the use of enzymes for the conversion of starch to sugar; one filed by Schultz et al. (1939) describing the use of a *Bacillus mesentericus* "extract," and the other by Dale and Langlois (1940) claiming the use of fungal saccharifying enzymes.

In the mid-1950s, microbial enzymes started being used extensively in several applications. Large-scale enzyme preparations, obtained via microbial fermentation thus prominently entered the industrial world (Underkofler et al. 1958). The 1960s saw the dawn of *Bacillus* as a production workhorse. Toward the end of the decade, *Bacillus*-derived proteases took hold as essential components of laundry detergents (Roald and De Tieme 1969). At about the same time, high temperature-resistant amylases, useful in the

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saccharification process, were identified in *Bacillus licheniformis* and *Bacillus amyloliquefaciens*. At first, due to the insufficient genetic characterization, the strains used in large-scale fermentation were isolated via a labor-intensive and time-consuming approach of mutagenesis and screening. Most likely, several thousand mutants were tested for improved production characteristics, such as relief of catabolite repression, antibiotic resistance (most likely mutation in one or more ribosomal components), and sporulation deficiency (Ingle and Boyer 1976). The choice of sporulation mutations is particularly important since it allows extending production time in fermentors and, due to poor survival of nonsporulating cells in the environment, precludes isolation of production strains by competitors. The advent of genetic engineering allowed making rapid targeted changes in enzymes and accelerated construction of *ad hoc* production strains starting from laboratory strains, allowing budding industrial biotechnology companies, such as Genencor, to introduce the first detergent alkaline protease produced by a recombinant microorganism in 1984 (E. Ferrari, unpublished).

For the reasons mentioned above, and for their ease of growth in large-scale submerged fermentation, members of the genus *Bacillus* play a very important role in the manufacture of a number of industrially important products. While the use of *Bacilli* has been explored for the synthesis of pharmaceutical products, their most important commercial role is in the production of industrial enzymes (Aehle 2007). It is estimated that in the current greater than $4-billion industrial enzyme market, *Bacilli* produce about 50% of the enzymes (G. Nedwin, personal communication). These products are employed in a variety of important commercial applications such as laundry, dishwashing, starch-derived ethanol and sweeteners, baking, animal feed, textile, and leather (for review see Aehle 2007).

Several traits make the genus *Bacillus* attractive for protein production especially since *B. subtilis* has a long history of safe use. *Bacillus natto*, a very close relative of the laboratory strain *B. subtilis*, has been used to obtain natto, a staple of Japanese cuisine from soybean fermentation, for over a thousand years (Nishito et al. 2010). Furthermore, what makes the use of *Bacillus* for the production of industrial enzymes particularly attractive is its ability to secrete proteins in the culture fluid. This is a necessary feature to keep the cost of the enzymes low, an essential aspect for this class of product. In fact, in most cases, the cost of enzyme production has to be below the $\$500\,kg^{-1}$ mark, hence the necessity to have low recovery-associated costs. Over the years, a number of tools have been developed to ease and speed up *Bacillus* genetic manipulation. The availability of the sequenced genomes of both *B. subtilis* (Kunst et al. 1997) and *B. licheniformis* (Rey et al. 2004; Veith et al. 2004) has allowed studies aimed at better understanding their behavior during growth and production (Buescher et al. 2012; Nicolas et al. 2012). Moreover, the well-characterized fermentation, its relatively short time, and

the possibility to use cheap feedstock add to the appeal of using these bacteria for the production of industrial enzymes.

This chapter is divided in two main sections: the first section focuses on the genetic tools and strategies useful for the efficient cloning and expression of proteins in *Bacillus*, while the second section provides an up-to-date status on fermentation and recovery of heterologous enzymes from *Bacillus*.

## 1.2 The Building of a Production Strain: Genetic Tools for *B. subtilis* Manipulation

Numerous genetic manipulation techniques of *B. subtilis* laboratory strains have been established over the years. These tools have helped in refining the genetic and biochemical characterization of this microbe. Hence, even if *B. subtilis* had never played a major role in the historical development of the genus *Bacillus* as an industrial workhorse, the availability of new or genetically engineered enzymes with new properties, and the need to express them at very high levels, has placed this laboratory microbe as a frontrunner in the expression of industrial enzymes. In fact, the only tool available to carry out the needed manipulations in the traditional industrial strains, namely *B. licheniformis* and *B. amyloliquefaciens*, was and is to a large extent protoplast transformation. But this approach is very time-consuming and not always reliable. Recently, however, in some instances, it has become possible to develop competence in *B. licheniformis* strains using the *comK* induction system described below (Diaz-Torres et al. 2003; Hoffmann et al. 2010). Given that the tools and ways to transform *B. subtilis* are simple, the building of a *B. subtilis* production strain for a secreted protein is relatively straightforward when the transcriptional and translational determinants for the synthesis of the protein of interest as well as a signal sequence to direct its secretion are available.

Some of the genetic techniques and tools currently available for building a *B. subtilis* production strain are briefly outlined in the next section.

### 1.2.1 Promoters

There are a number of promoters that can be used to direct transcription of any target protein. One of the best-characterized *B. subtilis* promoters is *aprE*, which is responsible for the transcription of the alkaline protease. It is yet difficult to explain why a promoter responsible for the expression of one of several scavenging enzymes is so complexly regulated (Ferrari et al. 1993). The transcription of *aprE* is controlled by at least two different repressors, AbrB and ScoC, and by a pleiotropic transcriptional activator, DegU (Henner et al. 1988), which can boost the transcription of the *aprE* mRNA by about 100-fold. The presence of both AbrB and ScoC assure that AprE is not synthesized before

the transition phase, e.g. before the culture enters the stationary phase. One advantage of using the *aprE* promoter for heterologous expression is the presence of a transcriptional leader sequence responsible for extending the half-life of its mRNA to about 25–30 minutes (Hambraeus et al. 2002). The mRNA stability is transferred to most genes hooked to this transcriptional leader, allowing robust expression.

Another widely used promoter is the amylase promoter, in its different versions, *amyE*, *amyQ*, and *amyL*, which are derived from *B. subtilis*, *B. amyloliquefaciens,* and *B. licheniformis*, respectively. The amylase promoter, albeit not under strict sporulation control, is temporally regulated and its transcription is turned on at the end of the vegetative growth, just before the cells enter the stationary phase. This is most likely due to the control exerted by catabolite repression in both *B. subtilis* and *B. licheniformis* (Nicholson et al. 1987; Laoide et al. 1989).

Two other promoters worth mentioning are the *sacB* and *sacC* promoters. The *sacB* promoter directs the transcription of a gene responsible for the conversion of sucrose to glucose and fructose and for the production of levans (Gay et al. 1983; Steinmetz 1993). The expression of the *sacB* gene is transcriptionally boosted by certain *degU* mutations, similar to the *aprE* promoter. However, *sacB* is not under sporulation control and is transcribed during vegetative growth. A useful synthetic promoter, widely used in research studies in *B. subtilis* is the *spac* promoter and all its derivatives. It is a hybrid promoter built by fusing a promoter taken from the *B. subtilis* phage SP01 and the lac operator sequence from *Escherichia coli* (Yansura and Henner 1984). This promoter is very useful to understand the possible toxicity of an expressed gene because it allows, to a certain extent, to modulate the transcription of any gene fused to it.

### 1.2.2 Vectors for Building a Production Strain

There are three types of plasmid vectors that can be used for carrying out genetic manipulations in *B. subtilis*: replicating, temperature sensitive (Ts), and integrative. We will limit the description of the vectors to their use in cloning/expression experiments and will refer the curious reader to three extensive reviews on this subject (Bron 1990; Janniere et al.; 1993; Perego 1993).

Most replicating plasmids are inadequate when building an expression strain due to their inherent instability: in some cases these plasmids are lost during an overnight incubation, even when the strain expressing them is grown under selective pressure conditions. Their usefulness is, therefore, limited to initial cloning and expression testing tasks. Furthermore, since only multimers are effective in transformation of competent *B. subtilis* cells (see section on transformation), "shuttle" vectors carrying both an origin of replication for *B. subtilis* as well as for *E. coli* were developed. One drawback of using shuttle vectors

is that often *Bacillus* genes, especially the secreted ones, are toxic to *E. coli*, therefore the outcomes of this approach need to be monitored carefully.

To build expression strains it is, therefore, preferable to work with Ts or integrative vectors that allow stable integration of the desired expression construct into the chromosome. A temperature-sensitive origin of replication, such as the one from pE194Ts (Bron 1990), forces the plasmid to integrate into the chromosome, upon raising the temperature under selective pressure, provided that the vector carries a region of homology with the host DNA. The use of these Ts vectors is a necessary step when transforming protoplasts, which are incapable of integrating incoming DNA directly in their chromosome in contrast to competent cells. Furthermore, when working with a *Bacillus* strain that can be made competent, it is sufficient to create a circular expression cassette carrying: (i) a fragment of DNA homologous to the chromosome of the recipient host; (ii) an antibiotic-resistance locus for selection; and; and (iii) the construct with the gene to be expressed. In this case, because of the multimeric requirement, one must resort to the *in vitro* amplification steps described in Figure 1.1.

### 1.2.3 *B. subtilis* Competent Cell Transformation

The ability of *B. subtilis* to differentiate in a physiological state, known as "competence," associated with the ability to take up exogenous DNA in response to the exhaustion of nutrients in the environment, has been widely exploited for the genetic manipulation of this industrially relevant microorganism. Under laboratory growth conditions, when nutrients become limiting and cells enter stationary phase, a limited fraction of the cell population switches to the competent state for DNA transformation (Hamoen et al. 2003). Since the pioneering work of Anagnostopolous and Spizizen (1961), different protocols for the preparation of competent *B. subtilis* cells have been developed. The majority of them relies on a two-step procedure: growing cells to an early stationary phase, at 37 °C, in minimal medium with 0.5% glucose as the sole carbon source, then, diluting the cells in a poorer minimal medium containing, in most cases, a lower concentration of amino acids. After 90 minutes of incubation, the cells are highly competent and ready to be transformed by addition of purified chromosomal or plasmid DNA. In optimal conditions, only about 10–20% of the cells in the culture will develop competence (Somma and Polsinelli 1970).

Remarkably, only multimeric plasmids, either produced by *rec*$^{+}$, *rec*B-C$^{-}$, or *rec*F$^{-}$ *E. coli* hosts strains (Bedbrook and Ausubel 1976), or generated *in vitro* can be used to successfully transform *B. subtilis* competent cells at high frequency. Though the requirement for multimerization does not allow the use of a ligation mixture to directly transform *Bacillus* competent cells; multimers created via PCR (Shafikhani et al. 1997) or the use of commercial kits (e.g. Templiphi, sold by GE Healthcare Lifesciences) are sufficient to transform

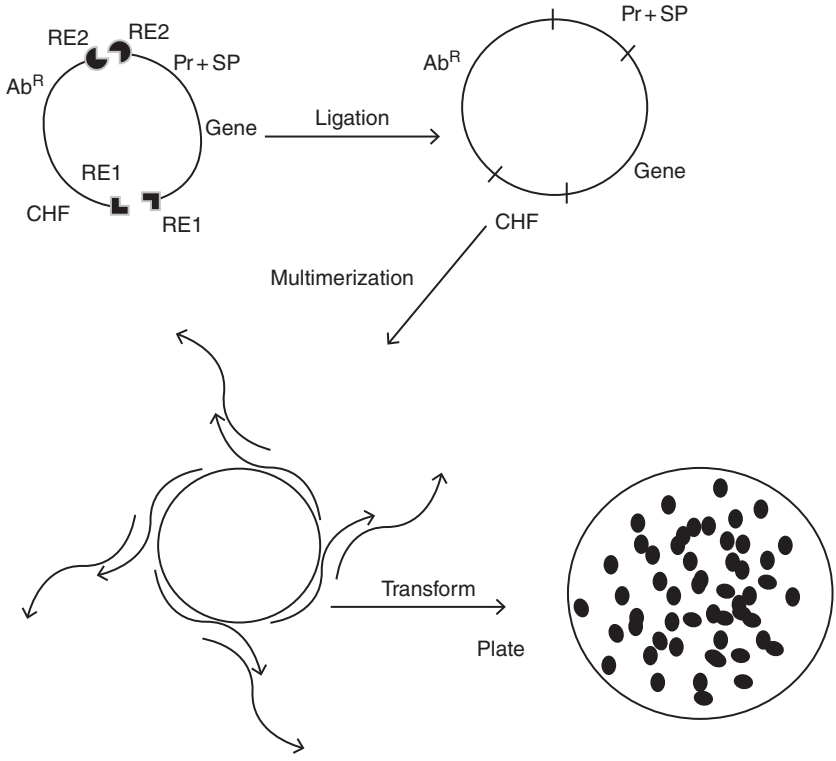


**Figure 1.1** *In vitro* amplification steps. REs, restriction enzymes; Pr + SP, promoter and signal peptide; CHF, chromosome homology fragment; and $Ab^R$, antibiotic resistance or any other selectable marker.

*Bacillus* competent cells with ligation mixtures and thereby skip the initial *E. coli* cloning step.

The selection of cells reaching competency within a population is random and dependent on the variable expression of a single protein, the transcriptional regulator of competence ComK (van Sinderen et al. 1995). The expression of this master regulator of competence is controlled, via a quorum-sensing-associated mechanism, both at the transcriptional and posttranslational level and is subject to an auto-regulatory positive feedback loop (van Sinderen and Venema 1994).

The role of ComK as the master regulator of competence has been exploited for the induction of competence and the development of "super-competent" *B. subtilis* cells. An extra copy of the *comK* gene, placed under the control of the xylose-inducible promoter PxylA, was integrated in the *lacA* locus of the chromosome (Hahn et al. 1996, Zhang and Zhang 2011). Xylose-induced competent cells can be transformed with efficiencies greater than $1 \times 10^7$ transformants $\mu g^{-1}$ of multimeric plasmid DNA.

### 1.2.4 Protoplasts-Mediated Manipulations

Use of protoplast-mediated techniques requires methods that allow conversion of cells to protoplasts and subsequent reversion to the bacillary form (regeneration) (Bourne and Dancer 1986). *B. subtilis* cells growing exponentially in rich medium can be easily converted to protoplasts by lysozyme treatment at 37 °C in an osmotic stabilizing medium. Protoplast formation can be monitored by microscopic observation and generally begins within 30 minutes after addition of lysozyme. The incubation period in *B. subtilis* can generally be prolonged to 60–90 minutes to ensure complete protoplast formation (Chang and Cohen 1979). However, for some bacilli, such as *B. licheniformis*, a long incubation period in the presence of lysozyme can ultimately affect the efficiency of regeneration.

### 1.2.5 Genetics by Electroporation

High transformation efficiencies in *B. subtilis* have also been obtained by electroporation, a technique in which the application of an electric pulse to the cells alters the membrane potential causing a temporary breakdown of the cell membrane permeation barrier that allows the entry of DNA into the cells (Tsong 1992). The procedure varies depending on the strain employed and is very sensitive to different parameters, such as field strength, growth and electroporation medium composition, concentration of competent cells, plasmid variety, and so forth (Lu et al. 2012). The addition of DL-threonine and glycine to the growth medium made it possible to obtain reliable electro-competent *B. subtilis* str. 168 cells that could be efficiently electro-transformed (McDonald et al. 1995). A hyper-osmolarity electroporation method developed by Xue and coworkers (1999) using high concentrations of sorbitol and mannitol gave $1.4 \times 10^6$ transformants $\mu g^{-1}$ of plasmid DNA. In addition to the introduction of circular replicating DNA, methods for successful electroporation with linear integrative DNA have been recently described (Yang et al. 2010; Cao et al. 2011; Meddeb-Mouelhi et al. 2012; Wang et al. 2012).

## 1.3 *B. subtilis* Secretion System and Heterologous Protein Production

There is a large body of excellent reviews dealing with the *Bacillus* Sec-dependent secretion machinery (Simonen and Palva 1993; van Wely et al. 2001; Tjalsma et al. 2004; Harwood and Cranenburgh 2008) as well as the TAT secretion pathway (van Dijl et al. 2002); hence, we will not review the subject here. We will only point out that the translocase complex of *B. subtilis* is homologous to the system found in *E. coli* (de Keyzer et al. 2003). SecY, SecE, and SecG proteins form the core of a heterotrimeric integral membrane

pore that interacts with SecA, an ATPase that drives translocation (Meyer et al. 1999). However, in Gram-positive bacteria, unlike the Gram-negative ones, proteins exported through the cytoplasmic membrane are released directly into the external environment. This ability to export high amounts ($>20\,g\,l^{-1}$) of proteins into the growth medium (Schallmey et al. 2004) renders *B. subtilis* an ideal host for the production of industrial enzymes. In fact, from a commercial point of view, the purification of proteins from the culture supernatant rather than from the cytoplasm is considerably more cost-effective, less time-consuming, and often leads to improved structural authenticity (Pohl and Harwood 2010). Nevertheless, the secretion of heterologous or mutagenized proteins is frequently inefficient because of a variety of secretion bottlenecks, not fully elucidated. These could include poor membrane targeting, inefficient membrane translocation, slow or incorrect polypeptide chain folding, and degradation by extracellular proteases (Li et al. 2004). While the degradation of the protein of interest by the extracellular proteases is, at least in part, under control thanks to the availability of strains deleted for genes encoding multiple proteases (Wu et al. 2002), there is no definite solution to the export blocks. However, the current understanding of the secretion machinery does not allow making rational changes that would result in solving this problem. It is likely that changes in the Sec components would affect the fitness of the cell, given their important role in targeting a large number of vital components to the membrane/cell wall apparatus. It is difficult to imagine that such a weakened cell could perform well under stressful fermentation conditions; hence, most approaches should focus on introducing changes in the protein to be exported. One such approach would be the testing of different signal peptides that are necessary to efficiently direct the protein to the translocase (Kakeshita et al. 2011). As recently revealed by a systematic study in which all *B. subtilis* Sec-type signal peptides were screened for their ability to direct heterologous protein secretion, an optimal fit between the signal peptide and the mature protein is required for efficient secretion (Brockmeier et al. 2006). Target proteins must fold rapidly as they emerge from the Sec translocase. Proteins that fold slowly or partially in fact expose protease-sensitive sites that are recognized and cleaved by WprA, HtrA, and HtrB, the "quality control" proteases expressed by *B. subtilis* to monitor proteins at the membrane and wall interface (Jensen et al. 2000).

Another solution devised in the case of mutagenized commercial proteases was the work in which the proregion of the subtilisin in question was mutagenized via a site evaluation library (Estell and Ferrari 2009; Ferrari et al. 2010). Each of the 84 triplets of the pro-region was mutagenized using 84 libraries of *in vitro*-generated primers (one library for each residue). The anticipated outcome was to obtain pro-region libraries in which each targeted codon contained the triplets encoding for all the 20 natural amino acids. *Bacillus*-competent cells were transformed with the mutagenized constructs and

protease expression in the variant cells was tested. Every mutation introduced in some of the residues close to the autoproteolytic site (Power et al. 1986) was deleterious to protease expression, probably due to the interference with the self-maturation process. However, changes at other sites boosted expression up to 100% of the initial titer. The rationale behind this outcome or whether this approach can be applied in a broader context is not yet obvious.

In conclusion, while achieving the secretion of heterologous proteins appears, at present, to have a hit or miss outcome, some recent studies seem to suggest that there are ways to overcome this problem.

### 1.3.1 *Bacillus* Fermentation and Recovery of Industrial Enzyme

This section examines an industrial process used to produce an engineered thermostable α-amylase for anti-staling in baking applications. A description of this process was chosen because it delivers critical enzymes for the baking industry, e.g. α-amylases that maintain bread freshness for a longer time that reduces bread waste. However, the baking process involves raising the internal bread temperatures to at least 100 °C, and typical enzymes are inactivated around 85 °C. Therefore, thermostable amylases are desired for this application. A production process begins with the engineering of an α-amylase for thermostability and concludes with the enzyme product in the form of a spray-dried powder. Various examples from the literature are highlighted to provide specific process details. Rather than describing an optimal production process, the goal of this example is to provide the reader with a flavor of the options to consider when choosing between various types of processes for the production of a protein product from *B. subtilis*.

A thermostable amylase may be discovered as a novel enzyme from nature, selected from existing enzyme databases, or engineered by using either rational or combinatorial approaches. An example of enzyme engineering is described in the European patent application from Genencor International, Inc. entitled, "Thermostable amylase polypeptides, nucleic acids encoding those polypeptides and uses thereof," EP2292745A1 (Gernot et al. 2011). A plasmid containing the variant gene encoding the desired thermostable amylase is then transformed into a protease-deficient sporulation mutant of *B. subtilis*, according to the methods of Wells et al. (1983) as described in detail in the earlier section of this chapter.

The engineered *B. subtilis* strain is exposed to a representative production environment after preliminary screening and characterization. Optimization of fermentation conditions includes the development of an appropriate medium. Ideally, the organism's requirements for the basic elements, such as carbon, nitrogen, phosphorus, sulfur, magnesium, potassium, and trace elements, are met in a cost-effective manner that allow for optimal growth and product formation rates. These nutrients can be provided using complex (plant, microbial,

or animal derived) or chemically defined sources that also include reducing and oxidizing agents (Theil 1998). Medium optimization can be performed using various stochastic and statistical design techniques (Kennedy and Krouse 1999; Weuster-Botz 2000). Considerations to include when evaluating a medium recipe (as well as other fermentation conditions) entail examination of the potential trade-offs between fermentation production metrics and the ease of downstream processing. For the purpose of this α-amylase application, a defined media is chosen for the fermentation step. Specific fermentation details can be found in the research by Huang et al. (2004).

### 1.3.2 Fermentation Stoichiometry

Knowledge of the chemical composition of the production microorganism can assist fermentation process development toward several objectives, namely, optimizing media composition, rationalizing carbon and nitrogen sources for generating cells and products, and estimating respiration (oxygen, carbon dioxide) and volumetric rate parameters. The composition of a typical microbial cell is shown in Table 1.1 (although ash/minerals content is expected to be much higher) and has a molecular formula of $CH_{1.8}O_{0.5}N_{0.2}$, molecular weight (MW) = 24.6 (Ingram et al. 1983). A similar composition has been observed for *B. subtilis* ($CH_{1.7}O_{0.5}N_{0.2}$, MW = 24.5) (Sauer et al. 1996). General equations for aerobic cell growth, product formation, and cell maintenance have been described in the literature (Hong 1989). Briefly, the equation for cell mass formation is

$$S + n_1N + o_1O_2 \rightarrow x_1X + e_1CO_2 + w_1H_2O \tag{1.1}$$

**Table 1.1** Typical composition of a microbial cell (approximately 70% water).

| Molecule | Dry cell weight (%) |
|---|---|
| Protein | 55 |
| RNA | 20 |
| Lipids | 9 |
| Glycogen | 3 |
| DNA | 3 |
| Liposaccharide | 3 |
| Peptidoglycan | 3 |
| Metabolites | 3 |
| Metal ions | 1 |

where $S$ is the key (limiting) substrate, typically a carbon source; $N$ is the nitrogen source; $X$ is cell mass; and $n$, $o$, $x$, $e$, and $w$ are stoichiometric coefficients. For cell mass formation from glucose in defined media, the equation is

$$C_6H_{12}O_6 + 0.76NH_3 + 2.03O_2 = 0.63C_6H_{10.8}O_3N_{1.2} + 2.22CO_2 + 3.7H_2O \quad (1.2)$$

where the stoichiometric coefficient 0.63 is calculated from metabolic flux experiments. A similar equation can be written for product formation, e.g.

$$S + n_2N + o_2O_2 \rightarrow p_2P + e_2CO_2 + w_2H_2O \quad (1.3)$$

where P is product of interest and $p$ is a stoichiometric coefficient. This equation can be written as follows for an α-amylase molecule such as the thermostable version from *Geobacillus stearothermophilus* with the GenBank Accession Number AAA22227.1 (Suominen et al. 1987).

$$C_6H_{12}O_6 + 1.1NH_3 + 1.4O_2 = 0.73C_6H_{8.9}O_{1.7}N_{1.5} + 1.6CO_2 + 4.4H_2O \quad (1.4)$$

where the stoichiometric coefficient 0.73 is calculated from metabolic flux experiments. Finally, an equation for cell maintenance can be used to describe the consumption of substrates for purposes not resulting in cell mass or product:

$$S + n_3N + o_3O_2 \rightarrow \text{maintenance} + e_3CO_2 + w_3H_2O \quad (1.5)$$

This equation becomes the following when adapted to the α-amylase example:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O \quad (1.6)$$

These equations assume that the only fermentation products containing carbon are the cell mass, α-amylase, and carbon dioxide, though there are often additional side products that divert carbon and energy away and can be taken into account to improve the accuracy of the model.

The theoretical cell mass and product yield coefficients derived from Eqs. (1.2) and (1.4) are

$$Y_{x/s} = \frac{x_1 M_x}{M_s} = \frac{0.63 \times 147.8 \text{ g mol}^{-1}}{180.2 \text{ g mol}^{-1}} = 0.52 \quad (1.7)$$

and

$$Y_{p/s} = \frac{p_2 M_p}{M_s} = \frac{0.73 \times 129.1 \text{ g mol}^{-1}}{180.2 \text{ g mol}^{-1}} = 0.52 \quad (1.8)$$

respectively, where $M_x$ is the MW of cell mass, $M_p$ is the MW of product, and $M_s$ is the MW of substrate.

These equations have uses throughout fermentation development, such as a comparison of the fermentor's product yield to its theoretical value (see the following section), and the calculation of the cumulative heat of reaction to provide an understanding of the fermentation cooling requirements.

### 1.3.3 Fermentor Kinetics and Outputs

The proper selection of a fermentation process involves a consideration of a number of factors. The microorganism may prefer aerobic or anaerobic conditions, the product may be extracellular or intracellular (or sometimes the cell mass itself), and it may be produced in a growth or nongrowth associated manner (Asenjo and Merchuk 1991; Van't Riet and Tramper 1991; Arbige et al. 1993). In addition, fermentation process selection must be considered in the context of the entire production process since this upstream step can significantly affect downstream processing.

The following section will focus on the *fed-batch* fermentation process due to its extensive use in industry and its ability to generate high-production rates. The reader can find a detailed description of this and other fermentor processes in the literature where the equations for total mass and species balances around the fermentor are presented (Chotani et al. 2007). Additional equations of interest include the specific substrate consumption rate, $q_s$, which can be defined as follows:

$$q_s = \frac{\mu}{Y_{x/s}^{max}} + \frac{q_p}{Y_{p/s}^{max}} + m \tag{1.9}$$

where μ is the growth rate of the cell mass, $Y_{x/s}^{max}$ is the maximum growth yield, $q_p$ is the specific product formation rate, $Y_{p/s}^{max}$ is the maximum product yield, and $m$ is the maintenance coefficient. The specific product formation rate, $q_p$, can be expressed by the Leudeking–Piret equation (Atkinson and Mavituna 1983):

$$q_p = \alpha \times \mu + \beta \tag{1.10}$$

where $\alpha$ is a growth rate-associated coefficient and $\beta$ is a nongrowth rate-associated coefficient of product formation.

In our specific example, the engineered α-amylase strain (biocatalyst) is fermented for 27 hours at which time the dissolved oxygen level decreases below 20%, and the fermentation is ended. The cell density, α-amylase, and broth glucose concentration profiles are shown in Figure 1.2a. The fermentation finished with a cell density of 17.6 g $l^{-1}$ and an α-amylase concentration of 7.3 g $l^{-1}$. No residual glucose was detected in the final fermentation broth, and the total glucose added to the fermentor (including batched and fed) was equivalent to

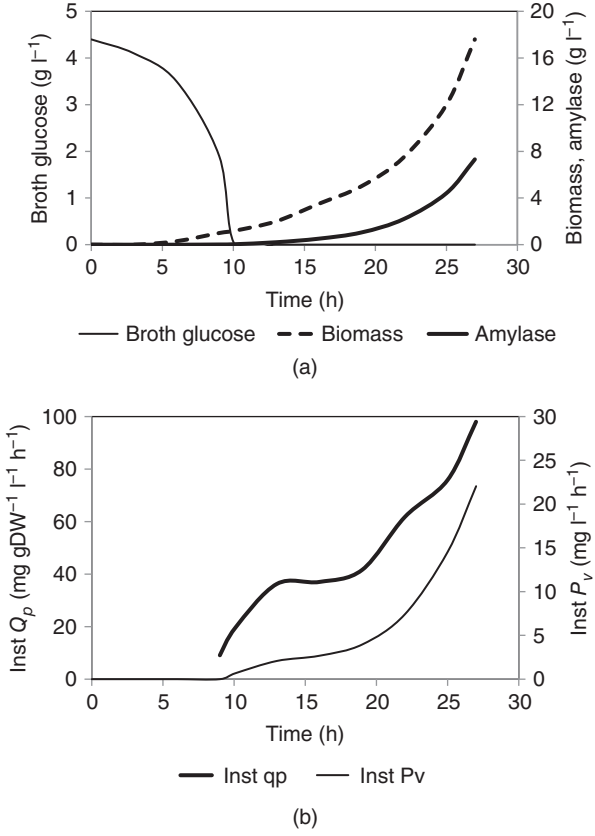


**Figure 1.2** Selected fermentation trends illustrating (a) broth glucose, biomass, and α-amylase concentrations over time. (b) Oxygen uptake rate (OUR), instantaneous volumetric productivity ($P_v$), and hinstantaneous specific productivity ($Q_p$).

68.3 g $l^{-1}$. Titer, fermentor productivity, and yield are fermentation metrics used to evaluate the economics of this unit operation and the performance of the biocatalyst. The product concentration in the broth (titer), $C_p$, at time $t$, is

$$C_p = \frac{\int_0^t (q_p \times X \times V)dt}{V_t} = \frac{\text{cumulative product mass}}{\text{cumulative volume}} = 7.3 \text{ g l}^{-1} \text{ at 27 hours} \quad (1.11)$$

where $X$ is the cell mass concentration in the fermentor broth, $V$ is the volume of the culture, and $V_t$ is the final fermentor broth volume. The average

volumetric productivity, $P_v$, is

$$P_v = \frac{\int_0^t (q_p \times X \times V)dt}{V_t \times t} = \frac{C_p}{t} = \frac{\text{cumulative product mass}}{\text{cumulative volume} \times \text{time}}$$
$$= \frac{7.3\ \text{g l}^{-1}}{27\ \text{hours}} = 270\ \text{mg (l h)}^{-1} \quad (1.12)$$

Equation (1.12) can be modified to estimate instantaneous productivity by replacing $V_t$ with the average working volume, $\overline{V}$, or nominal productivity by replacing $V_t$ with nominal fermentor volume. The cumulative observed cell mass yield, $Y_{x/s}^{obs}$, at time $t$ is

$$Y_{x/s}^{obs} = \frac{X_t \times V_t}{\left[S_0 \cdot V_0 + S_f \int_0^t F(t)dt\right]} = \frac{\text{biomass}}{\text{substrate mass consumed}}$$
$$= \frac{17.6\ \text{g l}^{-1}}{68.3\ \text{g l}^{-1}} = 0.26 \quad (1.13)$$

The cumulative observed product yield, $Y_{p/s}^{obs}$, at time $t$ is

$$Y_{p/s}^{obs} = \frac{C_p \times V_t}{\left[S_0 \times V_0 + S_f \int_0^t F(t)dt\right]} = \frac{\text{product mass}}{\text{substrate mass consumed}}$$
$$= \frac{7.3\ \text{g l}^{-1}}{68.3\ \text{g l}^{-1}} = 0.11 \quad (1.14)$$

where $S_0$ is the substrate concentration at time zero, and $V_0$ is the volume at time zero. These observed cell mass and product yields were 50% and 21% of the theoretical maximum values (Eqs. (1.7) and (1.8)), respectively.

Additional calculations provide information about the biocatalyst performance. The ability of the biocatalyst to generate product can be quantified by the specific productivity metric, $\overline{q}_p$:

$$\overline{q}_p = \frac{P_v}{\overline{X}}$$
$$= \frac{\text{cumulative product mass}}{\text{average working volume} \times \text{average grams dry cell weight} \times \text{time}} \quad (1.15)$$

Specific productivity can also be quantified on cumulative cell mass basis or instantaneous product formation rate basis by changing numerator and denominator of Eq. (1.15). The instantaneous fermentor productivity ($\overline{P_v}$), and biocatalyst specific productivity ($\overline{q}_p$) profiles are shown in Figure 1.2b. Other measurements provide information of the metabolic activity and include the oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) and are described in detail elsewhere in the literature (Chotani et al. 2007). These

metrics help characterize biocatalyst performance by quantifying the interplay between metabolic activity and product formation over the course of the fermentation.

Additional end-point calculations provide important metrics for an overall fermentation process evaluation. A mass balance over the fermentor indicates a significant fraction of the glucose provided for cell mass and product was also used for maintenance.

$$\text{Maintenance glucose} = 68.3\ \text{g l}^{-1} - \frac{17.6\ \text{g l}^{-1} \times 180.2\ \text{g mol}^{-1}}{0.63 \times 147.8\ \text{g mol}^{-1}} - \frac{7.3\ \text{g l}^{-1} \times 180.2\ \text{g mol}^{-1}}{0.73 \times 129.1\ \text{g mol}^{-1}} = 20.3\ \text{g l}^{-1}$$

Thus, $\frac{20.3\ \text{g l}^{-1}}{68.3\ \text{g l}^{-1}} = 0.3$ is the fraction of glucose used for maintenance (0.5 for cell mass and 0.2 for α-amylase). This value can range between 0.05 and 0.9 for biological systems producing heterologous protein products. This information can also be presented as the glucose carbon fate (Figure 1.3), which indicates the overall efficiency of product generation. These data indicate that fermentation costs could be lowered by genetic changes to the biocatalyst and/or process changes that improve cell mass and/or α-amylase yield on carbon.

### 1.3.4 Downstream Processing

Downstream processing typically refers to the separation, purification, and formulation of fermentation products. The overall objective is to (i) recover the product in an efficient manner and (ii) formulate it in a cost-effective manner for the properties required by the application. Numerous recovery processes exist since the required characteristics of the products can vary widely. However, they typically include some modular combination of the following procedures: fermentor harvest, cell separation from the broth, cell

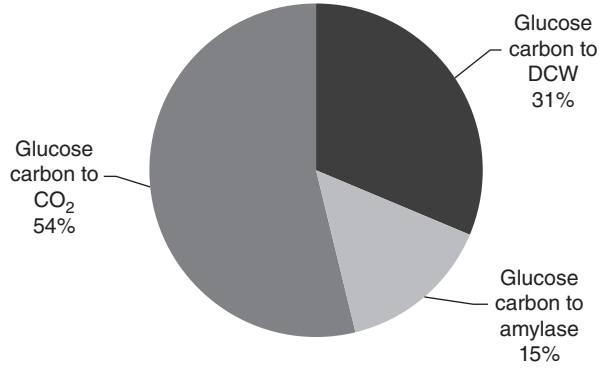


**Figure 1.3** The fraction of glucose carbon residing in dry cell weight (DCW), α-amylase, or carbon dioxide ($CO_2$) after 27 hours of fermentation.

lysis, product extraction, crude and refined separation steps, concentration, purification, formulation, and/or drying. Factors taken into consideration include the type and physiological state of microorganism used for production, the fermentation media, and the physical and chemical properties of the product. The final product form can be as simple as fermentation broth or require more sophisticated procedures such as those utilized in making an enzyme granule.

The recovery of proteins and enzymes requires particular care since they can become inactivated by physical or chemical denaturation. They can be produced as either intracellular or extracellular products. Intracellular product recovery typically involves some type of cell disruption technique prior to a separation process. For extracellular products, cell disruption is avoided since it can liberate intracellular components that hinder separation processes and/or induce issues related to regulatory compliance. In any of these cases, cell separation is typically one of the initial processes and is accomplished in industry using centrifugation and/or filtration techniques. What follows is a discussion of filtration methods. The reader is referred to equipment manufacturer's manual for an in-depth discussion of centrifugation theory and practice.

Filtration processes include various types of equipment that are operated in batch, continuous, or semicontinuous mode, such as membrane modules, rotary vacuum drum filters (RVDFs), filter presses, and belt filters. The membrane's mode of operation can be described as dead-end filtration since the only fluid flow is through the membrane. A membrane acts as a separating boundary for pressure and concentration-driven mass transfer between feed and filtrate. Darcy's law relates the physical properties within this process to the fluid flow:

$$J = \frac{\mathrm{d}V}{A\mathrm{d}t} = \frac{\Delta p}{\nu_0(R_\mathrm{m} + R_\mathrm{c})} = \frac{\Delta p}{\nu_0\left(R_\mathrm{m} + \alpha\rho_\mathrm{c}\frac{V}{A}\right)} = \frac{\text{fluid volume}}{\text{membrane area} \times \text{time}} \tag{1.16}$$

where $J$ is the solvent flux, $V$ is the fluid volume, $A$ is the membrane area, $\Delta p$ is the transmembrane pressure (TMP), $\nu_0$ is the permeate viscosity, $R_\mathrm{m}$ is the resistance of the membrane, and $R_\mathrm{c}$ is the resistance of the filter cake. The resistance of the filter cake is usually the dominating resistance to permeability and can be cast in terms of the specific cake resistance, $\alpha$, the mass of dry filter cake per unit volume of permeate, $\rho_\mathrm{c}$, as shown in Eq. (1.16). Integration of both sides of Eq. (1.16) gives

$$\frac{tA}{V} = \frac{\rho_\mathrm{c}\alpha\nu_0}{2\Delta p}\left(\frac{V}{A}\right) + \frac{\nu_0 R_\mathrm{m}}{\Delta p} \tag{1.17}$$

where $\alpha$ can be determined from the slope of a plot of $\frac{tA}{V}$ versus $\frac{V}{A}$. Filter aids are used for RVDF and filter press systems to improve permeability through the cell cake and filtrate clarity.

In addition to permeate flux rate, other important metrics include product yield and purity. Recovery process product can be improved by increasing the separation selection for the product (physical) and by controlling degradation losses (chemical or biochemical) within the unit operation. For example, proteases from *Bacillus* can undergo self-degradation during recovery processes, and so concentration, temperature, and pH must be appropriately controlled. Product purity is typically dictated by the end-user application and can be affected by numerous upstream conditions including the fermentation media composition and physiological state of the cells.

Following fermentation, the α-amylase-containing broth is subjected to tangential flow microfiltration (MF) for cell separation (Caridis and Papathanasiou 1997; Keefe and Dubbin 2005). While MF equipment costs can be relatively high compared to other cell separation technologies, advantages include the potential for high throughputs and purity as well as a better contained and cleaner process. MF also fits well into the theme of integrated process development. For example, the replacement of centrifugation and rotary drum vacuum filtration steps with a MF step can consolidate a two-step process to one, eliminate the need for adding filter aids that can cause broth disposal issues, improve downstream ultrafiltration (UF) fluxes, and provide higher product yields by improving the selectivity of the separation and by maintaining biological activity. MF membranes are typically defined by pore sizes ranging between 0.01 and 0 μm and operated at TMP values of 0.1–3 bar. They can be constructed from numerous materials such as synthetic polymers, ceramics, carbon and stainless steel. As opposed to polymeric spiral or hollow fiber membranes, ceramic and steel membranes provide open, tubular channels that avoid clogging and facilitate cleaning and sterilization. Their use is commonplace in industry as a result of their durability (years of use before replacement), though fouling must be minimized to maintain flux. However, unlike dead-end filtration processes, turbulent crossflow rates ($\geq 5\,\mathrm{m\,s^{-1}}$) can assist in minimizing the membrane cake layer formation and thereby reduce fouling. For illustrative purposes, it will be assumed that an average MF permeate flux rate of $20\,\mathrm{l\,m^{-2}\,h^{-1}}$ is observed over a 36-hour period with a single pass α-amylase transmission rate of 65% over the range of concentrations in question. Thus, a MF unit containing three membrane stages can be used to recover α-amylase over a 12-hour period with the following membrane load:

$$20\,\frac{\mathrm{l\,broth}}{\mathrm{m^2\,h^{-1}}} \times 12\ \mathrm{hours} = 240\,\mathrm{l\,m^{-2}},$$

and yield:

$$1 - (1 - 0.65)^{3\ \mathrm{stages}} = 0.96$$

leading to a 96% product recovery. Purity is observed at 90%, where the impurities consist primarily of salts, cell-derived polysaccharides, and some proteins and peptides.

Next, the permeate stream from the MF stage is concentrated using UF. The considerations for UF systems are similar to those described for MF systems since they utilize similar materials and configurations. The filtration unit consists of plate-and-frame style cassettes containing polyethersulfone membranes with a nominal molecular weight cut-off value of 30 kDa. UF concentration results in comparable flow rates, yields (98%), and purity levels (90%) to the MF process, concentrating the MF-permeate α-amylase concentration from 7.3 to 65 g $l^{-1}$ (for other products there can be up to a 20-fold increase from the final fermentor concentration). Finally, the same UF equipment is used to perform diafiltration (DF) to reduce the solution conductivity to 4 mS $cm^{-1}$ while improving purity to 95% by removing salts (98% yield).

Purification is usually achieved by less expensive techniques for industrial products, if it is performed at all. Crystallization of the α-amylase was chosen for this example though precipitation and extraction also meet this criteria. Crystallization is initiated by adding solid sodium chloride to the DF retentate to a final concentration of 400 mM. This solution is stirred at 25 °C for 16 hours before separating the crystallized enzyme using a filter press (Becker and Lawlis, Jr. 1991). The crystallization step increases the purity to 99% at the expense of a relatively low yield (80%).

The final product form can be a liquid, granule, or powder. The choice is typically dictated by the requirements of the application, and each has its own considerations to take into account. For the α-amylase example, a spray-dried powder (Neubeck 1980; Becker and Crowley 1998) was chosen since this form is commonly used for baking applications. A spray dryer feed solution is fed into the chamber at a rate of 1.7 $cm^3$ $s^{-1}$. Inlet air at a constant flow rate of 2.3 m $s^{-1}$ and 220 °C is used as the drying media. Process measurements include the powder moisture content and the remaining α-amylase activity (Nath and Satpathy 1998; Samborska et al. 2005). The spray-drier is able to produce 1.6 kg $h^{-1}$ of powder with a residual moisture level of 6% and 90% of the initial α-amylase activity remaining.

**Table 1.2** Example data for an α-amylase downstream production process illustrating yield and purity values for individual process unit operations.

| Process step | Yield (%) | Purity (%) |
|---|---|---|
| Microfiltration | 96 | 90 |
| Ultrafiltration | 96 | 90 |
| Diafiltration | 98 | 95 |
| Crystallization | 80 | 99 |
| Spray drying | 90 | – |
| Total | 65 | 99 |

Table 1.2 shows individual and cumulative process step yield values (65%), as well as purity levels (99%). Further integrated process development work could be directed toward improving the entire production system yield as well as product quality characteristics.

## 1.4 Summary

The systematic approach for developing *Bacillus* as host of choice for expression and secretion is a useful cell factory design model. The advances in *Bacillus* molecular genetics and cell engineering in the last three decades have reshaped enzyme production. It has become possible to clone engineered gene sequences encoding efficient enzymes and express them in *Bacilli* suitable for large-scale industrial fermentation processes. Enzyme productivity has steadily been increased through efficient promoters, regulatory gene mutations, deletions, and multiple copy insertions. Industrial protein engineering continues to tailor enzyme properties such as optimum temperature, pH, stability, and substrate interaction. The development of nonpathogenic and nontoxigenic microbial production strains, such as *B. subtilis* and *B. licheniformis*, well characterized by generally accepted safety evaluation procedures (e.g. Pariza and Johnson 2001) has found wide acceptance by regulatory agencies such as the US FDA (Olempska-Beer et al. 2006) and US EPA (http://www.epa.gov/opptintr/biotech/pubs/rulesupc.htm). The general acceptance of the safety of microbial enzymes tested against international standards (JECFA 2006) has allowed increased enzyme manufacture and use worldwide over the last few decades. Industrial bioprocessing will expand as gene sequencing and engineering of production microorganisms continue to become faster.

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